MACROGLUBULINS IN NORMAL PIG SERUM

MACROGLOBULINS IN NORMAL PIG SERUM

A thesis submitted to the University of Adelaide for the degree of Master of Science.

by

WARWICK SOUTER, B.Sc., A.U.A.

* * *

Department of Microbiology,

University of Adelaide.

* * *

TABLE OF CONTENTS

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief it contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

(W. SOUTER)

ABSTRACT

The sera of most mammals contain, in addition to the by now well established 19 S macroglobulins, IgM and α_2M, globulins of even higher sedimentation coefficient such as 22 S, 29 S, 34 S and 42 S. These components have been reported in particular in the serum of patients with Waldenstrom's macroglobulinemia, a pathological condition associated with an elevation in concentration of serum macroglobulins. The exact chemical composition of these macroglobulins with sedimentation greater than 19 S remains obscure. Pig serum contains relatively high concentrations of a 29 S macroglobulin and therefore provides suitable material for an examination of the structural relationships between this component and other proteins present in porcine serum. The 29 S macroglobulin has been shown to be an immunoglobulin according to the following criteria: 1. It is a protein. 2. It has a β mobility by electrophoretic analysis. 3. It is labile to reduction with sulfhydryl reagents (e.g. 2 mercapto ethanol); and 4. It shares antigenic similarity with IgG globulin. The latter property is the strongest evidence that the 29 S macroglobulin is an immunoglobulin, particularly as the antigenic similarity appears to lie in the light polypeptide chains of both molecules.

A unique feature of the 29 S macroglobulin is its

lability towards reduction with potassium borohydride under controlled experimental conditions when the molecule dissociates into 7 S subunits. Under identical conditions of treatment 19 S macroglobulins (IgM) do not dissociate as has been clearly established by the use of radio-labelled preparations of IgM. However, both the 29 S and IgM macroglobulin dissociate into 7 S subunits upon reduction with 2-mercapto ethanol.

Homogeneous preparations of porcine IgG and IgM have been isolated as have the light and heavy chains from both of these globulins. Antisera have been prepared in rabbits directed against porcine IgG and specifically against the γ and μ chains of these preparations.

Methods have been developed for the isolation of limited quantities of the 29 S macroglobulin but the difficulties encountered have prohibited its preparation in sufficient amounts to allow for a comprehensive study of the physical and chemical characteristics of the material. However, immunoelectrophoretic studies have shown that 29 S macroglobulin cross reacts with rabbit anti-porcine IgG but does not precipitate with rabbit anti-porcine γ or μ chains. In similar manner a mixture of 29 S + 19 S macroglobulins develops only one precipitin line on immunoelectrophoresis

against rabbit antisera to porcine μ chains. These results together with those of potassium borohydride reduction, clearly indicate that the 29 S macroglobulin is a unique entity and is not a polymeric form of either IgG or IgM immunoglobulins.

Preliminary evidence is provided to suggest that the 29 S macroglobulin may be endowed with antibody activity and that it appears early in the immune response of rabbits following injection with T4 bacteriophage and of mice with Salmonella typhimurium M206.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

In 1890 it was shown that immunity to tetanus is a function of a substance or substances in the blood. These substances were named antibodies.

Antibodies are produced by all vertebrates as a defence against invasion by certain foreign substances, known collectively as antigens. The most remarkable aspect of this phenomenon is the specificity of the antibody for the antigen injected. That is, the antibody formed will combine only with the antigen injected or with other substances whose structure is closely related. This phenomenon, taken together with the observation that a great variety of substances are known to be antigenic, indicates that the immunological system can synthesize a very large number of different antibody molecules distinguished by their binding specificity. Despite this remarkable diversity, all antibodies have a basically similar peptide chain structure which is common to man and all vertebrates.

All antibodies are found in a group of related serum proteins known as the immunoglobulins.

Definition of the chemical basis of combining specificity is complicated by the size of antibody molecules and more particularly by their chemical heterogeneity. This structural

complexity in normal immunoglobulins and specific antibodies has stimulated renewed interest in those homogeneous proteins which are found in mice and men in association with myelomatosis and certain related lympho-proliferative disorders. These are believed to be individual species of the normal immunoglobulin population with peptide chains sufficiently homogeneous to permit detailed chemical analysis. Studies on these proteins have provided much detailed information about immunoglobulin structure and in particular have shown that large segments of immunoglobulin molecules have a relatively conservative structure with variability mostly confined to portions of the molecule known to carry the antibody combining sites.

It has been stated that the immunoglobulins are found to be a very complex mixture of molecules; however, this complexity is not only due to the presence of antibodies of different specificity in response to various antigens. The situation is further complicated in that there are four main classes of immunoglobulins distinguishable from one another in size, carbohydrate content and amino acid analysis. These classes are designated the symbols 1gG, 1gM, 1gA and 1gD. Ishizaka et al (1966) have identified an additional class (1gE) which carries the properties of the reaginic antibodies which mediate various immediate hypersensitivity

responses in man. Fahey (1965) has suggested that myeloma proteins, Bence-Jones proteins and normal urinary components which are structurally related to the antibodies should also be classed as immunoglobulins. Recently there have been examples of myeloma proteins exhibiting specific antibody activity (Eisen et al, 1967).

The nomenclature for immunoglobulins used throughout this work is that agreed upon by the World Health Organization in Prague in 1964 for human immunoglobulins. This system of nomenclature will also be used for the designation of heavy chains, i.e. the heavy chains are designated by small Greek letters corresponding to the Roman capital used for the immunoglobulin classes.

Immunoglobulin class	Heavy chain
γG or IgG	γ (gamma)
γA or IgA	α (alpha)
γM or IgM	μ (mu)

All antibody molecules are made up of a basic unit of four polypeptide chains consisting of two heavy (molecular weight 50,000-70,000) and two light chains (molecular weight about 20,000) covalently linked by interchain disulphide bonds. This structure was first proposed by Porter in 1962

for rabbit 1gG antibody and has subsequently been found to apply to the immunoglobulins of all vertebrate species so far examined. The general four-chain structure of antibody molecules was inferred from the properties of peptide chains separated from reduced immunoglobulins and also by analysis of fragments obtained by splitting antibodies with proteolytic enzymes. In fact most of our knowledge of antibody structure comes from this type of study of the IgG molecule.

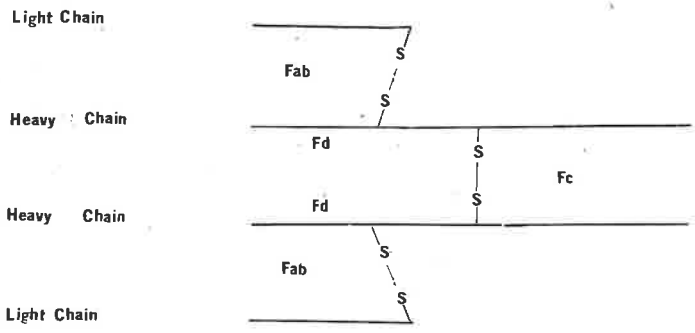
Immunoglobulin G molecules can be split by proteolytic enzymes such as papain, which breaks the molecule into three pieces of about equal size. Two, known as Fab ("fragment antigen binding"), appear to be identical, and the third, known as Fc (fragment crystalline), is quite different. Fab is so named because it will still combine with the antigen although it will not precipitate with it. Each Fab fragment carries one combining site; thus the two fragments together account for the two combining sites that each 1gG antibody molecule has been deduced to possess.

The proteolytic enzyme pepsin splits the IgG molecule at a position adjacent to, but on the C terminal side of the interchain disulphide bond linking the two heavy chains. The products of pepsin digestion are $(Fab^1)2$ a molecule

which is slightly larger than two Fab fragments and is bivalent (i.e. contains two antibody combining sites), and peptides which arise from the hydrolysis of the Fc piece. When IgG is treated with a disulphide bond splitting agent such as 2 - mercaptoethanol 4.5 to 5 disulphide bonds per molecule are split and the chains can be dissociated. One thiol group is formed per light chain and this suggests one bond between the light and heavy chains. Because the inter-chain bonds are more labile than the intra chain bonds it was suggested that the other three easily reduced bonds might be links between the heavy chains. However Nisonoff (1965) has since shown that there is one bond between the heavy chains which is very labile to low concentrations of 2 - mercaptoethanol. When this bond is broken the whole molecule falls into halves. He therefore suggested that there is only one disulphide bond between the heavy chains and the other two disulphide bonds which are broken are labile intra chain bonds. This single disulphide bond lies between the sites of papain and pepsin attack.

The position is not entirely clear however, as Cebra (1967) has shown that if IgG is hydrolyzed very slightly by insoluble papain with breakage of only three to four peptide bonds, there is no splitting of the molecule into fragments unless a disulphide bond splitting agent is added. If

detergent rather than 2 - mercaptoethanol is added, slow dissociation occurs yielding $(Fab)_2$ and Fc, but this dissociation can be prevented by a thiol-blocking reagent. Hence the following model has been postulated.



According to this model, papain acts between 2 half-cystines which form an intra-chain bond and in the presence of a detergent, a disulphide interchange occurs leading to the formation of a new bond between the heavy chains and hence to $(Fab)_2$. This also agrees with the finding that 2 new thiol groups are found in the Fd fraction (that part of the heavy chain in the Fab fragment) when it is prepared from Fab by gentle reduction. One of these 2 new thiol groups is presumably from the light-heavy disulphide bond and the other from the intra chain bond.

The light chains are the common structural features of different immunoglobulins and whether obtained from IgG, IgM, IgA, IgD or IgE each will have similar molecular weight,

electrophoretic mobility and antigenic specificity. The heavy chains of the five classes, however are distinct and differ in all three properties. The heavy chains of IgG, IgM, IgA, IgD and IgE are referred to as γ, μ, α, δ, ε respectively. Differences as great as 50% are found between the content of some amino acids in the μ chain and those in the γ chain (Chaplin et al, 1965). The antigenic cross reaction between the immunoglobulins is eliminated if antibodies directed to the light chains are removed by adsorption with free light chains prepared from any of the immunoglobulins. This suggests that very different primary structures will be found in the five types of heavy chain. This is supported by the comparison of the peptide maps obtained by tryptic hydrolysis of immunoglobulin heavy chains which reveal few peptides in common between γ, μ and α chains (Lamm et al, 1966).

The classes of immunoglobulins may be subdivided into subclasses, or divisions based principally on antigenic characteristics which reflect variations in amino acids. These subclasses have been principally identified in IgG.

The heavy chains of subclasses of IgG show the antigenic characteristics of all γ chains but have additional distinctive features. In human IgG for example, four such

subclasses IgG1, IgG2, IgG3 and IgG4 are recognised, using as reference myeloma globulins each of which is homogeneous and an example of one or other subclass. They have been named Ne, We, Vi and Ge (Grey et al, 1964) and differ from one another in their amino acid sequences at certain points on their polypeptide chains. The light chains are common to all classes and subclasses, but are themselves present in two antigenically unrelated forms known as K and L chains (Cohen and Porter, 1964). Further subdivisions also exist because it is known that both isolated heavy and light chains show electrophoretic heterogeneity when electrophoresed in starch or acrylamide gel containing 8M urea at neutral pH. For instance Sjoquist (1962) was able to show that the γ chain as well as the light chains gave a number of distinct bands in either type of gel. Feinstein (1966) has suggested that each electrophoretic component of the light chain differs from the next one by one charge unit. This variation of net charge is almost certainly a consequence of the multiplicity of sequences in the N-terminal half of both heavy and light chains where many variations in amino acid sequence and composition occur.

As well as these four kinds of structural variation - class, subclass, type and electrophoretic banding there is

a fifth kind of variability, allelic. There are several alternative forms of a given chain (recognized by antigenic specificity) only one of which is present in animals homozygous for this character. Heterozygotes on the other hand possess two such structural forms. Thus in any individual, the immunoglobulins will be present in the serum in at least five classes, each with several subclasses and each subclass will exist in multiple electrophoretic forms. All these different groups will be present in two types, depending on the characteristics of the light chain in the molecule, and again each variant will be present in one or two different allelic forms. The exact nature of these differences arising from variations in peptide composition and the resultant antigenic differences can only be determined by studying the actual sequence of amino acids in the peptide fragments or polypeptide chain.

The most detailed sequence data available is for the light chains of human and mouse gamma globulin, although at this time only five complete light chain sequences have been resolved. These sequence studies have been carried out on Bence-Jones protein - an abnormal protein which appears in the urine of about half of all myeloma patients. Bence-Jones protein light chains are believed to be identical with the light chains of the myeloma protein in the serum of the same

patient. Such studies have supported the idea that variation in the amino acid sequence in light chains occurs at the amino end of the chain. Of the 107 amino acid residues in the N terminal half of the chain substitution occurs in at least 40 positions. This situation clearly opens the way for the existence of molecular variation on a very large scale.

Since heavy chains from IgG contain approximately 460 amino acid residues, sequence studies of heavy chains are less advanced than those with light chains. Most information available in this case has come from attempts to determine the sequence of the constant part of the heavy chain. The whole of the Fc section of heavy chain from normal IgG has provided a relatively stable sequence. By contrast considerable amino acid substituion has been recorded in the Fab region of IgG. This is to be expected if antibody specificity resides in the Fab section of IgG.

The genetic mechanism concerned with the biological origin of the unique situation in the immunoglobulin molecules whereby the molecules are composed of constant and variable sections of amino acid sequence is not known. A continuation of the structural studies at present being undertaken may eventually provide a clear answer as to which,

if any, of the present speculative hypotheses is correct.

Immunoglobulin IgA occurs not only in serum - but also in saliva and colostrum. As late as 1962 there was doubt as to the possible antibody activity of these globulins although several investigators had reported antibody activity in serum fractions which contained IgA and probably other globulins. Activity has now been demonstrated in IgA from man (Heremans, 1963), horse (Klinman, 1966), bovine (Wilkinson, 1966), rabbit (Onoue, 1964), and guinea pig (Rothamn, 1965). So far agglutination (Kunkel, 1963), precipitation (Winter, 1965) and neutralization of some viruses (Rossen, 1966) have been demonstrated in serum IgA and endocrine IgA also appears to have antibody activity.

The chemical and physical study of IgA has not as yet been undertaken as extensively as the work on IgG, however the amount of information available is increasing. In 1965 Tomasi showed that while serum and salivary IgA are similar to each other, they differ significantly in some of their chemical and immunological properties. Serum IgA has an $S^{o}_{20,w}$ of 6.9 compared with different forms of salivary IgA where molecules with an $S^{o}_{20,w}$ value of 11.4 predominate. Deutsch (1963) has described one particular IgA-myeloma protein which had a molecular weight of 400,000 and a

sedimentation coefficient of 9.6 S and which could be dissociated by reduction. However, rabbit secretory IgA described by Cebra (1966) showed no tendency to dissociate on mild reduction and thus it resembled the human IgA immunoglobulin isolated either from saliva or colostrum by Tomasi (1965). Recent determinations of the molecular weight of secretory IgA immunoglobulin and its heavy α chains, and of the weight ratio of α to light chains in the molecule suggest that the colostral protein exists as a dimer complexed through a secretory or Tpiece. These dimers are themselves assembled according to the basic pattern of gamma globulins. In other words the secretory IgA consists in the main part of four pairs of light and α chains. However, an antiserum raised against the 11 S IgA derived from saliva shows immunological differences between serum 7 S IgA and the salivory 11 S. Hence it seems that the 11 S IgA is not merely a polymer of 7 S since it has this antigenic difference. Whether the portion of IgA which is antigenically different is a piece incorporated during the local synthesis of IgA in the gland or is added by the epithelial cell in the process remains to be determined. Tomasi (1965) suggested the piece is probably involved in transport or polymerization of IgA. Further light was shed on this question by South (1966) who, with her co-workers observed that IgA can be

selectively transported into saliva from serum, and that, as suggested by Tomasi, salivary IgA has immuno-chemical characteristics suggesting that it is composed of two antigenically different entities. It appears that salivary IgA differs from serum IgA only in that an additional protein is attached to the IgA molecule in its passage into secretions. This additional protein can occur independently of IgA in a gamma-globulinemic saliva, in mammary tissues and in saliva of many normal children. Although unable to directly demonstrate any transport role for this IgA - attached protein, South has tentatively called it the "transport piece."

When the electrophoretic mobilities of the immunoglobulins are examined there is found among the faster moving fractions a protein which has a molecular weight of about 900,000 and whose sedimentation constant is 19 S. This is the so called macroglobulin (IgM) fraction. The structure of IgM has yet to be clearly elucidated and in contrast to IgG it is apparent that at this stage very few facts relating to the structure of IgM are available. As with IgG, the most convenient material to study is protein derived from patients with a pathological disorder, in this case macroglobulinemia. This is because it is available in large quantities and is relatively homogeneous. (Miller and Metzger, 1965)

Miller and Metzger have worked extensively on these pathological macroglobulins and they suggested in 1965 that IgM is built up of 5 subunits each consisting of two heavy and two light polypeptide chains. The light chains of IgM and IgG appear to be identical in size and composition whereas the heavy chains of IgM appear to be larger than those of IgG. It has been calculated that the IgM heavy (μ) chains have a molecular weight of 65,000 to 70,000. The chains do not react with anti γ chain serum and are therefore antigenically distinct and hence amino acid composition differences occur in some segments of the chains. Suzuki and Deutsch have reported that each of the 5 subunits of IgM consists of three light chains and 2 heavy chains. These workers suggested that the difference in results arose because in the work of Miller and Metzger the results are based on sedimentation and diffusion data obtained by carrying out the analyses in a relatively low ionic strength buffer and that these conditions could have led to a charge effect resulting in lowered sedimentation and possibly a higher diffusion constant for their protein. Despite this evidence the general consensus of opinion at the present time favours the two light, two heavy chain composition for each subunit. The 5 subunits have a molecular weight of approximately 200,000 and are believed to be linked by disulphide linkages.

In contrast to IgG the nature and position of these linkages is unknown. Miller and Metzger (1965) have shown that on reduction of IgM, 49-50 new sulphydryls per molecule of IgM were formed and that 40 of the new sulphydryls were associated with the heavy chains and 10 with the light chains.

Onoue et al (1965) and Metzger (1967) suggested that each macroglobulin molecule has 5 antibody combining sites. By equilibrium dialysis, an IgM antibody (against benzenearsonate) was shown by Onoue (1965) to have 5 ligand-binding sites per molecule, or one per 7 S monomer. Metzger (1967) showed a similar result using a myeloma protein which he concluded had antibody activity against human IgG. These results seemed surprising since the predicted value on the basis of the structure of IgG antibody would be 10 combining sites per IgM molecule. In 1968 Merler, Karlin and Matsumoto injected rabbits with an oligosaccharide and isolated the macroglobulin directed against this antigen. Equilibrium dialysis studies on this system suggested that the macroglobulin did in fact have a valence of 10. Although a discrepancy appears to exist, this could be explained in terms of the size of the antigen used. Obviously this would limit the number of molecules which are able to occupy potential combining sites. It seems probable therefore that there are

are 10 potential combining sites per IgM molecule.

Since both IgG and IgM globulins have antibody activity but substantial differences in structure it is of interest to compare their presence and function in the general immunological picture.

It is generally found that the first antibodies detected in serum after immunization are IgM molecules (Fahey, 1965). The level of IgM antibody usually declines and often disappears completely in several weeks to be replaced by IgG antibody which may persist for long periods of time. Although this pattern appears to be a general one there are variations in the duration of IgM antibody response depending on the kind of antigen, the dosage and the species (Pike, 1967). More recent observations suggest, however, that the sequential synthesis of IgM and IgG may be more apparent than real. Using more sophisticated techniques for detecting antibody, Freeman and Stavitsky (1965) have shown the simultaneous appearance of IgG and IgM antibodies in rabbits after injection of human serum albumin and bovine gamma globulin. Hence although synthesis of IgG and IgM may begin at about the same time, IgM will appear to predominate in early sera if the methods used for detection of antibody activity favour the detection of IgM. (Freeman and Stavitsky, 1965; Ostler et al, 1966).

Many factors influence the nature of the antibody produced and the most significant of these is the nature of the antigen. Protein antigens have usually been found to stimulate a preponderance of IgG after the IgM has decreased or disappeared (Grey, 1964), however, lipo polysaccharide somatic antigens of enterobacteria stimulate predominantly IgM antibodies in man and the rabbit (Grey, 1964; Pike and Schulze, 1964). There is evidence to suggest that the physical state of the antigen may also be a significant factor in determining the nature of the antibody produced. Ada et al (1965) found that rats, immunized with either the monomer or polymer forms of flagellin tended to produce more IgM in response to the polymer form. Similar responses have been found by Linquist and Bauer (1966) using aggregated bovine serum albumin compared with native albumin. It has been noted by Pike and Schulze (1964) that bacterial lipo polysaccharide elicited IgM antibodies only, but when larger amounts of antigen were injected over longer periods of time, considerable amounts of IgG were demonstrable. It appears therefore that the dosage can affect the nature of the antibody produced. Further support for this suggestion is found in the work of Svehag and Mandel (1962) who observed that a relatively small dose of poliovirus in rabbits elicited only IgM, but when a much larger dose was given the initial

IgM response was followed by an IgG response. It seems, therefore, that in some cases the dosage of antigen may affect the nature of the antibody formed. However, it should be realized that the relative effectiveness of detecting different classes of antibody must also be considered. From the examples given it is apparent that IgM and IgG antibodies can be produced following the injection of any antigenic material although it seems likely one or the other may predominate in a particular situation. Further comparison between IgG and IgM antibodies can be made by considering some of the important biological reactions in which they participate. For example, both IgG and IgM antibodies are capable of causing agglutination. Greenbury et al (1963) compared rabbit I^{131} labelled IgG and IgM antibodies raised against human erythrocytes and found that on a weight basis over 100 times as much IgG as IgM was required to give 50% agglutination. On a molecular basis they calculated that 19,000 molecules of IgG per cell were required for 50% agglutination, whereas only 25 molecules of IgM per cell were required. Most reports support this suggestion that smaller numbers of IgM than IgG molecules are required to mediate immune reactions. One exception to this general finding is the report by Heremans (1963) in which human IgG and IgM displayed approximately the same agglutinating activity for Brucella.

The firmness with which a particular antibody combines with its specific antigen is a property often referred to as its avidity. The literature pertaining to this subject is difficult to interpret because of the varied criteria used for measuring avidity. IgG and IgM have been shown to exhibit differences in avidity, in some cases IgM being thought to be more avid than IgG and in others the reverse situation appears to hold. At this stage no clear cut generalizations can be made from the rather confusing data which exists.

It is of interest to compare the situation for agglutination with that which applies in the so called precipitin reaction. Several investigators have reported little or no precipitation with IgM antibodies to serum albumin (Reddin et al, 1965) although these antibodies were readily detected by passive haemagglutination. It has been shown by Weidanz (1964) that both IgM and IgG give precipitin lines in gels with Salmonella endotoxin and several other antigens. Further studies show that there are variations in the capacity of IgM and IgG to agglutinate and precipitate various antibodies. As yet there is no clear cut explanation for these observed differences. The probability that the IgM molecule has five or more combining sites may account for its marked agglutinating ability but this hypothesis does not explain

the precipitin behaviour.

The inability to explain the differing behaviour of IgG and IgM is, at least in part, a reflection of our present inadequate knowledge of the basic chemical and physical structure of these molecules. Further variations in the relative ability of IgM and IgG to carry out a biological function attributed to antibodies may be seen by comparing their respective abilities to fix complement. It seems that IgG antibodies are more effective than IgM antibodies in fixing complement (Bellanti et al, 1965). This has been shown to be true to varying degrees in a number of different systems. Since the lysis of erythrocytes is dependent upon the presence of complement it is an appropriate system to compare the relative efficiency of IgM and IgG molecules to bring about this lysis. One would expect that the type of antibody more effective in fixing complement would also have the greater lytic action. However, it seems likely that the reverse is true. Stelos and Talmage (1957) reported that IgM rabbit antibodies for sheep cells were from 50 to 100 times more efficient in causing hemolysis than were IgG molecules when compared on the basis of combining power. The efficiency has also been compared on a molecular level. Wigzell (1966) injected mice with sheep red blood cells and was able to show that mouse IgM is 100 to 1000 times more

efficient than IgG. Electron microscopic examination of sheep cells lysed by antibody and complement revealed the sites of damage as holes in the cell membrane. It was calculated that 2 to 3 molecules of IgM antibody were sufficient to make 1 hole, whereas about 1000 times as many IgG molecules were required for this effect. Observations by Goodman (1959) revealed a lack of correlation between the amount of complement fixed and the hemolylic activity of serum. Since the antibody with the greater molecular weight fixed less complement per hemolytic unit, it appeared that this antibody utilized complement more efficiently in the hemolytic process than did the antibody of lower molecular weight. This difference in the hemolytic efficiency of IgM and IgG has been apparent in the application of the plaque technique for detecting antibody for erythrocytes produced by single cells described by Jerne (1963) and in the adaptation by Landy (1965) of this technique for the detection of cells producing antibodies to bacterial antigens.

In summarising this comparison between the functioning of IgG and IgM, as already indicated, there is conflicting evidence in some areas, and in others the data is subject to different interpretations. It is possible to conclude, however, that IgG antibodies, which are usually more apparent in later stages of immunization are highly effective precipitin

and, in most instances, account for the major portion of the complement-fixing activity of serum. They are effective in neutralizing viruses, endotoxins and enzymes and their ability to form a stable union with antigen increases with time after immunization. In contrast to IgG, IgM molecules which are often the first to be detected are most active in agglutination and lytic reactions. They are not as readily detected by precipitation and complement mediated lysis as are IgG molecules. They also have virus neutralizing capacity, but they apparently fail to neutralize toxins and enzymes (e.g. lysozyme). In some instances they have been dissociated from antigen less readily than IgG but in other instances the reverse has been observed. These observed differences between IgG and IgM do not show any simple relationship between the two types of antibody. Possibly in the future when the complete structures of the antibodies have been elucidated it will be possible to relate this structure to the observed immunological activities.

This study is centered around the higher molecular weight antibodies. The macroglobulin fraction in serum has a sedimentation rate between 17 S and 20 S and makes up 2 to 5 per cent of the total serum proteins. Here the sedimentation rate of the antibodies has been expressed in terms of the sedimentation constant. The sedimentation

coefficient of a molecule represents the rate of sedimentation of the molecule in solution in cm per sec., per unit centrifugal field of force (dynes/gram). The absolute sediment constant is derived from an observed sedimentation coefficient by correction to conditions in water at a temperature of (usually) 20°C. Sedimentation constants are expressed in Svedberg (S) units where one Svedberg (S) = 10^{-13} times the absolute sedimentation constant.

It has been shown in pig serum that the 19 S immunoglobulins have antibody like properties, hence throughout the context of this thesis the terms IgG and IgM will be used to define the 7 S and 19 S immunoglobulins respectively, unless otherwise stated in the text. The term 29 S will be used to define the higher molecular weight component illustrated on ultracentrifugation of partially purified normal pig macroglobulin.

One of the most characteristic features of the macroglobulin is that the 19 S fraction is always associated with other components of still higher S rates which are present in smaller amounts. The study of one of these which occurs in normal pig serum and its relation to other serum components of the pig is the aim of this present study.

These higher molecular weight components are most readily

observed in the serum of different patients suffering from macroglobulinaemia. In general the predominating components in addition to the major 19 S fraction, in such a system have S values of 26 and 32 and in general the ratio of one to the other is remarkably constant from patient to patient. These components are usually associated with various other components of intermediate or higher sedimentation constants. Through ultracentrifugal recycling it is possible to concentrate these heavier components. Kunkel (1960) suggests that for this reason, these components are real entities and not necessarily polymeric forms in equilibrium with the 19 S components. Peterman and Braunsteiner (1954) however, reported one case of macroglobulinaemia where the ratio between the 19 S and the higher S rate components varied with concentration, suggesting some type of equilibrium. Suzuki and Deutsch (1966) subjected a 22 S component obtained from a pathological serum to various studies. The results of their work suggested that the 22 S material was a dimer of 19 S proteins and could undergo reversible molecular transitions which were both pH and temperature dependent. The first case where a concentration equilibrium exists appears to be an unusual one and certainly an exception since many other workers (Abrams, 1949; Deutsch, 1958 and Putnam, 1959) have noted the absence of any such equilibrium. Other studies

have shown that the heavier components do not dissociate in urea or acid buffers and in fact have a stability similar to the main 19 S component (Kunkel, 1959). However, sulphhydryl compounds such as 2 - mercaptoethanol will cause complete dissociation of both 19 S and heavier components to units with an S rate of approximately 7 S. It therefore seems possible that they represent higher polymers of a 7 S component linked by disulphide bridges similar to the situation in the 19 S material.

The aim of this study is to determine whether there is any antigenic relationship between the 29 S component of normal pig serum and other components of pig serum. Several hypotheses can be formulated with respect to possible relationships.

1. The 29 S component is a polymer of normal IgG present in the serum.
2. The 29 S component is a polymer of 19 S present in the serum.
3. The 29 S component is a separate entity and the relationship between it and 19 S is similar to the relationship between IgM and IgG. That is, it has light chains with common antigenic determinants to the light chains of 19 S, but has antigenically distinct heavy chains.

4. The 29 S is an antigen - antibody complex.

The work described in this present study is an endeavour to establish which, if any, of these possibilities is true.

Although serum proteins of higher molecular weight than IgM (molecular weight approximately 1,000,000) have been studied previously these investigations have been of a highly superficial nature (Deutsch, 1956; Korngold, 1957) and only arose as a biproduct of major studies by these workers on macroglobulins present in the serum of patients suffering from macro-globulinemia. No real attempt was made by either workers to relate the larger macroglobulins to other serum components and the only data obtained concerns the sedimentation coefficients of these components and their ability to dissociate and reaggregate under a variety of artificial conditions. Neither of these workers made any attempt to separate the macroglobulins of molecular weight greater than 19 S from the more abundant 19 S material and consequently any results concerning the antigenic specificity of the higher molecular weight components are open to a variety of interpretations.

CHAPTER II

MATERIALS AND METHODS

1. Collection of pig serum

The pig serum used was prepared from the blood of healthy pigs slaughtered at the Adelaide Metropolitan Abatoirs. The blood was stood at room temperature for one hour to allow clotting and then kept at 4°c for 30 minutes to one hour to encourage clot retraction for maximal recovery of serum. After this time the serum was withdrawn using a glass tube connected via a 2 litre buchner flask to a vacuum pump. The serum was then spun in the M.S.E. centrifuge at 3,000 r.p.m. for 20 minutes. The clear straw coloured liquid was removed from the packed cells and deep frozen in approximately 50 ml samples. These samples were only thawed once. All serum samples were obtained by pooling the blood from at least four animals.

2. Gel Filtration Chromatography

The method of serum fractionation by gel filtration used in this study was essentially that of Killander and Flodin (1962). The material used, Sephadex G-200 (A.B. Pharmacia, Uppsala, Sweden) is a cross linked polydextran. The Sephadex was soaked in 0.1 M tris - hydroxy - methyl - amino - methane (TRIS) - HCl buffer, pH 8.0 for 24 hours to allow swelling.

(This buffer was used frequently throughout this study and will subsequently be referred to as "tris buffer".) The turbid supernatant was removed by decantation and the material packed into a vertical column measuring 4 cm x 50 cm in the following manner. The column was closed at its base, a pad of glass wool was placed at the bottom of the column which was layered with about 1 cm depth of Sephadex G-25 and the column was one third filled with tris buffer. A thin suspension of the gel was introduced and allowed to settle onto the Sephadex G-25.

Excess buffer was drained from the outlet and the process of filling and draining was continued until the column was packed to within 5 cm of its top, care being taken to prevent drying. A reservoir was then placed on top of the column and the gel was washed with three times its own volume of buffer. The gel was allowed to equilibrate overnight. Before adding the serum sample, excess buffer was removed from the top of the gel by pipette and the sample was slowly applied and allowed to settle into the gel. The sides of the column above the gel were washed with buffer and a one litre reservoir placed on top of the column and filled with tris buffer.

Five ml. aliquots controlled by an electronic volume measuring cell were collected in a fraction collector. (Paton Industries, Beaumont, South Australia). The optical densities

of these fractions were read at a wave length of 280 millimicrons in 1 cm quartz cells in a Shimadzu spectrophotometer. An elution curve, plotting optical density of eluate against tube number, was drawn. Fractions were pooled and concentrated by dialysis against solid sucrose, then against tris buffer and finally concentrated by pressure dialysis, using a membrane thimble (Sartorius Membrane - filter).

3. Immuno - electrophoresis

The method followed in this study is essentially that described by Scheidegger (1955). Bacto-agar (Difco Laboratories, Detroit, Michigan, U.S.A.) was made to 1.6% gel with distilled water. This stock gel was maintained at 4^{o}c until required (1 in 10,000 parts of merthiolate was added to prevent contaminating bacterial growth.) For use, the gel was melted and diluted while hot with an equal volume of 0.1 M veronal buffer pH 8.6. The hot solution of gel was pipetted onto 3 inch x 1 inch glass slides, 3 ml per slide. The slides had previously been coated with 0.05% agar. When the gel had set two holes, 1 m.m. in diameter were cut 1½ inches from the end of the slide and ½ inch apart and the sample pipetted into the holes. The gel was then subjected to electrophoresis (50 volts) for 2 hours at room temperature. To prevent the gel drying out at the cathode end, a layer of agar about ½ inch wide was placed on the gel. After electrophoresis a longitudinal trough was

cut midway between the wells and filled with antiserum. The slides were placed overnight in a humid chamber to allow development of precipitin bands.

To preserve a permanent record, the slides were soaked in 0.15 M NaCl for about 5 days with daily changes of the saline; washed with distilled water for about 5 days and then placed in a dessicator to dry out (1 in 10,000 parts of merthiolate was added to the washing solutions to prevent contaminating bacterial growth). The slides were then stained for 15 minutes in amido black 10B and the excess dye removed by repeated washings with washing solution (ethanol, distilled water and glacial acetic acid in the ratios 5:5:1). The slides were finally rinsed with distilled water and dried in the incubator for 30 minutes at 37^{o}c.

4. Ion Exchange Chromatography

The method described by Turner and Rowley (1963) for the fractionation of serum proteins by di-ethyl-amino-ethyl (D.E.A.E.) cellulose ion exchange chromatography was employed in this study.

The D.E.A.E. cellulose was pretreated and washed by the method of Peterson and Sober (1960). The cellulose was packed into a column, 1.2 cm x 50 cm and equilibrated with 0.01M phosphate buffer pH 7.0. Pig serum was dialysed against

a large volume of this phosphate buffer and then 2 mls. of dialysed serum were slowly layered on top of the cellulose. The sample was eluted batchwise using 0.01M, 0.02M, 0.1M and finally 1.0M phosphate buffer.

The eluate from the column was collected in 3 ml. samples using a fraction collector (Paton Industries, Beaumont, South Aust.). These samples were assayed for protein content by measuring the extinction at 280 mμ and an elution pattern drawn from which the contents of the tubes were pooled as indicated on the appropriate graphs.

In the preparation of pig gammaglobulin, carboxy - methyl - cellulose (C.M.C.) was also employed. In this case a similar column was used and the C.M.C. was equilibrated with 0.02 M phosphate buffer, pH 6.0 and packed under 5 lb. pressure. Batchwise elution was carried out using phosphate buffers of the following composition 0.02M, pH 6.0; 0.05 M, pH 6.0; 0.1M, pH 6.2; 0.2M, pH 6.4; 0.4M, pH 6.6; 0.06M, pH 6.8 and 0.8M, pH 7.0. The resultant eluate was treated as previously described.

5. Density gradient ultracentrifugation

The separation of proteins into high and low molecular weight components has been described by Kunkel (1960). This method was employed in the present study.

Sucrose gradients with concentrations of 40, 35, 25 and 10 per cent sucrose in 4 M NaCl were prepared in ½ inch x 2 inch Beckman cellulose nitrate tubes. One ml aliquots of each of these concentrations were layered in the tubes starting with the least concentrated and injecting the next most concentrated aliquot under it. The solutions were then allowed to equilibrate for 24 hours at 4°c after which time each tube was layered with 1 ml of the serum sample. The tubes were assembled in a SW/39 swing-out rotor and centrifuged for 18 hours at 35,000 r.p.m. in a Spinco (Model L) centrifuge. After this time the sample was removed from each tube by dropwise bleeding. Aliquots of 4 drops were collected and diluted with 3 ml of 0.15 M NaCl to facilitate the assay of protein concentration by optical density measurement as previously described.

6. Preparative Zonal gel electrophoresis

In zonal electrophoresis for preparation of IgM macroglobulin and IgG from pig serum, the method of Smithies (1959) was employed using veronal buffer pH 8.4. The polyvinyl chloride (P.V.C.) block used in the present study measured 50 cm x 12 cm. Five ml. of sample was applied dropwise with a Pasteur pipette and 300v was passed through the block for 40 hours. This was carried out at 4°c. To extract the protein fractions after electrophoresis, 1 cm. wide sections

of P.V.C. were cut starting from the point of application. These were removed from the block using a specially shaped spatula and placed in numbered tubes. To each tube was added 5 ml. of veronal buffer and the tubes were carefully shaken to ensure all the P.V.C. was uniformly suspended. To extract the protein, a quickfit sidearm tube was used with a small scintered glass filter funnel. The sample was placed in the funnel and a vacuum was applied. The P.V.C. fractions were retained and after analysis of the protein content of the extracts a second washing was carried out on those fractions containing protein.

7. Quantitative assay of Protein content

Protein concentrations were determined by the Folin - Ciocalteau Method (Kabat and Mayer 1961) using dialysed pig serum as a standard.

8. Production of antisera

The schedule for production of the various antisera used in this study was essentially that described by Chase (1967).

Adult rabbits were injected with 3.0 mls of the appropriate protein plus 3.0 mls of Freund's complete adjuvant as follows. Two ml intra-peritoneally, a total of 2 mls subcutaneously at 5 different sites and 2 mls intra-muscularly (0.5 ml in each leg).

The animals were test bled 14 days later, given 2 mls of antigen intravenously and bled after a further 14 day interval.

Antisera was tested by immunoelectrophoresis and stored in the deep freeze.

9. Iodination of Pig Macroglobulin.

Macroglobulin (0.6 mgm/ml) was iodinated with I^{131} by the method of Hunter and Greenwood (1962) using chloramine T as an oxidizing agent. Specific activity of the labelled macroglobulin was 4.5 uc/mgm of protein.

CHAPTER III

ISOLATION OF IMMUNOGLOBULINS AND PREPARATION OF THEIR HEAVY CHAINS.

Preparation of normal Pig gamma globulin (IgG)

Two methods for preparation of pig gamma globulin were used in this study.

Method 1: Solid ammonium sulphate was added to 50 ml of normal pig serum to a final concentration of 50% weight to volume. The resultant precipitate was removed by centrifugation re-suspended in 0.1M phosphate buffer at pH 7.0, and then dialysed overnight against 2 litres of 0.01M phosphate buffer pH 7.0. 7 mls were placed on a D.E.A.E. column equilibrated with the same buffer. Elution of the sample was carried out as previously described (Page 30) under the heading "Ion Exchange Chromotography" and fractions were collected and optical densities read at 280 mu.

Two major peaks (Fig. 3.1) were observed and these were pooled separately. Both fractions were treated with solid ammonium sulphate to a concentration of 50% to precipitate the gamma globulins present in solution. The precipitates were spun down and re-suspended in 5 ml of physiological saline. The samples were then dialysed against saline for 5 hours.

Figure 3.1. Preparation of normal Pig gamma globulin.
The elution profiles of partially purified pig Serum (50% ammonium sulphate precipitate of 7 mls of pig serum) from a column of D.E.A.E. cellulose.
Buffer 0.01M phosphate pH 7.0
Column dimensions: 1.2 cm x 50 cm.

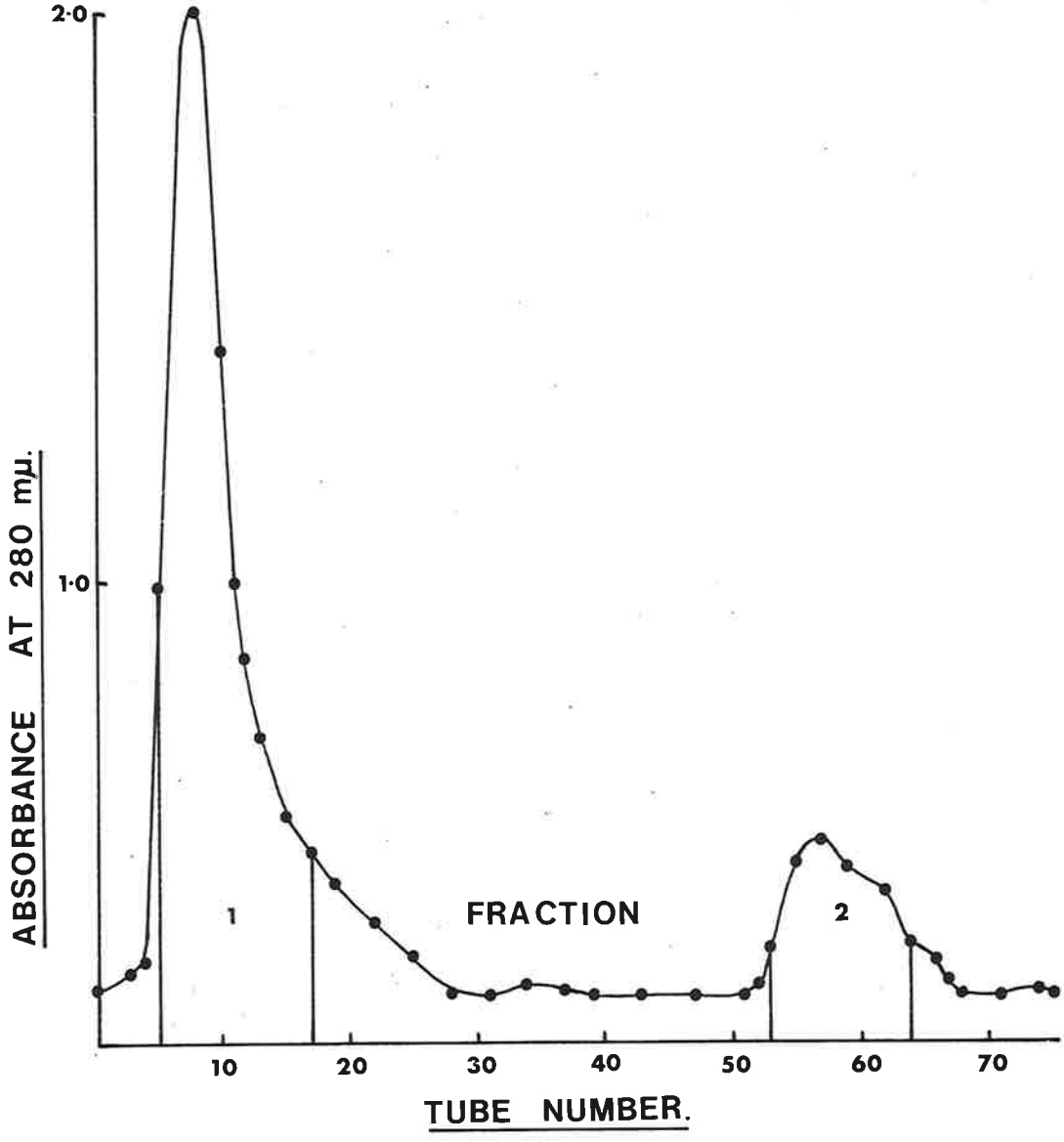
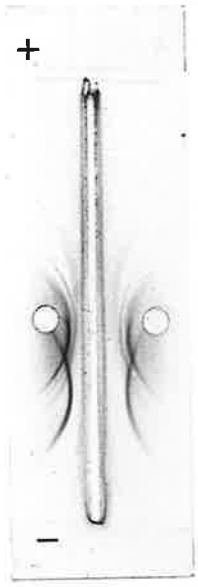

2·0
1·0
ABSORBANCE AT 280 mμ.
1
FRACTION
2
10
20
30
40
50
60
70
TUBE NUMBER.

Figure 3.2. Preparation of normal Pig gamma globulin.

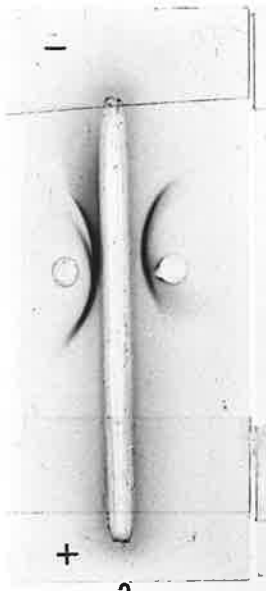
1. Precipitate from 50% ammonium sulphate solution in both wells, against rabbit anti pig serum.
2. Supernatant from same solution in both wells, against rabbit anti pig serum.
3. and 4.

   Fractions from C.M.C. column against rabbit anti pig serum. Wells from left to right correspond to fractions I, II, III and IV, (Fig. 3.3).

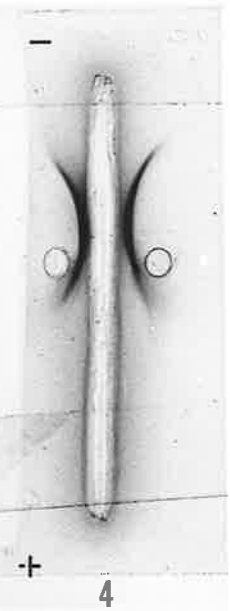


1

2



3



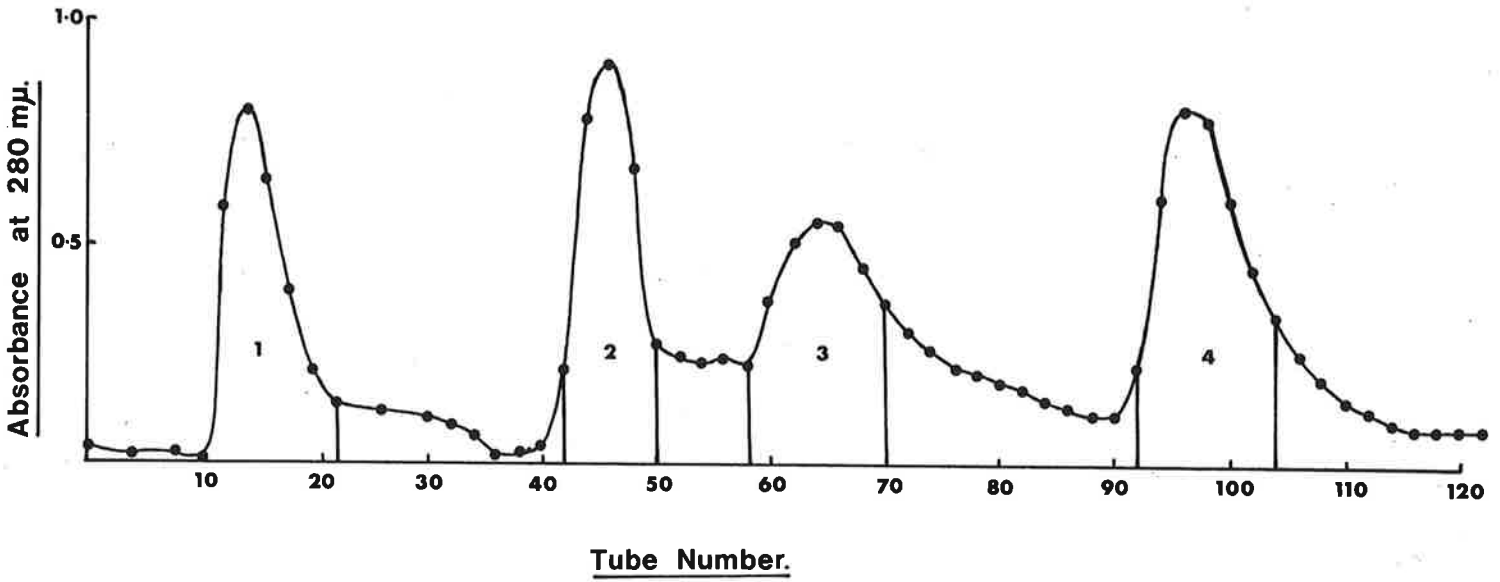
4

Figure 3.3. Preparation of normal Pig gamma globulin.

The elution profiles of pig serum, partially purified by passage through D.E.A.E. cellulose (pool of fractions from Peak I in Fig. 3.1) from a column of carboxymethyl cellulose. The C.M.C. was equilibrated with 0.02 M phosphate butter, pH 6.0.

Elution was batchwise using phosphate buffers of the following composition 0.02 M, pH 6.0; 0.05 M, pH 6.0; 0.1 M, pH 6.2; 0.2 M, pH 6.4; 0.4 M, pH 6.6; 0.6 M pH 6.8; 0.8 M, pH 7.0.

Column dimensions: 1.2 cm x 50 cm.


1·0
0·5
Absorbance at 280 mμ.
1
2
3
4
10
20
30
40
50
60
70
80
90
100
110
120
Tube Number.

Immunoelectrophoresis of both fractions were carried out against rabbit - anti whole pig serum (Fig. 3.2.).

To purify fraction I obtained from the D.E.A.E. column, 3 ml of the solution which had been dialysed against saline was dialysed against 2 litres of 0.02 M phosphate butter pH 6.0 for 2 hours.

This sample was then applied to a carboxy-methyl cellulose (C.M.C.) column and eluted batchwise as previously described (Page 30). The resultant fractions (Fig. 3.3) were pooled, and each pool concentrated separately against solid sucrose and then dialysed against saline.

Immunoelectrophoresis was carried out on fractions thus obtained against rabbit - anti whole pig serum. Fraction I showed a line of contamination and was discarded whereas Fractions II, III and IV showed one IgG precipitin band. These fractions were pooled and concentrated by pressure dialysis.

The pure IgG was frozen and stored and subsequently used as an immunogen and in the prepatation of heavy (γ) chains.

Method 2: After the IgG had been fractionated on D.E.A.E. as in method 1, the following procedure was carried out as an alternate method of purification. Fraction I (Fig.3.1) which contained 37 mgm of protein was pooled and dialysed against veronal buffer pH 8.4 overnight, concentrated to a volume of 5 ml and applied to a P.V.C. block. The sample was subjected to horizontal electrophoresis for 40 hours. After this time the gel was sliced into fractions, the protein eluted as described and the protein content estimated by the Folin-Ciocalteau method previously described. The resultant protein concentrations were plotted against fraction number (Fig. 3.4). The four fractions indicated were pooled separately and concentrated by pressure dialysis.

The total amount of protein in each fraction was determined. Fractions I and III were found to form a single line on immunoelectrophoresis against rabbit - anti whole pig serum and were therefore pooled for use as immunogens. A total of 19.1 mgm of pure gamma globulin was obtained.

Preparation of Pig Macroglobulin (IgM)

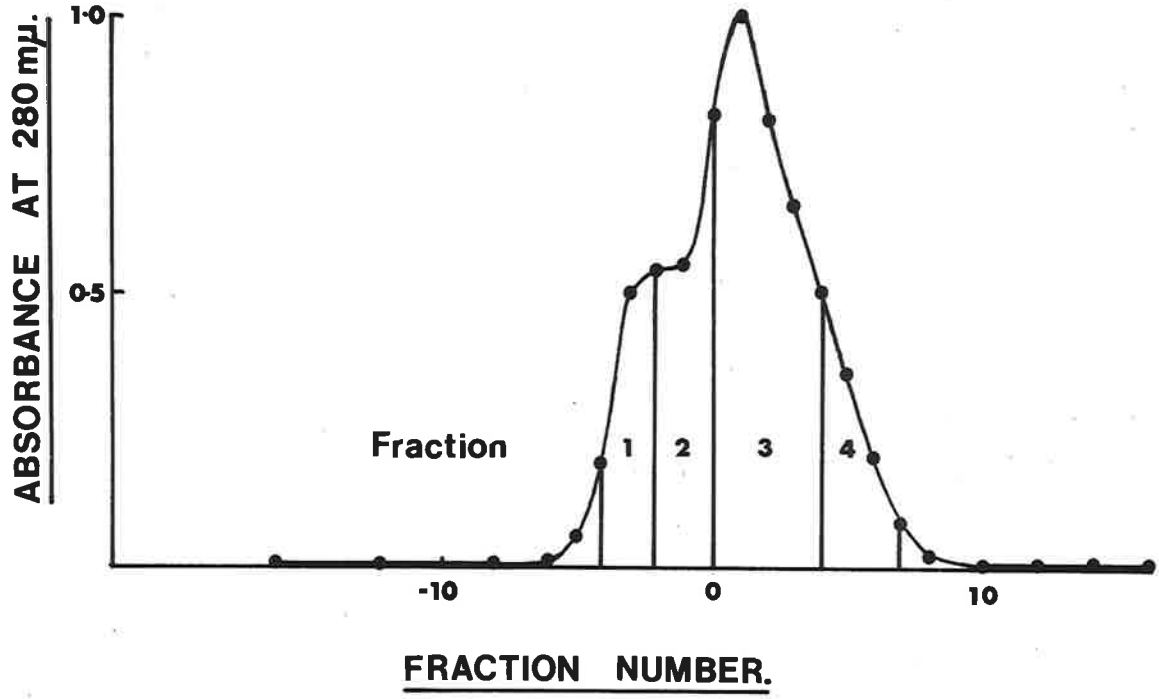
Two different methods were used to prepare pig IgM from normal pig serum. The methods are essentially the same, differing only in the first step.

Figure 3.4. Preparation of normal Pig gamma globulin.

P.V.C. zone electrophoresis of pig gamma globulin, partially purified by passage through D.E.A.E. cellulose. (pool of fractions from Peak I in Fig. 3.1).

Method of Smithies (1959) was employed using Veronal buffer, pH 8.4.

Protein content of fractions was measured using the Folin - Ciocalteau method (Kabat and Mayer, 1961).


ABSORBANCE AT 280 mμ.
1·0
0·5
Fraction
1
2
3
4
-10
0
10
FRACTION NUMBER.

Method 1: Pig serum was spun in the Spinco Model L centrifuge for 4 hours at 40,000 r.p.m. and the resultant clear pellet which formed on the bottom of the tubes was suspended in tris buffer.

Method 2: This method involved the precipitation by 50% ammonium sulphate of the globulin fraction of the serum. This was carried out at 4°c by adding small amounts of solid ammonium sulphate to the serum with continual stirring. The precipitate thus obtained was then resuspended in a minimal volume of tris buffer. This protein solution was dialysed for two days against running tap water, resulting in the precipitation of the desired euglobulin fraction. This precipitate was isolated by spinning a 3,000 r.p.m. for 30 minutes. This precipitate was resuspended in 10 ml of tris buffer. However, resuspension of these precipitates proved very difficult and resulted in a considerable loss of material. The precipitated globulins were then fractionated by passage through a G-200 Sephadex column (4 cm x 50 cm) in tris buffer This procedure yielded 2 major fractions shown in Fig. 3.5.

Immuno electrophoresis studies of this protein solution showed it to be a complex mixture of proteins. The fractions in peak 1. (Fig. 3.5) were pooled and subjected to ultracentrifugal analysis. This pool was found to consist of

Figure 3.5. Preparation of normal Pig macroglobulin.

The elution pattern off G-200 Sephadex of the protein sample obtained by re-suspension of the precipitate resulting from adding ammonium sulphate to pig serum.

Column dimensions : 4 cm x 50 cm.

Eluting solvent : Tris buffer pH 8.0.

Flow rate : 4 ml per hour

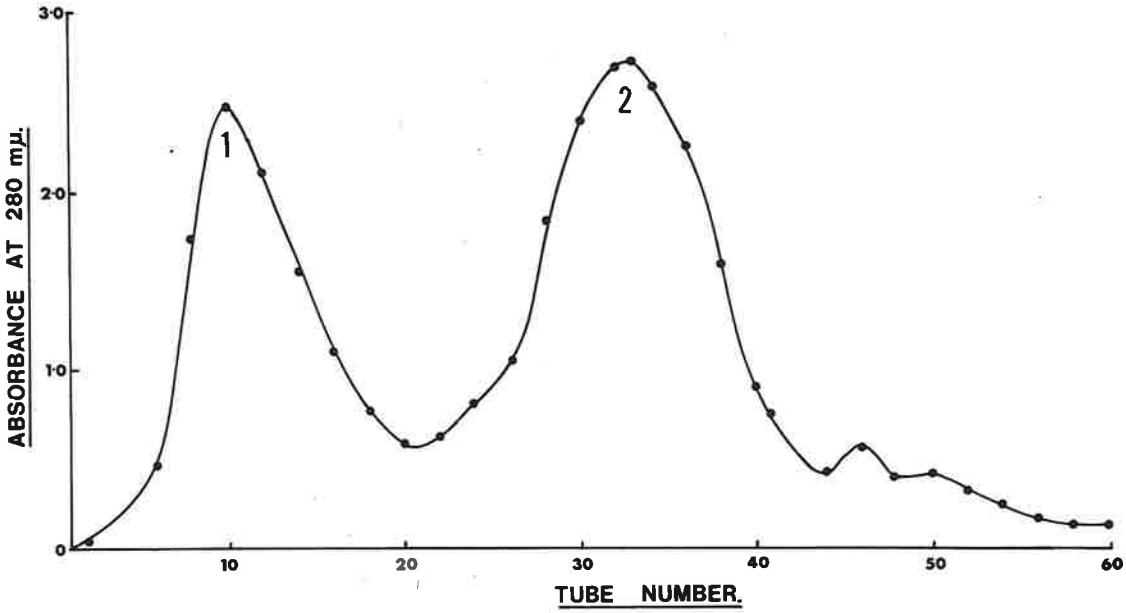

3·0
2·0
1·0
0
ABSORBANCE AT 280 mμ.
1
2
10
20
30
40
50
60
TUBE NUMBER.

Figure 3.6. Preparation of normal Pig macroglobulin

The elution pattern of the protein sample obtained by resuspension of the clear pellet formed by ultracentrifugation, from a column of Sephadex G-200 equilibrated with tris buffer.

Column dimensions : 4 cm x 50 cm.

Flow rate : 4 ml per hour.

Peak 1 : 19 S material

Peak 2 : lower molecular weight material

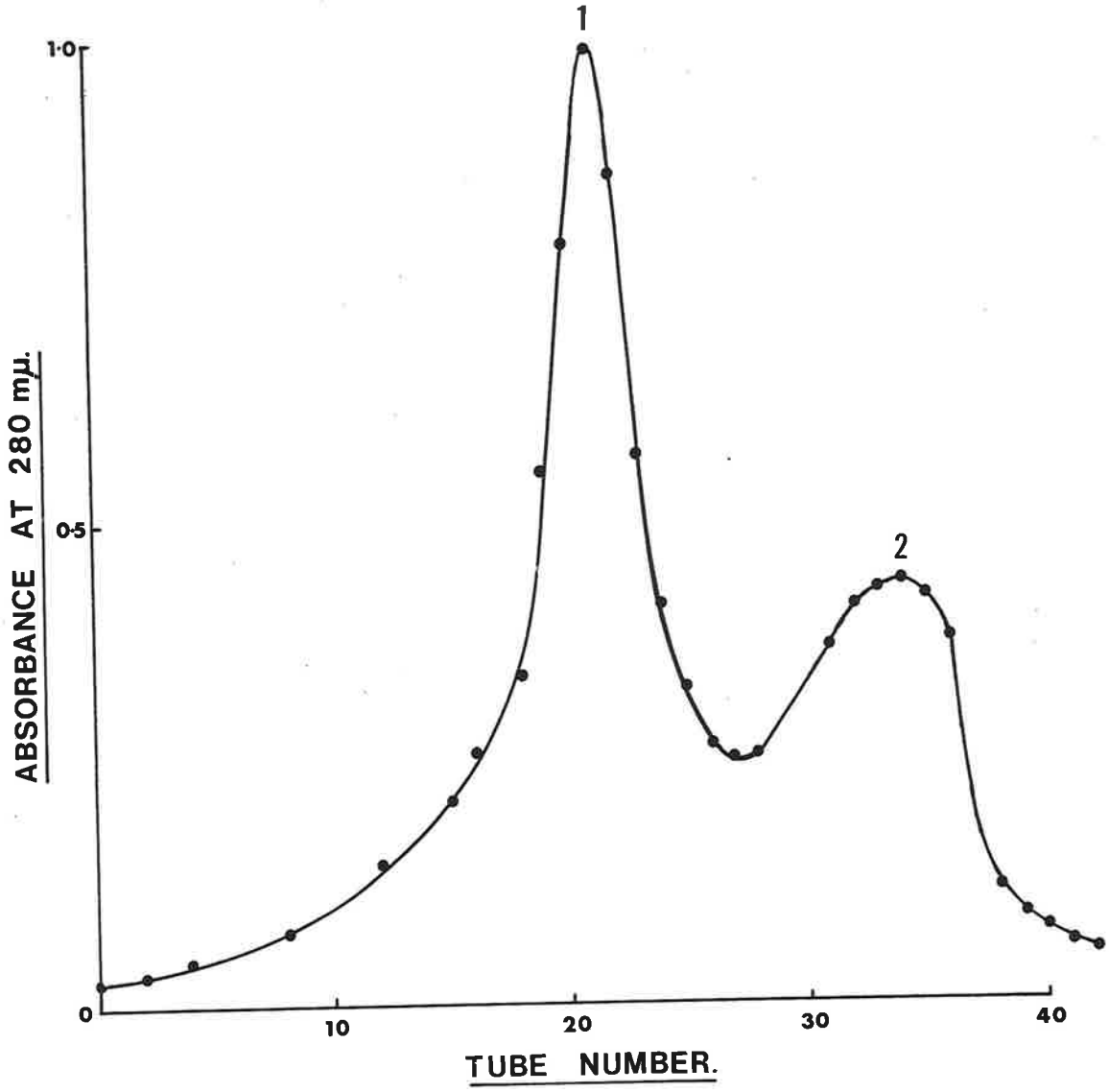

1
2
1·0
0·5
0
10
20
30
40
ABSORBANCE AT 280 mμ.
TUBE NUMBER.

Figure 3. 7. Preparation of normal Pig macroglobulin. Sucrose density gradient profile of pig macroglobulin sample. Sedimentation is from right to left and Peak I represents the 29 S component.
This sample is that obtained from the fractions pooled after elution from G-200 Sephadex (page 39).

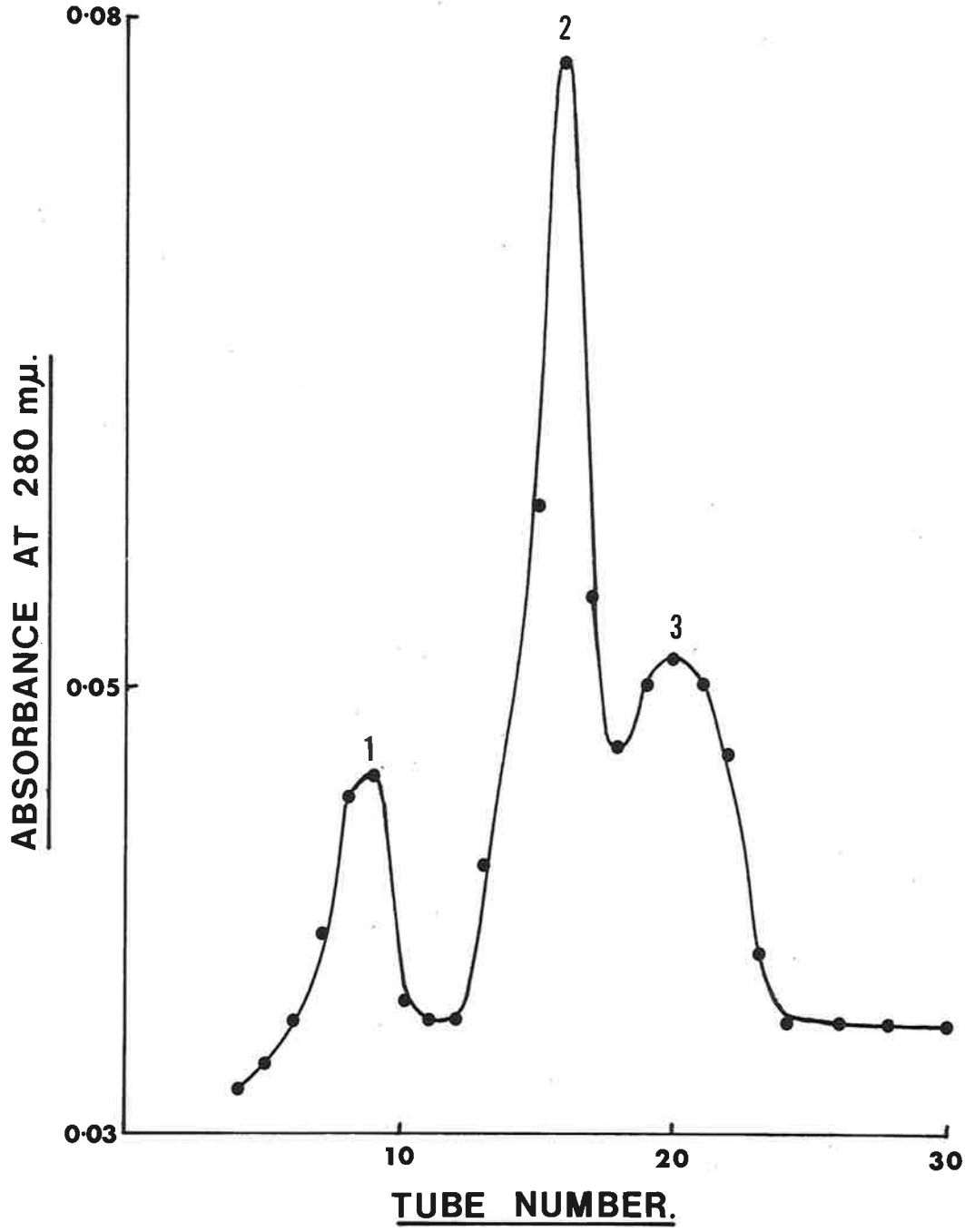

0·08
0·05
0·03
ABSORBANCE AT 280 mμ.
1
2
3
10
20
30
TUBE NUMBER.

Figure 3.8. Preparation of normal Pig macroglobulin. Sucrose density profile of pig macroglobulin sample after recycling to remove high and low molecular weight contaminants leaving the 19 S material. This sample is that obtained by pooling the fractions constituting peak 1 from Fig. 3.7 as described on page 39.

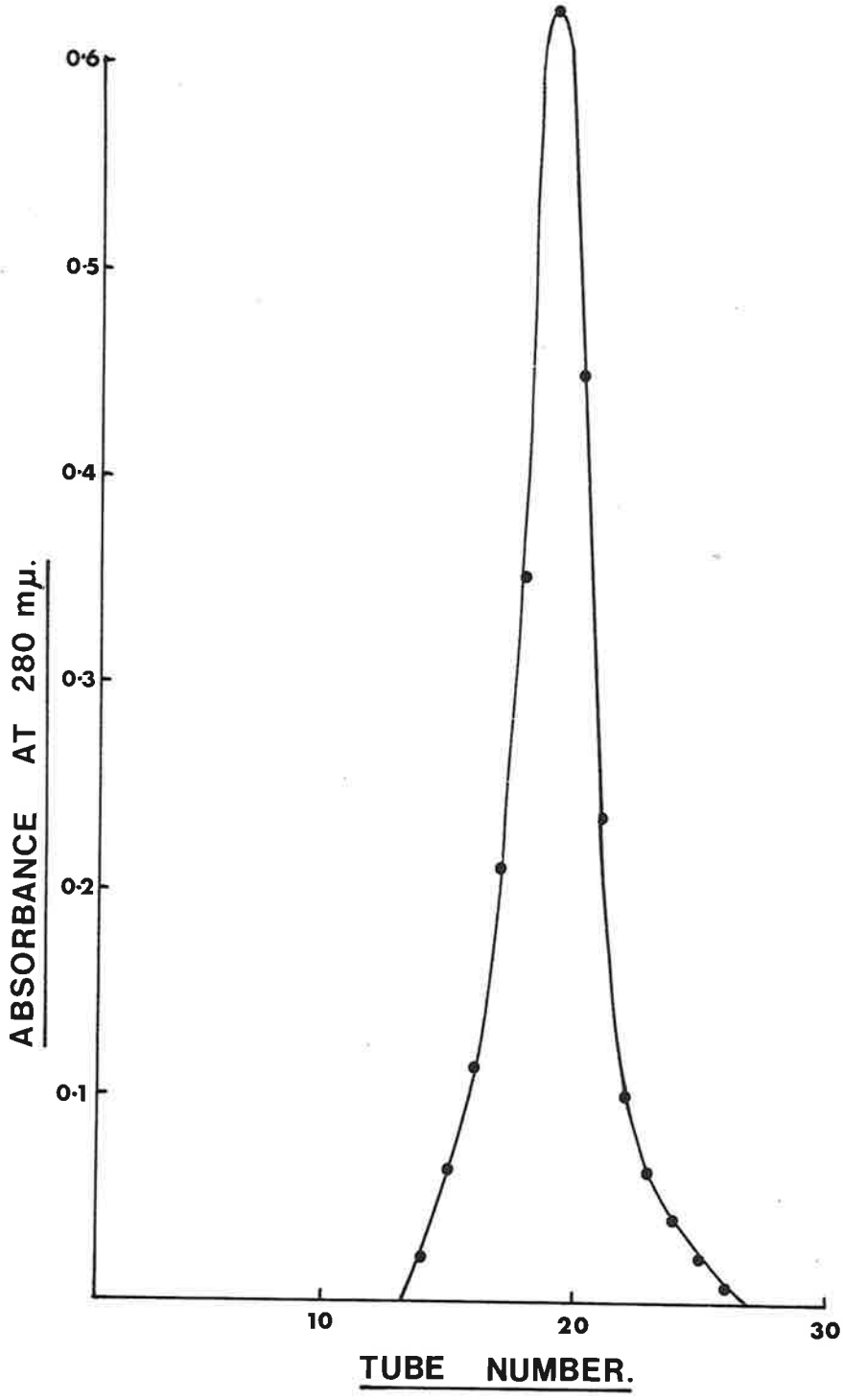
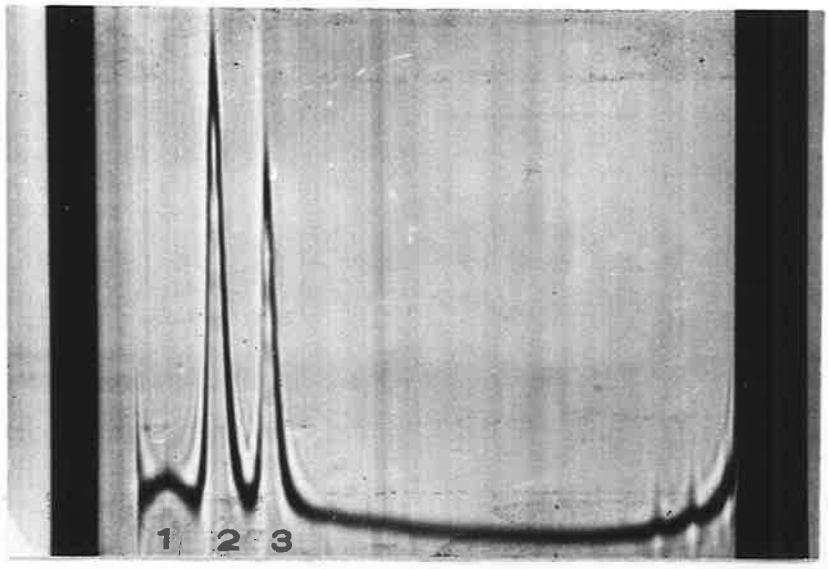

0·6
0·5
0·4
0·3
0·2
0·1
ABSORBANCE AT 280 mμ.
10
20
30
TUBE NUMBER.

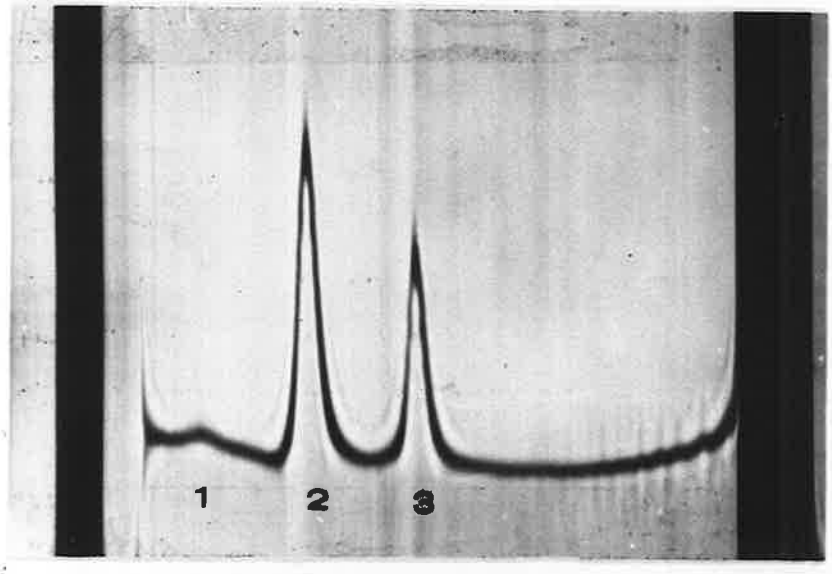
Figure 3.9. Ultracentrifugation pattern of normal pig serum pellet obtained by Method 1, page 38. Protein concentration of 7.7 mgm/ml.; photographs taken 7 and 19 minutes after reaching maximum speed of 50,740 r.p.m.; solvent tris - HCl. Sedimentation proceeded from left to right.

Peak 1. : S 20 = 5.95 : 4% of total protein.

Peak 2. : S 20 = 14.35 : 60% of total protein.

Peak 3. : S 20 = 29.55 : 36% of total protein.


1 2 3


1 2 3

3 main components, similar to those obtained by Method I and shown in Fig. 3.9.

This heterogeneous mixture of proteins was next applied to a G-200 Sephadex column which was equilibrated with tris buffer. The protein was eluted from the column using tris buffer. The resulting elution pattern (Fig. 3.6) showed that the protein was eluted in two major fractions. The fractions under the leading peak (Peak I) were pooled, concentrated by dialysis against solid sucrose, redialysed against tris buffer and then concentrated to a smaller volume by negative pressure dialysis. Ultracentrifugal analysis and examination of this solution by immuno-electrophoresis was carried out at this stage (Figs. 3.9 and 3.10). The slower moving components (Peak I) observed on ultracentrifugal analysis (Fig. 3.9) represent lower molecular weight contaminants and these were removed by running the sample overnight on a sucrose density gradient. This was carried out as previously described using the SW 39 rotor in the Spinco Model L ultracentrifuge and spinning for 18 hours at 35,000 r.p.m. A study of the resulting profile (Fig. 3.7) in most cases clearly showed the presence of a component of high molecular weight (Fraction 1), a main peak (Fraction 2) containing the required 19 S component and a slower moving peak (Fraction 3) comprising the lower molecular weight material. By

recycling the 19 S component (Fraction 2) on sucrose density gradients it was possible to obtain a pure sample of 19 S macroglobulin (Fig. 3.8). When this 19 S material was analysed by ultracentrifugation it formed a single peak, but immunoelectrophoresis showed the presence of two electrophoretically differing components (Fig. 3.10). One precipitin line represented the required IgM component, the other α_2M. The best method available to separate components of similar molecular weight but different electrophoretic mobility in adequate quantities is horizontal electrophoresis on a P.V.C. block. Protein concentration was plotted against Fraction number and the resulting profile is shown in Fig. 3.11. Fractions in tube numbers 9 to 17 were pooled and concentrated by dialysis against solid sucrose. The concentrated sample was examined by electrophoresis (Fig. 3.10).

This pure sample of IgM was stored at 4°c and subsequently used for the preparation of pure heavy chains.

Preparation of γ chains by reduction of Pig Gamma globulin.

Pure pig IgG was dispersed into its free chains by using 0.1M 2 mercaptoethanol to reduce the interchain disulphide bonds (Edelman and Poulik, 1961). IgG was dialysed against tris buffer for 3 hours at 4°c and the volume of dialysate was then reduced to 1.4 ml by pressure dialysis. 0.6 ml of 2 mercaptoethanol (1.16 ml of stock 2 mercaptoethanol per

Figure 3.10. Preparation of Pig macroglobulin.

1. Well a. Protein from passage through G-200 Sephadex column (Fraction I. as in Fig. 3.6.)

   Well b. As above.

   Antiserum in trough : rabbit anti pig serum.

2. Well a. IgM from P.V.C. block

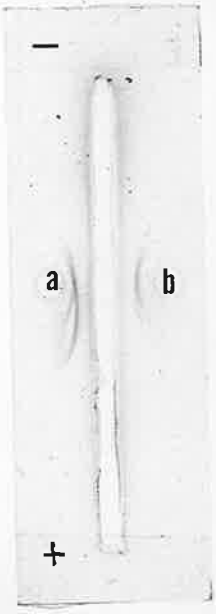
   Well b. As above.

   Antiserum : rabbit anti pig serum

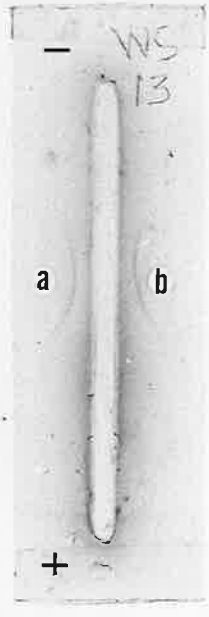
3. Well a. 19 S material from sucrose density gradient.

   Well b. As above

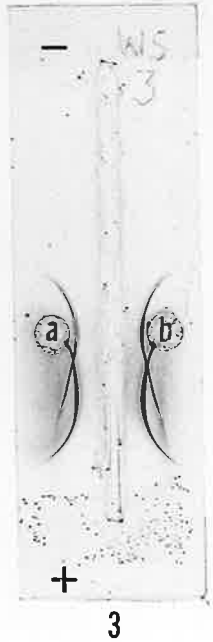
   Antiserum : rabbit anti pig serum.



1



2



3

Figure 3.11. Preparation of pig macroglobulin.

Zone electrophoresis of macroglobulin preparation on P.V.C. block.

Fraction I represents the macroglobulin fraction.

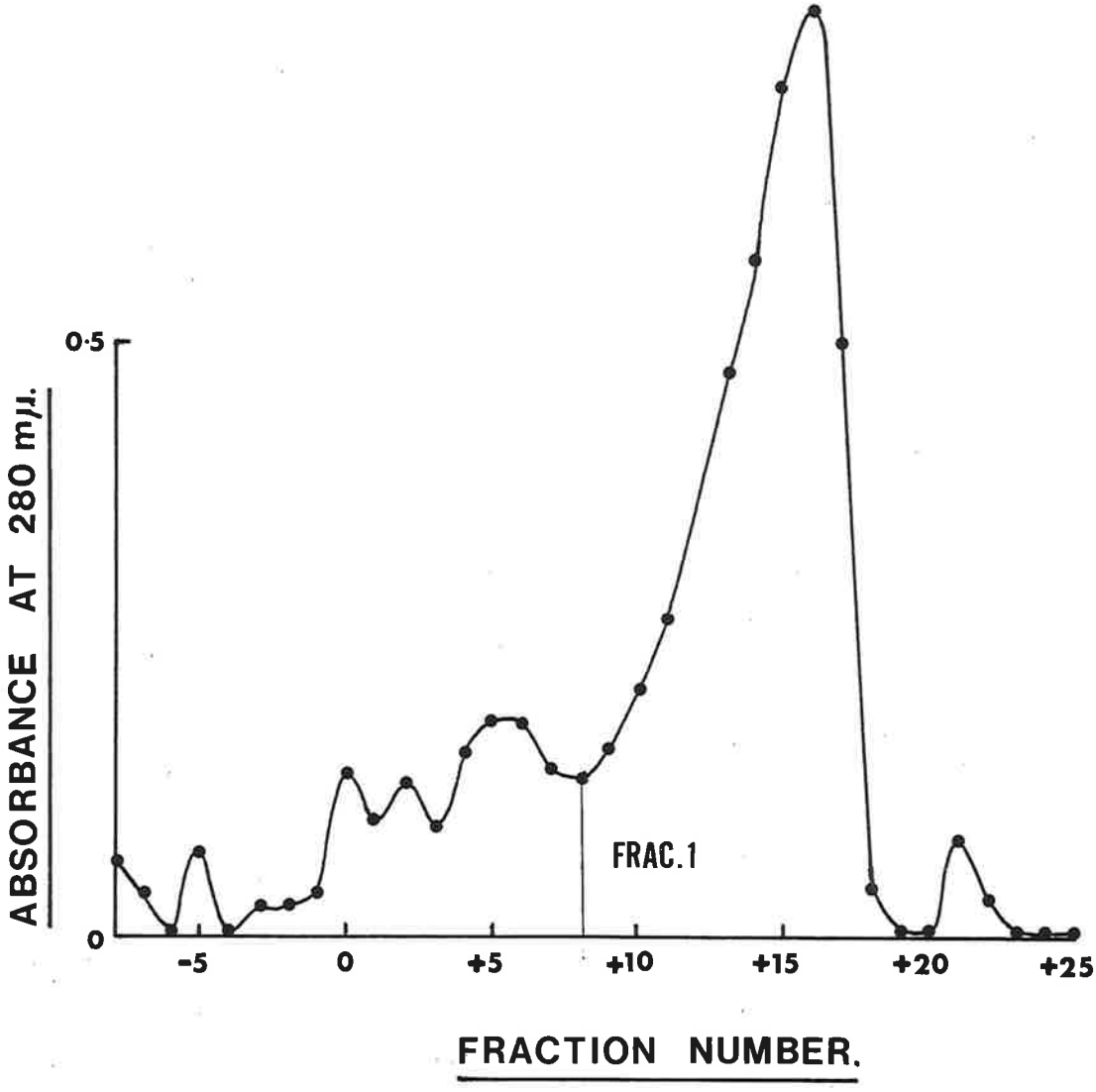

0·5
0
ABSORBANCE AT 280 mμ.
FRAC.1
-5
0
+5
+10
+15
+20
+25
FRACTION NUMBER.

Figure 3.12. Preparation of γ chains by reduction of pig IgG.

The elution profiles of reduced and alkylated protein from a column of Sephadex G-100 equilibrated with 6M urea in 0.05M formic acid.

Fraction I contains the IgG.

Column dimensions : 2cm x 50 cm.

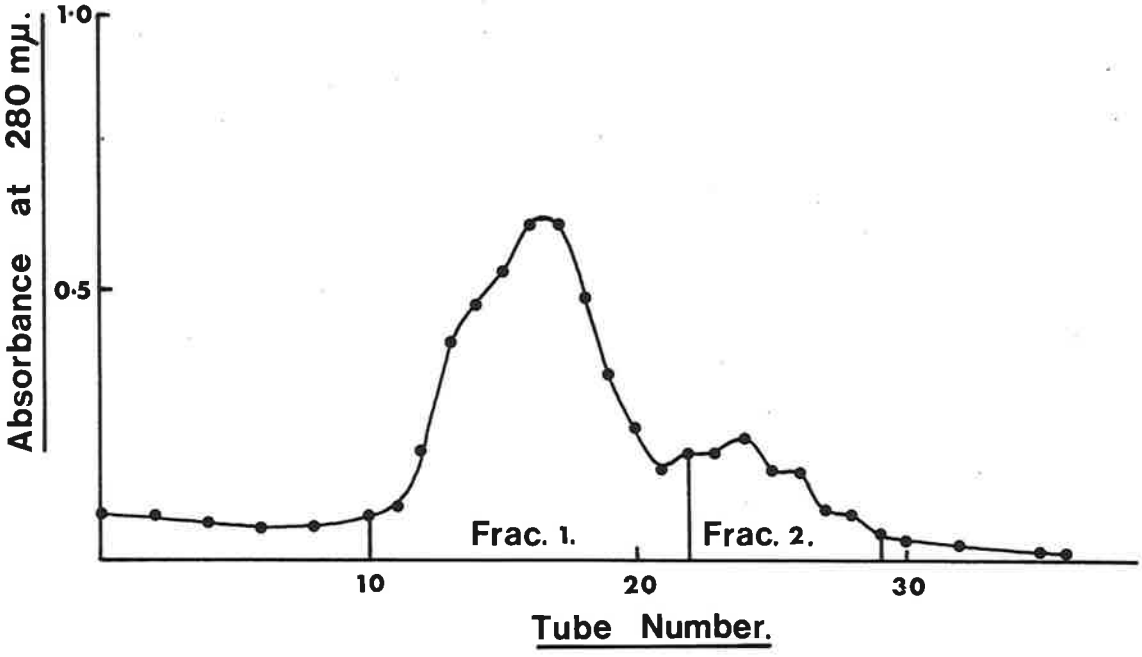

Absorbance at 280 mμ.
1·0
0·5
Frac. 1.
Frac. 2.
10
20
30
Tube Number.

Figure 3.13. Preparation of γ chains by reduction of pig IgG.

The elution profiles of reduced and alkylated protein from a column of Biogel P_2 eluted with ammonium carbonate.

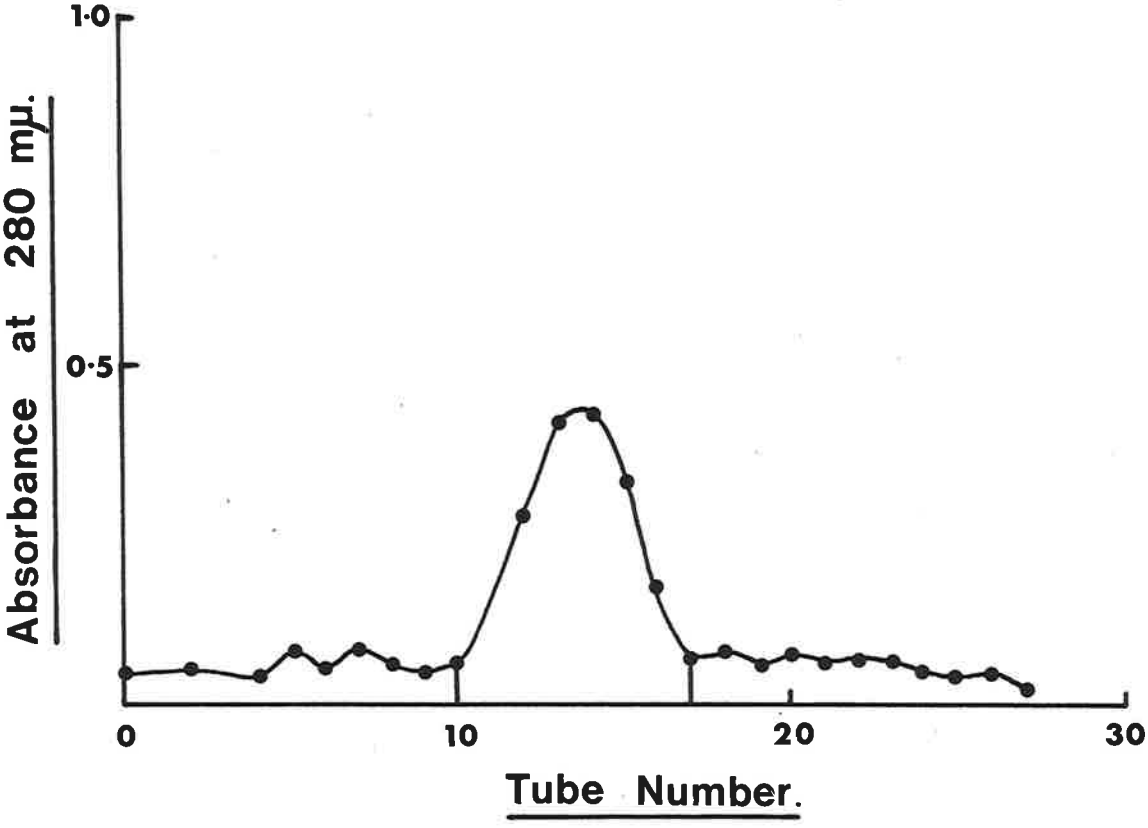
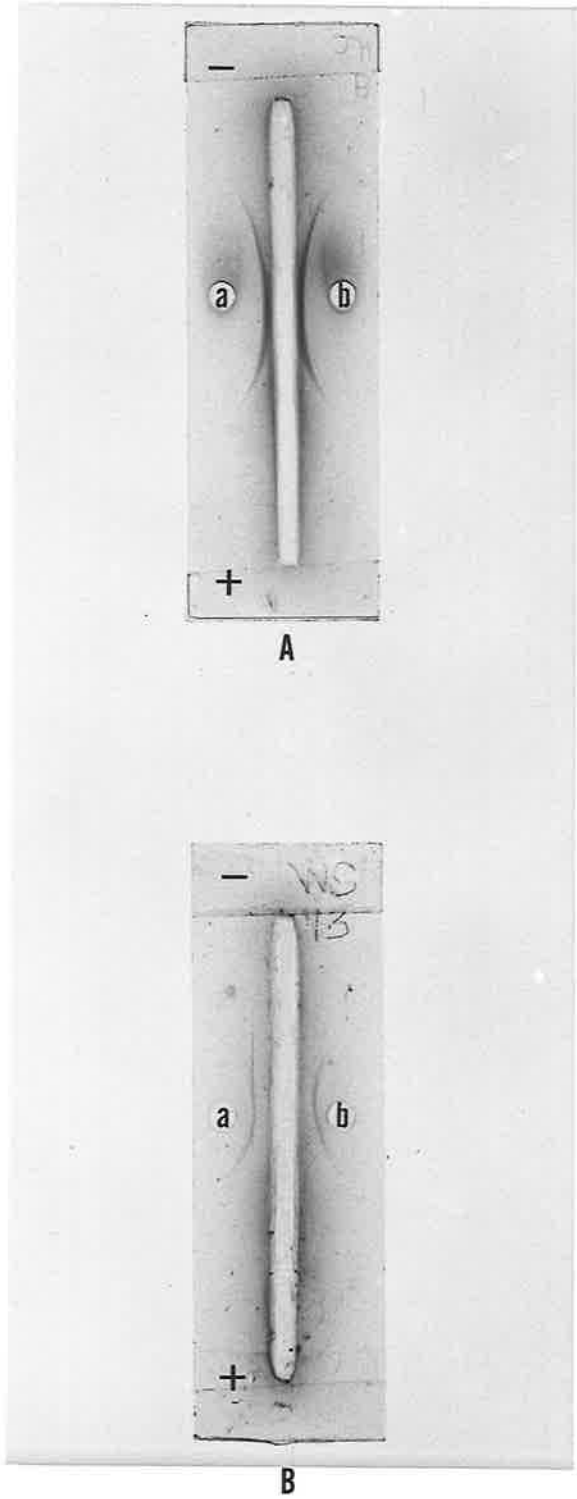

1·0
0·5
Absorbance at 280 mμ.
0
10
20
30
Tube Number.

Figure 3.14. Preparation of γ chains by reduction of pig IgG.

A. Wells a and b : final sample of γ chains.
Trough : rabbit anti pig serum.

Preparation of μ chains by reduction of pig IgM.

B. Wells a and b : final sample of μ chains.
Trough : rabbit anti pig serum.


–
a
b
+
A
–
a
b
+
B

25 ml of tris buffer) was added to the 1.4 ml of protein solution. The mixture was incubated for 1 hour at $37^{o}c$ and then chilled in an ice bath.

To alkylate the resulting free chains, 0.5 ml of 1.1M iodoacetamide (1.02 gm/15 ml tris buffer) was added and the reaction mixture stood for 1 hour in an ice bath. Finally the sample was dialysed overnight against 6M urea, 0.05 M formic acid buffer and applied to a column of G-100 Sephadex (50 cm x 2 cm) to separate the light and heavy chains. The column had previously been equilibrated with 6M urea in 0.05M formic acid. The elution profile is shown in Fig. 3.12. To ascertain the elution characteristics of the column a standard sample of pure, unreduced human gamma globulin was first applied to the column and the tube number in which this protein eluted was noted. The absence of protein in this tube when the reduced pig IgG was eluted confirmed that all the pig IgG had in fact been reduced. Fraction I (Fig. 3.12), which had a total volume of 20 ml was pooled and placed directly on a de-salting column (Biogel P_2) and eluted with ammonium carbonate. The major fraction indicated in Fig. 3.13 was pooled, freeze dried and the resulting solid material was then taken up in 2 ml of borate buffer at pH 8.0 and dialysed overnight at $4^{o}c$ against this buffer. Immunoelectrophoresis (Fig. 3.14) showed this to be a pure sample of γ chains which was subsequently used as an immunogen.

Reduction of IgM to its polypeptide chains.

The first method employed in an attempt to reduce pig IgM was that described by Edelman and Poulik (1961) using 0.2M 2 mercaptoethanol and subsequent alkylation with iodoacetamide. The treated macroglobulin was then dialysed against 1N propionic acid and run on a Sephadex G100 column previously equilibrated with 1N propionic acid. The resultant chromatogram (Fig. 3.15) showed clearly that only a small amount of the 19 S material had been reduced, and then only to fragments which eluted from the column in a position similar to that found for a human gamma globulin marker previously run on the column under similar conditions. These were subunits of pig IgM with a sedimentation coefficient between 7 S and 8 S and have been previously described for other macroglobulins (Suzuki and Deutsch, 1967; Miller and Metzger, 1965).

It was decided that more severe reducing conditions were required for this particular protein, hence reduction and alkylation was carried out as before but in this case using 0.95M 2 mercaptoethanol as described by Cohen (1963). The results obtained (Fig. 3.16) showed that under these conditions almost all of the 19 S material had been reduced but that the major product was still the 7 S subunits (identified using human IgG marker) of IgM with very little dissociation to free chains.

Figure 3.15. Preparation of μ chains by reduction of pig IgM.

The elution profiles of reduced and alkylated pig IgM from a column of Sephadex G 100 equilibrated with 1N propionic acid. (Reduction using 0.2M 2 mercaptoethanol).

Peak 1. : Represents unreduced IgM.

Peak 2. : Represents a small amount of reduced material.

Arrow shows position of human gamma globulin marker previously run on the column under similar conditions.

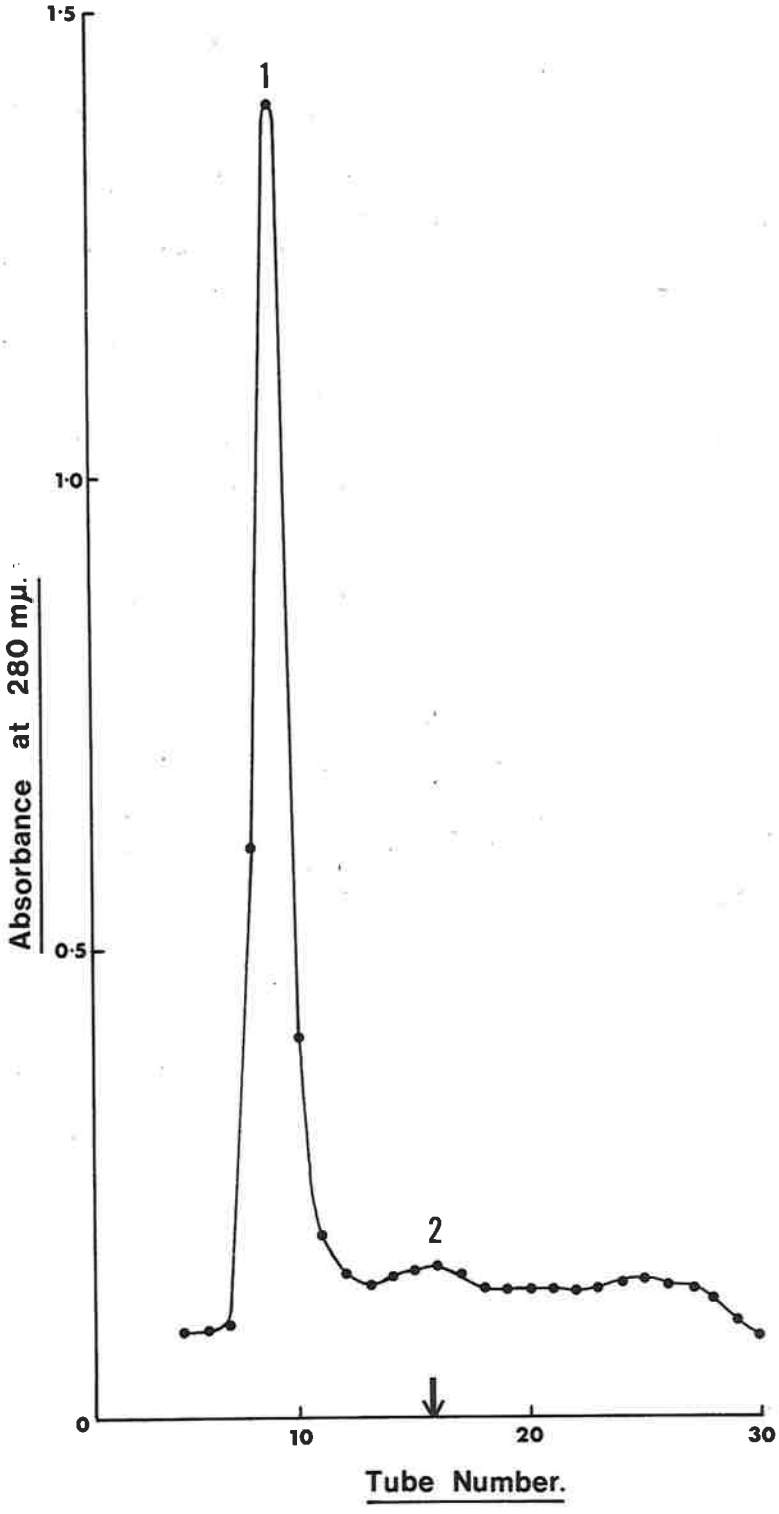

1·5
1·0
0·5
0
Absorbance at 280 mμ.
1
2
10
20
30
Tube Number.

Figure 3.16. Preparation of μ chains by reduction of pig IgM.

The elution profile of reduced and alkylated pig IgM from a column of Sephadex G-100 equilibrated with 1N propionic acid.

(Reduction using 0.95 M 2 mercaptoethanol)

Peak 1. : Unreduced IgM

Peak 2. : Reduced material (approximately 7 S).

Arrow shows position of human gamma globulin marker.

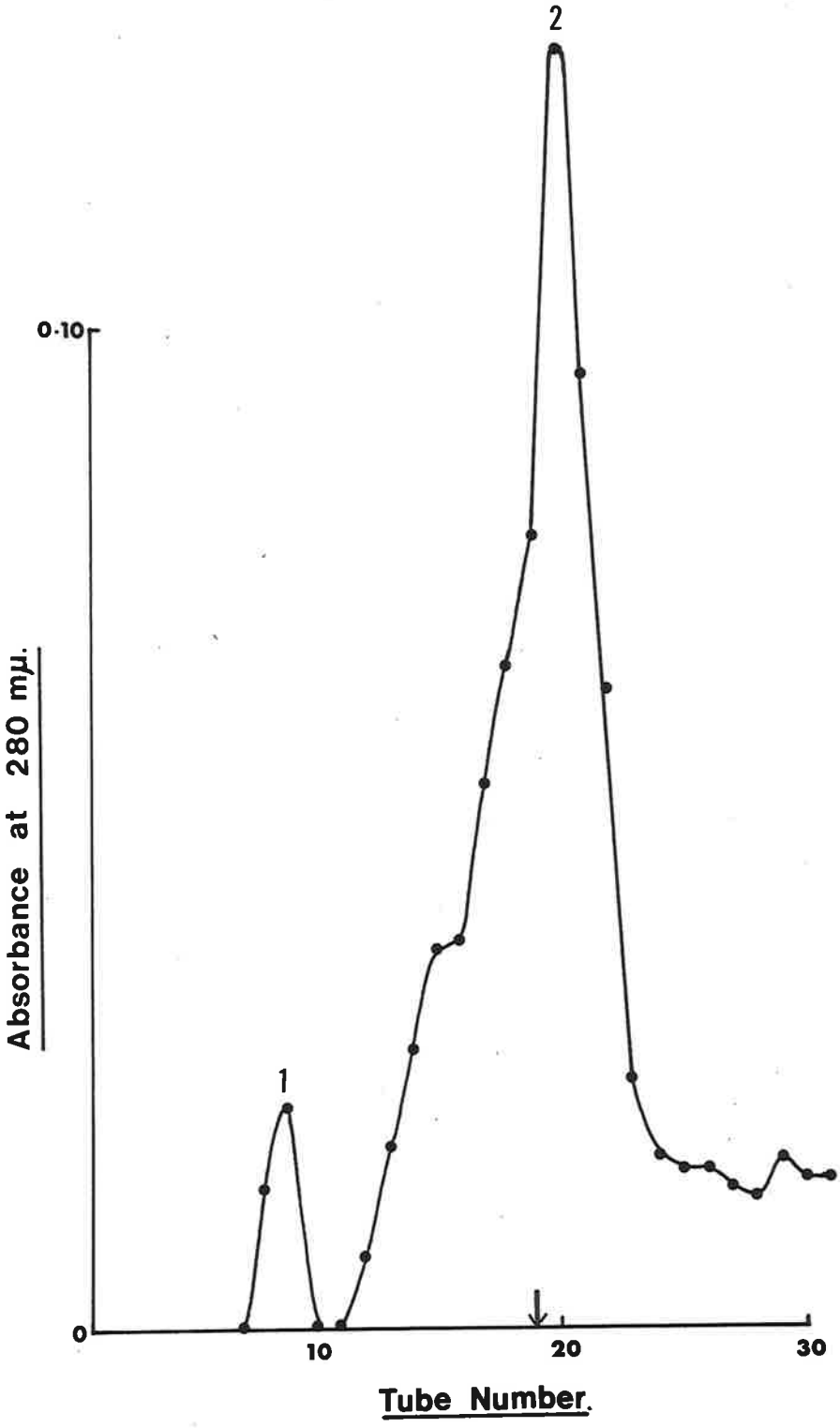
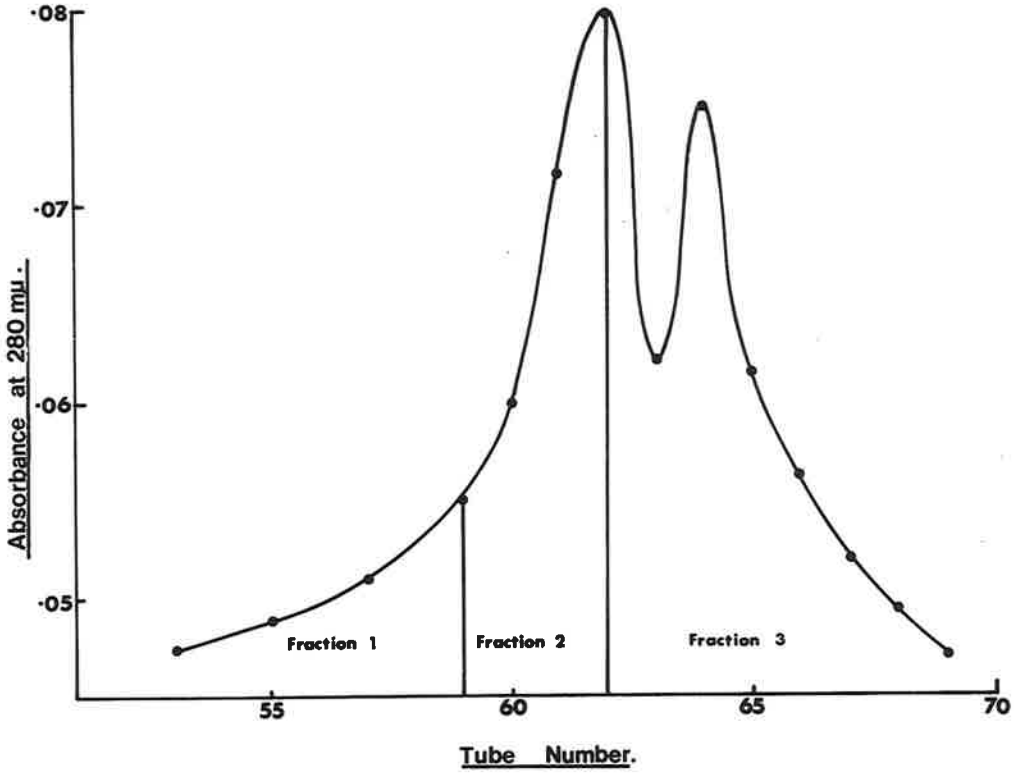

2
0·10
Absorbance at 280 mμ.
1
0
10
20
30
Tube Number.

Figure 3.17. Preparation of μ chains by reduction of Pig IgM.

Elution profile of reduced macroglobulin using the method of Potter, Appela and Geisser (1965) from a column of Sephadex G-200 equilibrated with 6M urea, 0.1 M acetic acid and 0.0014 M 2 mercaptoethanol.

Column dimensions : 3 cm x 100 cm

Fraction 2 represents the μ chains.


·08
·07
·06
·05
Absorbance at 280 mμ.
Fraction 1
Fraction 2
Fraction 3
55
60
65
70
Tube Number.

Finally the method of Potter, Appella and Geisser (1965) was employed. In this instance the macroglobulin solution was concentrated to a volume of 1.4 ml and this was made up to a total volume of 3 ml with 2 mercaptoethanol such that the solution was 0.3 M with respect to mercaptoethanol. Solid guanidine hydrochloride was then added to a final molarity of 7M. The reaction mixture was incubated at 37°c for 1 hour and then chilled in an ice bath. After incubation, recrystallized iodoacetamide was added to a concentration of 1.5M to alkylate and the mixture was stood in an ice bath for 30 minutes.

The mixture was then placed on top of a column of Sephadex G200, 3 cm x 100 cm. The column had been previously equilibrated with 6M urea, 0.1M acetic acid and 0.0014M 2 mercaptoethanol. This same solution was used to wash the mixture through the column. The 2 mercaptoethanol was used in this system to react with the cyanate groups formed by breakdown of urea in acid conditions. The heavy chain fraction (Fraction 2 in Fig. 3.17) was re-run on the column to purify, pooled and concentrated by pressure dialysis. The purity of the preparation was confirmed by immunoelectrophoresis against rabbit anti whole pig serum (Fig. 3.14)

Preparation of high molecular weight (29 S) component of normal pig serum.

Two methods were used in the preparation of the high

molecular weight component in normal pig serum. The methods differ only in the first isolation step.

Method 1.

This method involved the precipitation of the globulin fraction of pig serum by the addition of solid ammonium sulphate, with continual stirring to a final concentration of 50%. This precipitation was carried out at 4^{o}c. The precipitate thus obtained was resuspended in a minimal volume of tris buffer and then dialysed for two days against distilled water to precipitate the desired euglobulin fraction. This precipitate was spun down for 30 mins. at 3,000 r.p.m. and resuspended in tris buffer. Resuspension of these precipitates proved difficult and resulted in considerable loss of material hence the following alternative method of preparation was used.

Method 2.

In this case the required proteins were concentrated on the basis of their high molecular weight. Normal pig serum was spun in the Spinco Model L centrifuge for 4 hours at 40,000 r.p.m. and the resultant clear pellet which formed on the bottom of the tubes was suspended in tris buffer. Immunoelectrophoresis studies on this protein showed it to be a complex mixture of proteins. (Fig. 3.18).

This heterogeneous mixture of proteins was applied to a

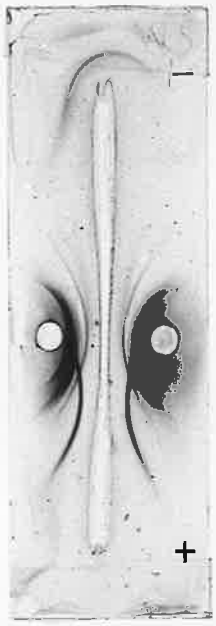
Figure 3.18. Preparation of high molecular weight (29 S) component of normal pig serum.

Slide I. In both wells resuspended protein pellet.

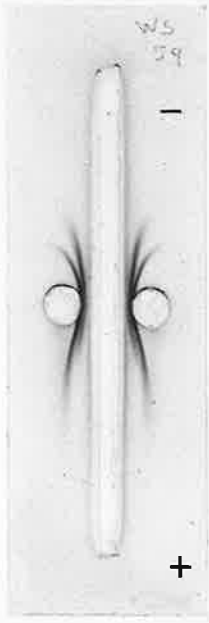
Trough : rabbit anti pig serum.

Slide 2. In both wells : protein solution after passage through G 200 Sephadex column (peak 1).

Trough : rabbit anti pig serum.



1

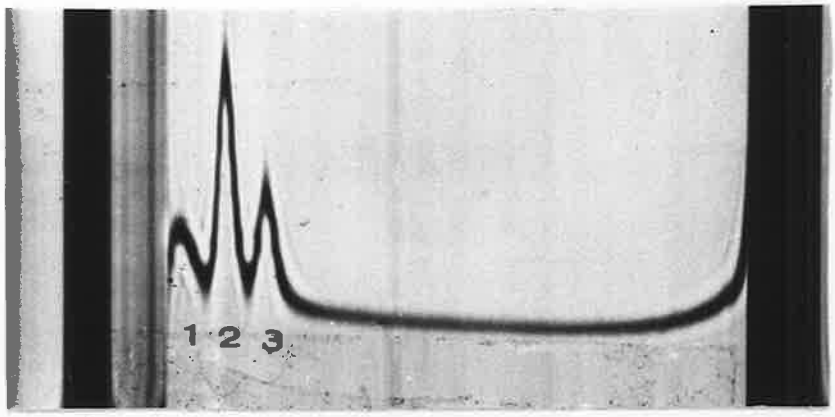


2

Fig. 3.19. Ultracentrifugal pattern of normal pig serum (partially purified). Protein concentration 7.7 mgm/ml. ; photographs taken 7 and 19 minutes after reaching maximum speed of 50,740 r.p.m. ; solvent tris-HCl. Sedimentation proceeds from left to right.

Peak 1.	: S 20 = 5.95	: 4% of total protein.
Peak 2.	: S 20 = 14.35	: 60% of total protein.
Peak 3.	: S 20 = 29.55	: 36% of total protein

(Repeat of Fig. 3.9)


1 2 3

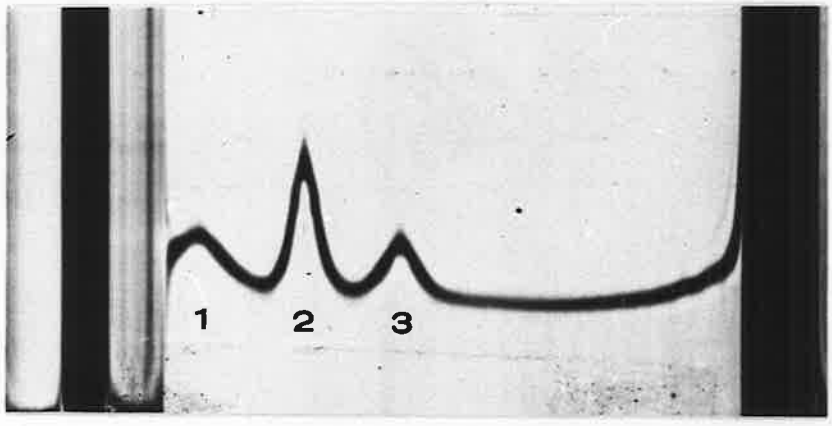
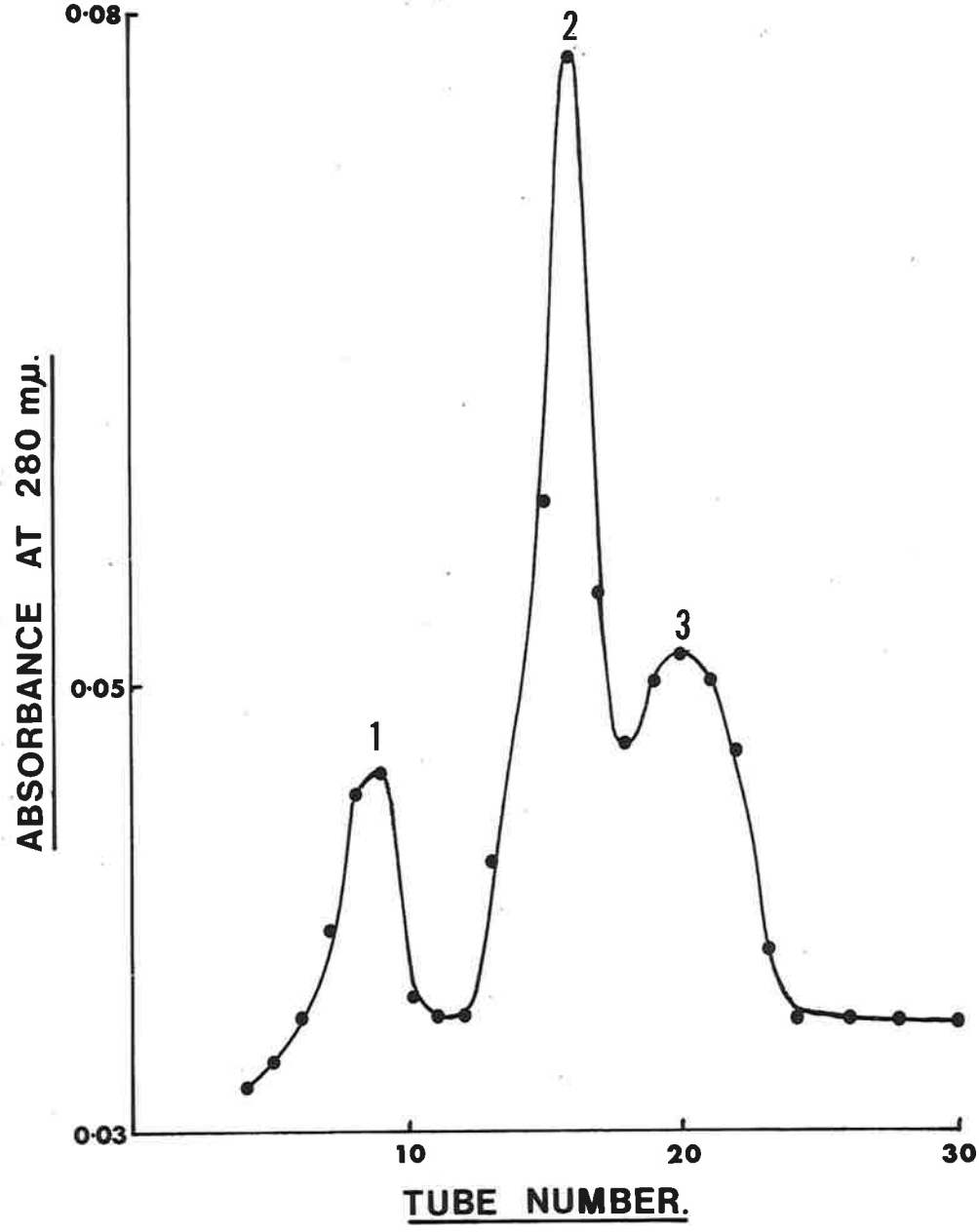

1 2 3

Figure 3.20. Preparation of normal Pig Macroglobulin.

Sucrose density gradient profile of pig macroglobulin sample.

Sedimentation is from right to left.

Peak 1 represents the 29 S material.


0·08
0·05
0·03
ABSORBANCE AT 280 mμ.
1
2
3
10
20
30
TUBE NUMBER.

Sephadex G-200 column which had been equilibrated with tris buffer as described previously. The same buffer was used for the elution of the protein. The resulting elution pattern showed that the protein eluted from the column in two major fractions. (Fig. 3.6) The fractions under the leading peak were pooled, concentrated by dialysis against solid sucrose, redialysed against tris buffer and then concentrated to a smaller volume by negative pressure dialysis.

Ultracentrifugal analysis and examination of the solution by immuno-electrophoresis was carried out at this stage. (Figs. 3.18 and 3.19). The slower moving components observed on ultracentrifugal analysis represent lower molecular weight contaminants and these were removed by separation on a sucrose density gradient. This was carried out as previously described using the SW39 rotor in the Spinco Model L overnight. A study of the resulting graph of optical density against fraction number (Fig. 3.20) in most cases clearly showed the presence of a component of high molecular weight (29 S) as well as the 19 S material and components of even lower molecular weight. This situation was confirmed by ultracentrifugal analysis.

To estimate the sedimentation coefficient of this leading peak, a control tube was included in the Spinco overnight run which contained an identical sucrose gradient overlaid with a protein with a known sedimentation coefficient of 28 S

(Swan mussel haemaglutinin). This was found to sediment in the same position as the leading peak of the partially purified pig serum sample run under identical conditions. By bleeding the tubes dropwise it was possible to remove the lower molecular weight components. It was originally thought that this method would also provide a possible means of separating the 29 S component from the 19 S material and hence obtaining a pure preparation of the 29 S component. However in practice this proved unfeasible because for complete separation of the 29 S component from the 19 S component on sucrose density gradients a very dilute solution of protein had to be used and hence the yield of 29 S material was very low. Thus, although density gradient ultracentrifugation could not be used as a preparative procedure for the 29 S component, the procedure still provided a useful method of analysis and a means of partially purifying the material. During preparation, large quantities (up to 15 ml) of concentrated protein solution which had previously been partially purified by passing through a Sephadex G 200 column were loaded onto many sucrose density gradients and the fraction containing the 29 S and 19 S components obtained free of lower molecular weight material.

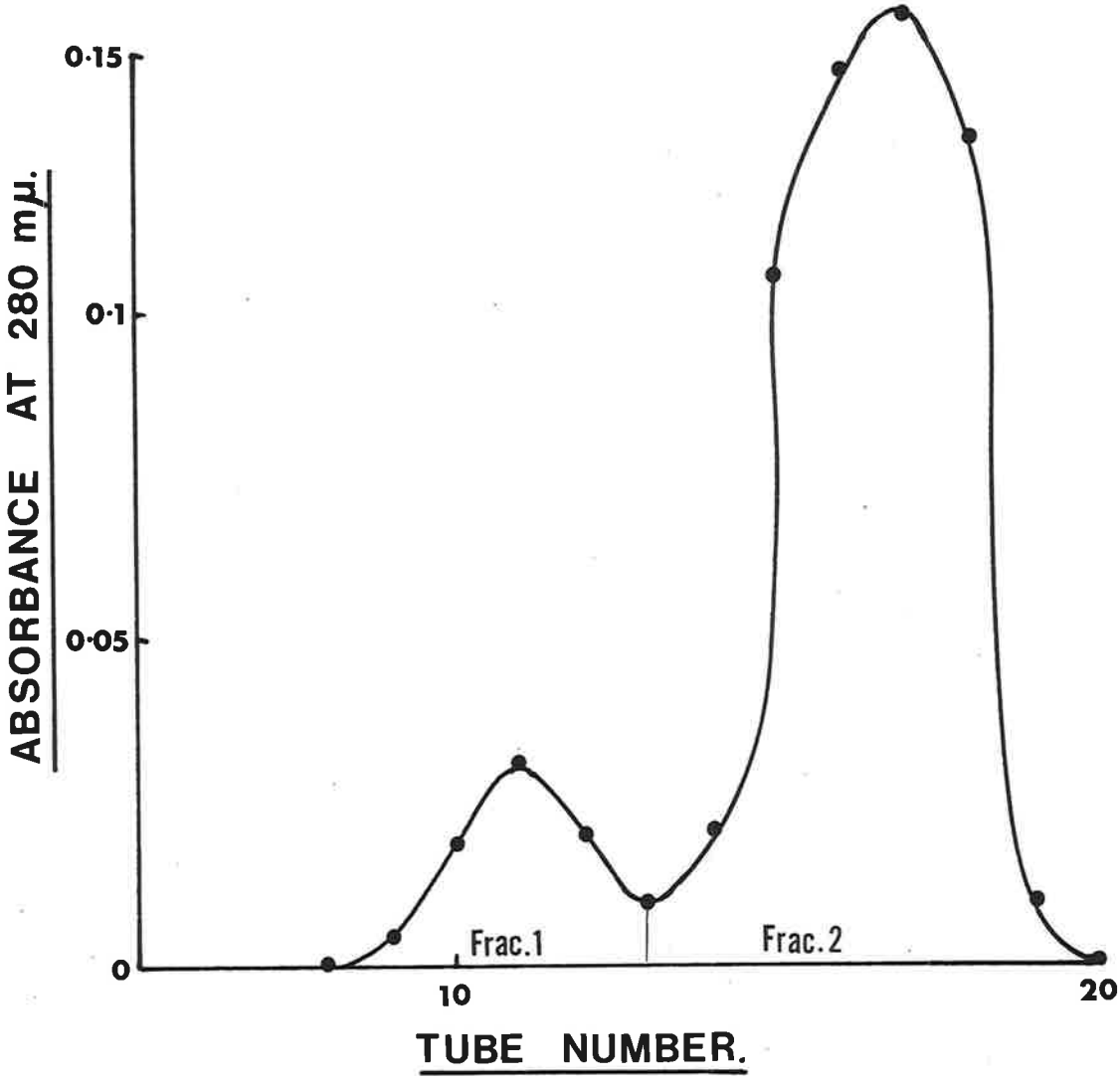
To separate the 29 S component from the 19 S fraction, Sepharose 4B was used. This gel provided an ideal method of separating these two components since it excludes molecules of

Figure 3.21. Preparation of the 29 S component of normal pig serum. The elution pattern of partially purified 19 S and 29 S protein solution from a column of Sepharose 4B equilibrated with tris buffer. Elution was with tris buffer also.

Fraction 1 : 29 S component.

Fraction 2 : 19 S component

Column dimensions : 100cm x 3cm.


0·15
0·1
0·05
0
ABSORBANCE AT 280 mμ.
Frac.1
Frac.2
10
20
TUBE NUMBER.

of molecular weight greater than one million. A column 50cm by 2cm was packed with Sepharose 4B, which had been washed several times with distilled water and equilibrated overnight with tris buffer. The solution containing the 29 S and 19 S components was appied to the column (in 5 ml samples), eluted with tris buffer and collected in 4 ml aliquots. The optical density of the protein solution in each tube was read at 280 m u and the readings were plotted against tube number. Figure 3.21 shows a typical result. A sample of swan mussel haemaglutinin (sedimentation coefficient 28 S) was run separately on the same column under the same conditions and this was found to have the same elution characteristics as the material in Fraction 1 obtained from mixture of 29 S and 19 S material. Fraction 1 therefore contains the 29 S component. The fractions making up this leading peak were pooled, concentrated by dialysis against solid sucrose and stored in the concentrated sucrose solution.

The methods employed in the isolation of this 29 S material involve the concentration of the required material at each stage. Unfortunately, as with many macromolecules, an increase in concentration brings about precipitation and a subsequent loss of material. Attempts to concentrate the 29 S material, not only after isolation from the 19 S component on a Sepharose column, but even at the earliest stages in the

preparation, resulted in the formation of a fine, fibrous precipitate which proved impossible to resuspend. Thus, while the separation of 29 S material from other serum components proved possible, the amounts obtained proved worthless as far as physico-chemical studies were concerned. It was found however, that the 29 S material was soluble in concentrated sucrose solutions and this proved the most useful method of storing the material. Although a concentrated sucrose solution provided an excellent storage medium it was of little use as a medium in which to carry out physico-chemical analyses. Consequently, throughout this study examination of the 29 S material has, through necessity, been undertaken in the presence of the 19 S macroglobulin.

CHAPTER IV

STRUCTURAL STUDIES ON THE 29 S MACROGLOBULIN

Introduction: In 1957 Deutsch and Morton utilized 2-mercapto-ethanol, a sulphydryl compound, to dissociate human 19 S macro-globulins. These sub-units have an S rate of approximately 7 and each macroglobulin is made up of 5 sub-units. In the presence of iodo-acetamide, stable monomeric units can be obtained on reduction. Reassociation of these sub-units in the absence of iodoacetamide can be brought about by dialysing away the mercaptoethanol. Deutsch and Morton also showed that if a mixture of 19 S, 25 S and 32 S macroglobulins were reduced with 2-mercaptoethanol then all of these components were dissociated into sub-units of between 6 and 7 S.

In view of the similarities between this system and the 19 S - 29 S macroglobulin mixture obtained from normal pig serum it seemed reasonable to expect a similar result if this protein mixture were treated with the appropriate concentration of 2-mercaptoethanol.

Studies on the effects of mercaptoethanol on the 29 S component.

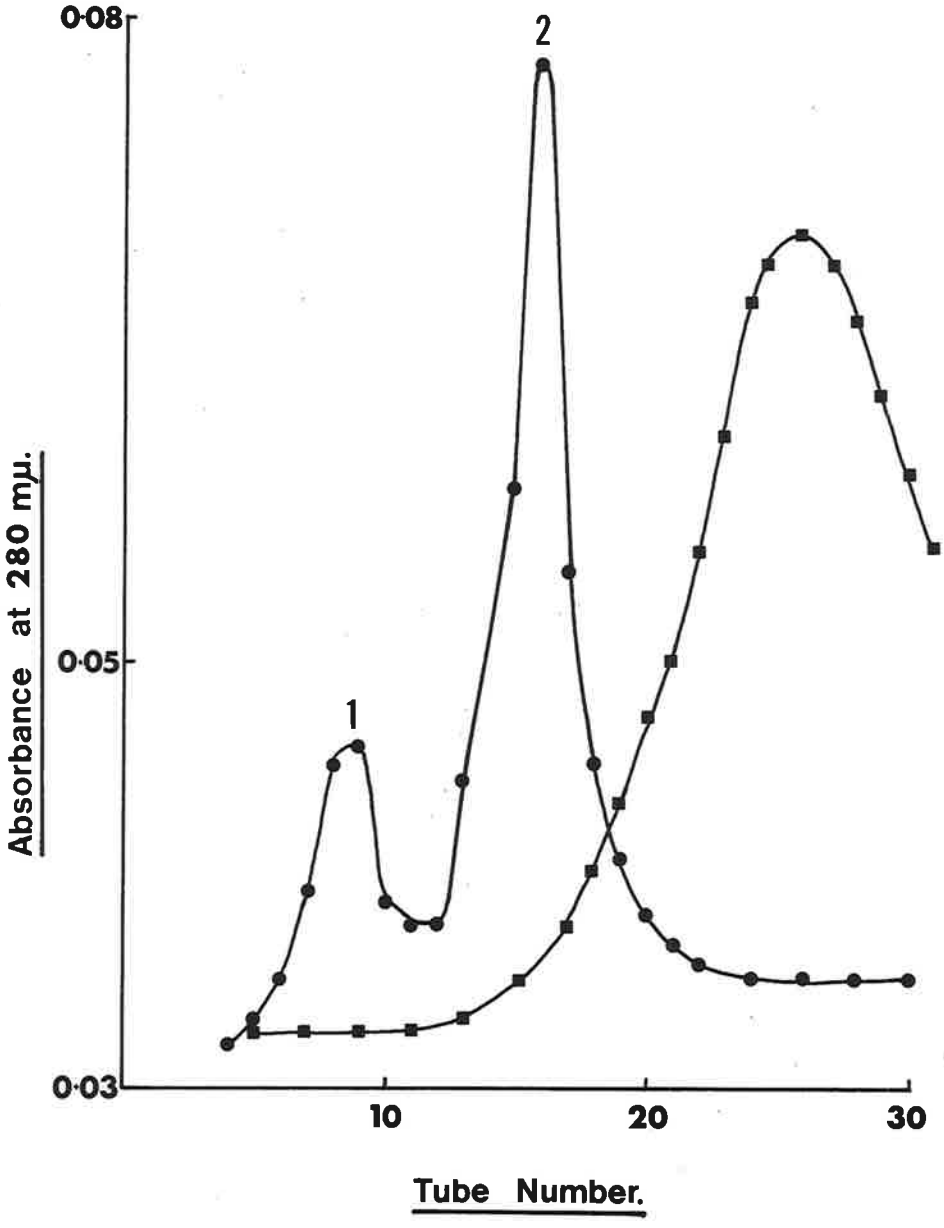
Method: Reduction of the partially purified mixture of the 29 S and 19 S components was first attempted using 0.2 mercapto-ethanol. After treatment with the reducing agent the solution was placed on a sucrose density gradient as described previously.

Figure 4.1. The effects of mercapto-ethanol on the 29 S component.

Sucrose density profile of a solution of 19 S and 29 S material before and after reduction with mercapto-ethanol. (Peak 1-29 S material; Peak 2 - 19 S material).

—●— : before reduction

—■— : after reduction


0·08
0·05
0·03
Absorbance at 280 mμ.
1
2
10
20
30
Tube Number.

The graph of the resultant optical densities plotted against fraction number showed that no appreciable reduction had occurred to either the 19 S or the 29 S components. It was decided therefore to attempt reduction with a more concentrated reducing agent. The procedure was repeated using 0.95M 2-mercaptoethanol. In this instance, after placing the solution on a sucrose density gradient the resultant plot of optical densities against fraction number showed the existence of only one peak and the position of this peak corresponded to that of material with a sedimentation coefficient between 6 S and 7 S (Fig. 4.1). The peak was not a particularly sharp one suggesting the possible existence of a heterogeneous mixture of units of about the same size.

It has been clearly established that human 19 S macroglobulin molecules are made up of 5 sub-units, each with a sedimentation coefficient of approximately 7 S (Deutsch and Morton, 1957). In the preparation of u chains described earlier it has been illustrated that pig 19 S macroglobulin is also made up of sub-units of similar molecular weight and sedimentation coefficient. It is likely that the 7 S material resulting from the reduction described above are of this type. However, since in this case both 19 S and 29 S components were present before reduction it appears that the 29 S material is a polymer of similar sub-units since there is only one apparent

product on reduction of the mixture of both the 29 S and 19 S components of partially purified normal pig serum. Furthermore, as is the case with the 5 sub-units of 19 S, it appears that the sub-units of the 29 S are also held together by disulphide linkages.

The Effect of Potassium Borohydride on the 29 S component of partially purified normal pig serum.

Potassium borohydride is a reducing agent which is generally used for the reduction of carboxylic acid groupings (-COOH) to alcohol groups. In this reaction the carbonyl group (-C=O) is reduced to an -OH group. Jacot-Guillarmod and Isliker (1965) have reported the use of potassium borohydride in attempts to reduce 19 S macroglobulin. When partially purified pig serum (a mixture of 19 S and 29 S material) was treated with 0.01M potassium borohydride the 29 S component appeared to be reduced to sub-units with an S value of approximately 7.

Method. Solid potassium borohydride was added to 1 ml. of the solution containing the 29 S and 19 S components of normal pig serum (total protein concentration of 4 mgm/ml) to a molarity of 0.01M with respect to potassium borohydride, pH 7.0. This reaction mixture was allowed to stand at room temperature for 2½ hours. The sample was then analysed in the ultra-centrifuge. Another sample was similarly treated

and after standing for 2½ hours at room temperature was dialysed against tris buffer for 92 hours to remove the potassium borohydride. After dialysis this sample was also analysed in the ultracentrifuge. Other sets of samples were treated in exactly the same manner but were analysed by placement on a standard sucrose density gradient. The results of these experiments are shown in figures 4.2, 4.3, 4.4 and 4.5.

Discussion: From the results obtained it appears that the 29 S component has been quantitatively broken down by the potassium borohydride to 7 S sub-units and that the 19 S material has been unaffected by this concentration of potassium borohydride. It is tempting to draw the conclusion that the 29 S material is simply an aggregate of 7 S units which are apparently different to those sub-units found in the 19 S material, and that the 29 S material is not a polymeric form of the 19 S material. However it is possible that potassium borohydride may first reduce the 29 S material to 19 S units then finally to 7 S so that the 19 S material is an intermediate in reduction to the 7 S units. This infers that the reduction of 29 S to 19 S and 19 S to 7 S occurs at similar rates. Hence it seemed necessary to determine whether any of the 19 S component is reduced to 7 S by 0.01M potassium borohydride under these conditions. This was done by using a pure sample of 19 S material labelled with I^{131} (page 34 and page 37).

Figure 4.2. The effect of Potassium Borohydride on the 29 S component of partially purified normal pig serum.

Ultracentrifugation pattern of normal pig serum components.

Protein concentration 7.2 mgm/ml. : solvent tris - HCl.

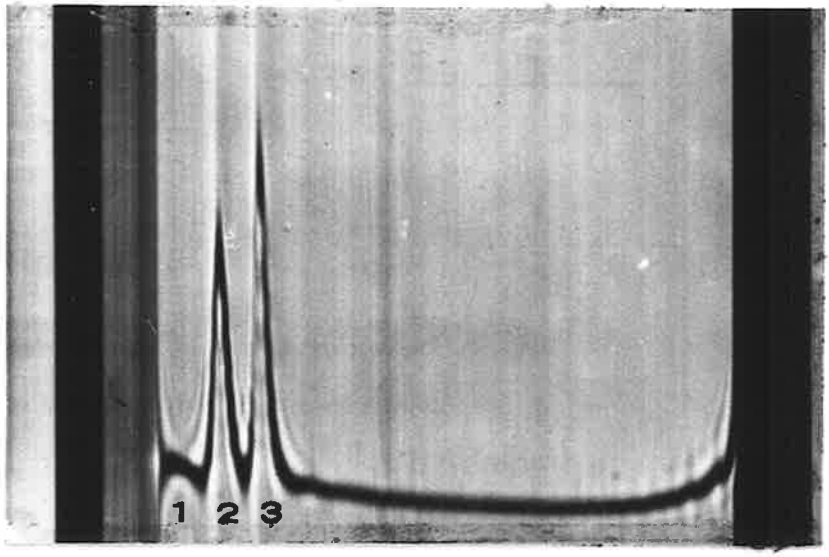
Sedimentation proceeded from left to right. Before treatment with potassium borohydride.

Photographs taken 6 (upper) and 18 minutes (lower) after reaching top speed.

Peak 1 : S20 - not known - 4% total corrected for radial dilution

Peak 2 : S20 - 18.4 S - 43% total corrected for radial dilution

Peak 3 " S20 - 29.8 S - 53% total corrected for radial dilution


1 2 3

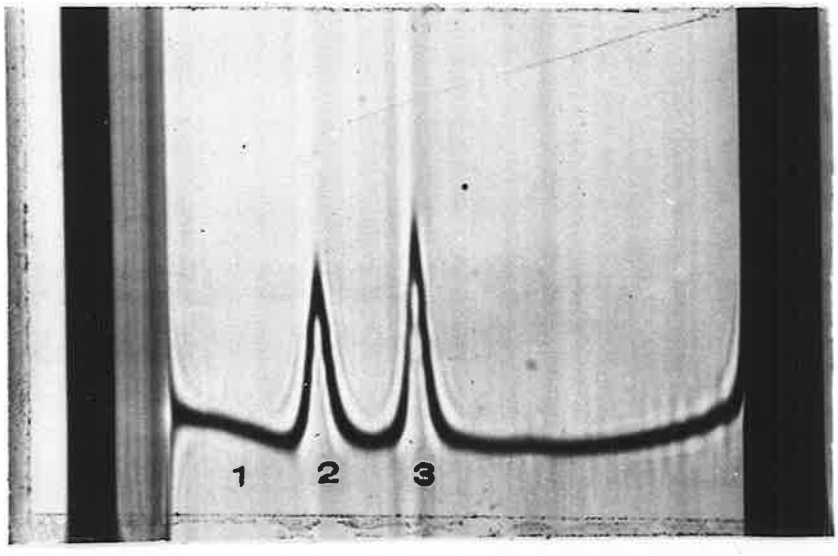

1 2 3

Figure 4.3. The effect of Potassium Borohydride on the 29 S component of partially purified normal pig serum. These photographs were taken after the protein solution had been reduced with 0.1 m potassium borohydride.

Protein concentration 8.8 mgm./ml ;

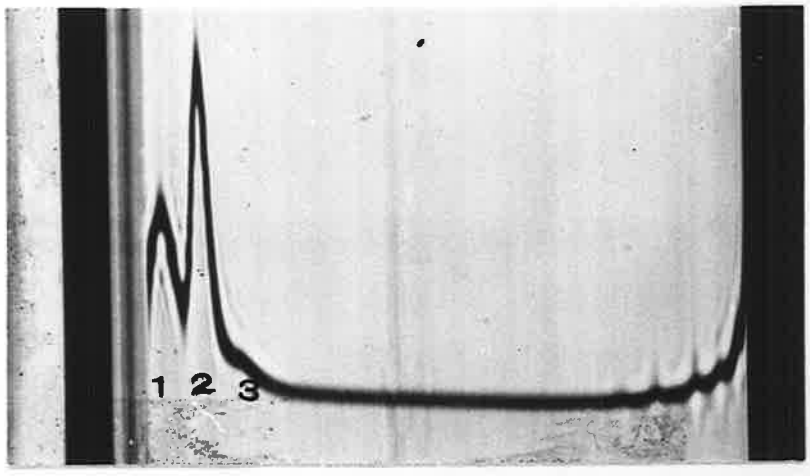
Sedimentation proceeded from left to right

Photographs taken 7 (upper) and 19 minutes (lower) after reaching top speed.

Peak 1: S20 5.1 S , 44% of total - corrected for radial dilution

Peak 2: S20 16.0 S , 50% of total - corrected for radial dilution

Peak 3: S20 25.4 , 6% of total - corrected for radial dilution


1 2 3

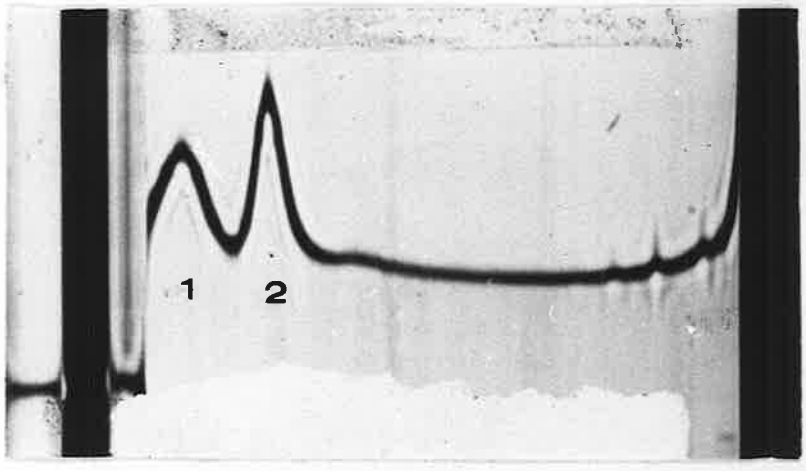

1 2

Figure 4.4. The effect of Potassium Borohydride on the 29 S component of partially purified normal pig serum.

Sucrose density profile of pig macroglobulin preparation before treatment with 0.1 M potassium borohydride.

Peak 1 corresponds to the 29 S component, Peak 2 to the 19 S component.

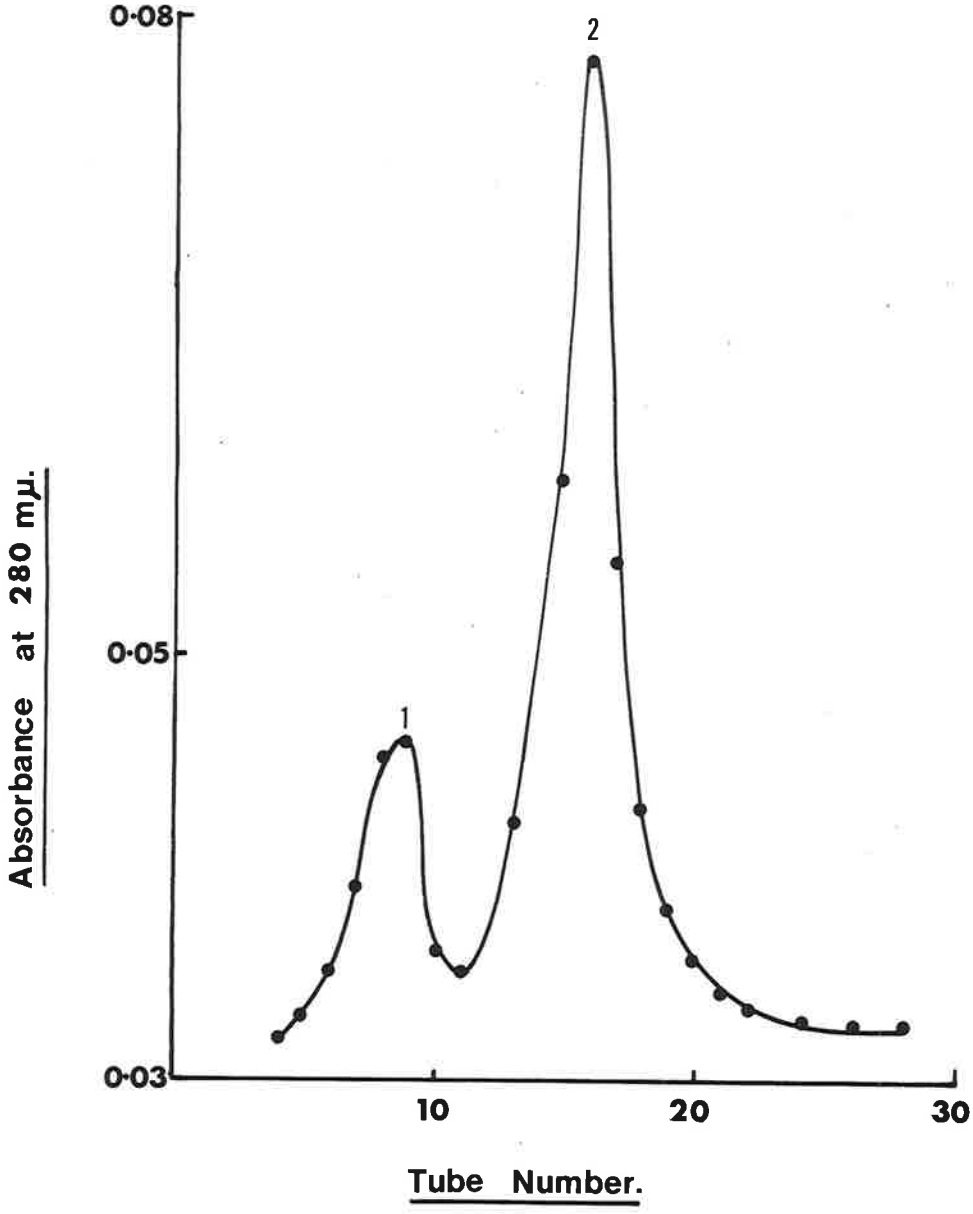

0·08
0·05
0·03
1
2
10
20
30
Absorbance at 280 mμ.
Tube Number.

Figure 4.5. The effect of Potassium Borohydride on the 29 S component of partially purified normal pig serum.

Sucrose density profile of pig macroglobulin preparation after treatment with 0.1M potassium borohydride.

Peak 2 corresponds to a component of low sedimentation constant (approximately 7 S), Peak 1 to the 19 S material.

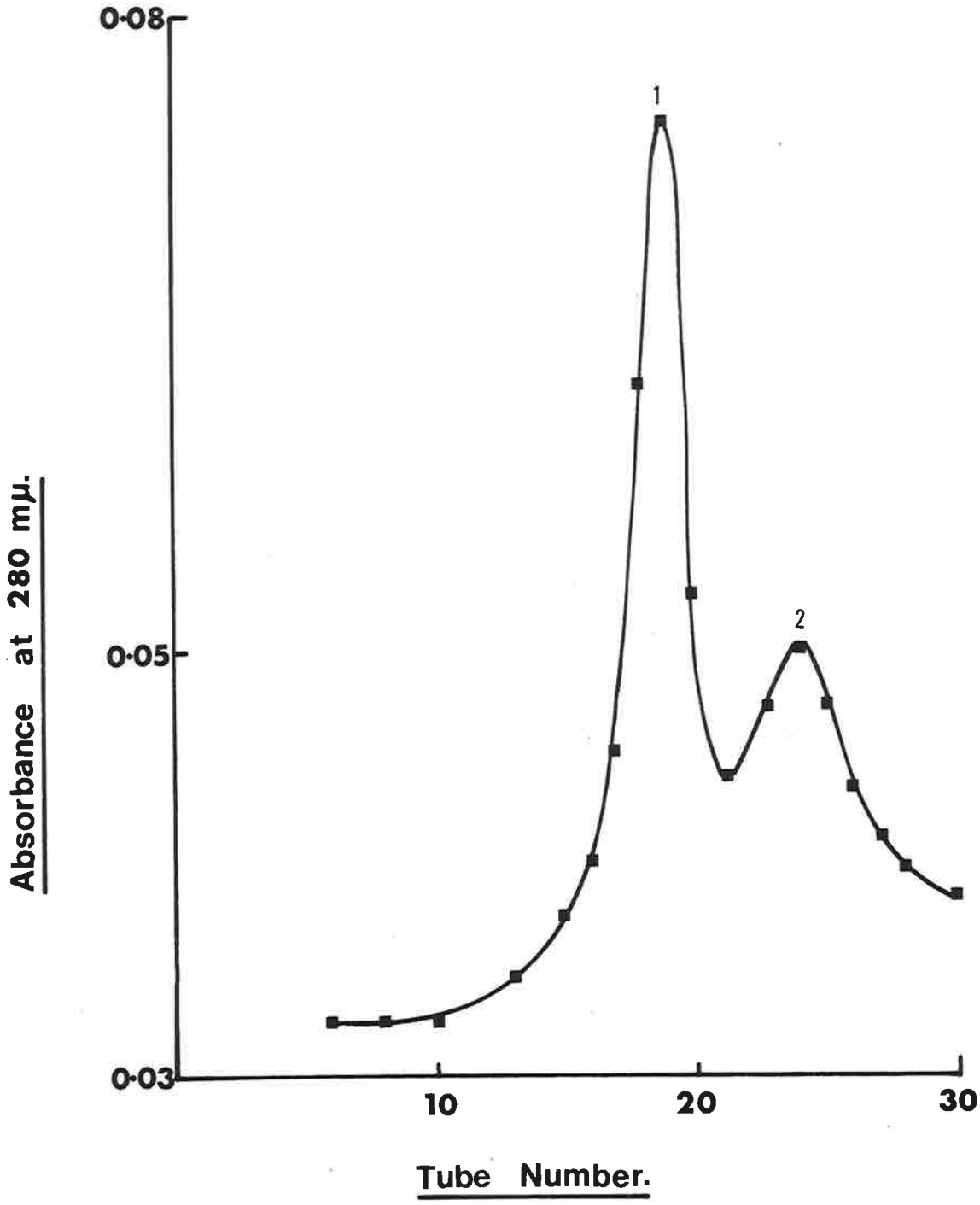

0·08
0·05
0·03
1
2
10
20
30
Absorbance at 280 mμ.
Tube Number.

Figure 4.6. Effect of Potassium Borohydride on the 19 S component of the mixture of 19 S and 29 S material.

Sucrose density profile of tube 1 (containing only labelled 19 S material).

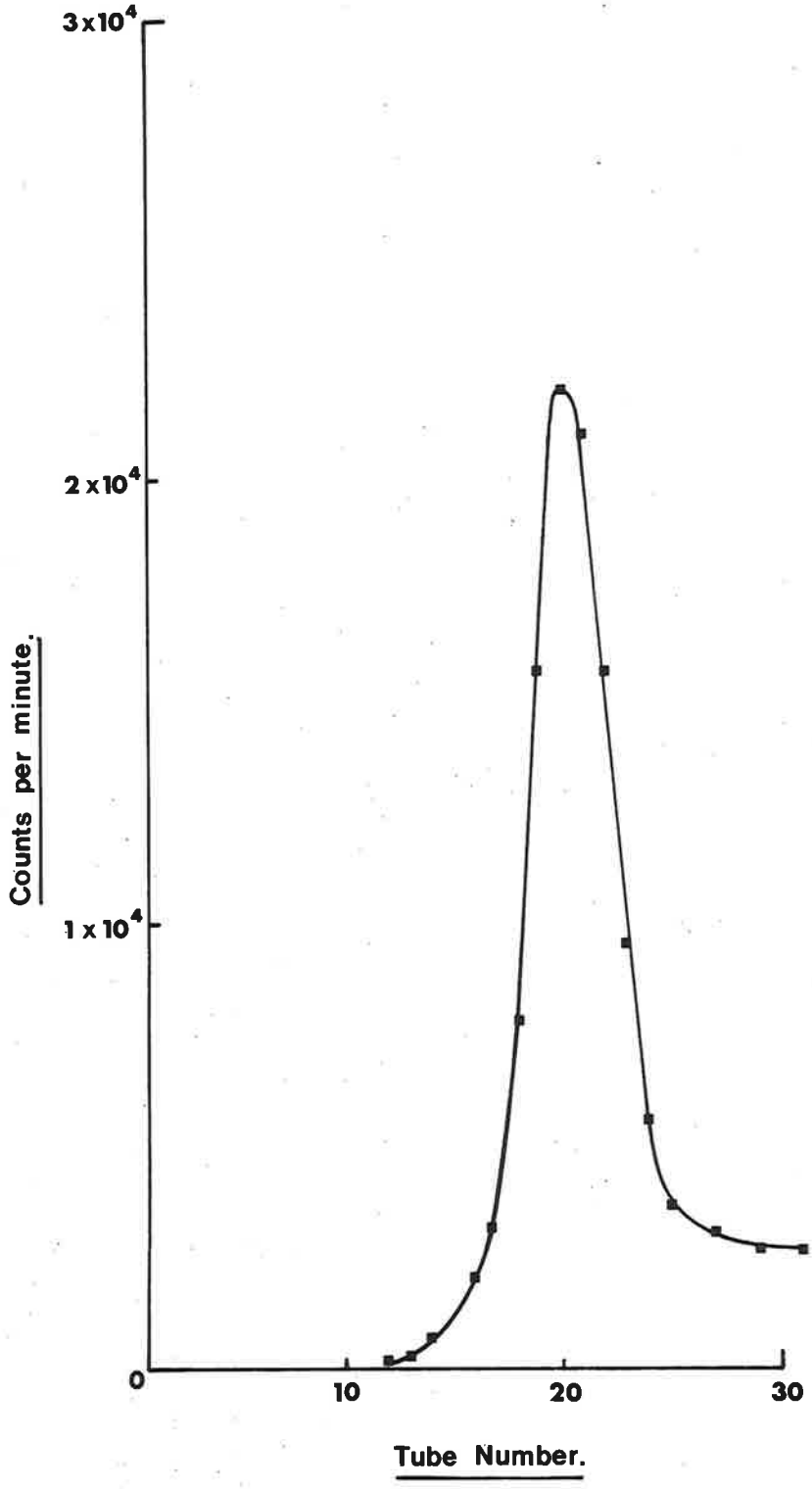
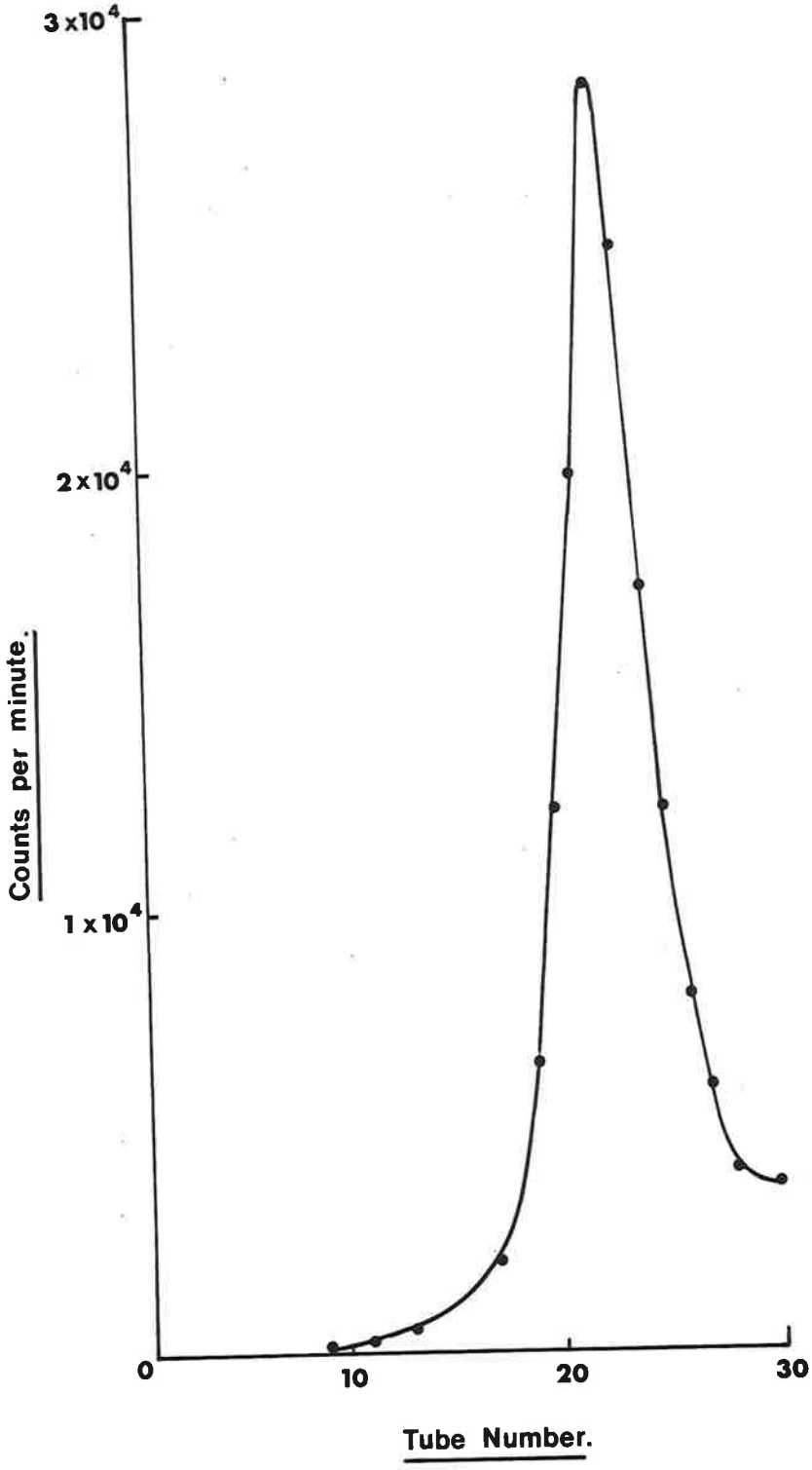

3x10^4
2x10^4
1x10^4
0
Counts per minute.
10
20
30
Tube Number.

Figure 4.7. Effect of Potassium Borohydride on the 19 S component of the mixture of 19 S and 29 S material.

Sucrose density profile of tube 2 (page 53). Containing labelled 19 S material plus native 19 S material and the 29 S component, reduced with potassium borohydride.


3 x 10^4
2 x 10^4
1 x 10^4
0
10
20
30
Counts per minute.
Tube Number.

A small quantity of the labelled 19 S material was added to the mixture of 29 S material and native 19 S material which was then treated with potassium borohydride.

Method: The following samples were placed on sucrose density gradients in each of the 3 tubes of the SW39 rotor of the Spinco Model L centrifuge.

Tube 1. : To check that its sedimentation properties had been unaffected by iodination, a 1 ml sample of labelled IgM was placed in tube 1.

Tube 2. : 0.8 mls of the native 29 S - 19 S protein mixture (3 mgm/ml) + 0.2 mls of labelled IgM, treated with potassium borohydride.

Tube 3. : 0.8 mls of native 29 S - 19 S protein mixture (3 mgm/ml) + 0.2 mls of unlabelled IgM, treated with potassium borohydride.

The tubes were spun overnight in the Spinco Model L ultracentrifuge at 35,000 r.p.m. The contents of each tube was collected in 4 drop aliquots through a hole pierced in the bottom of each tube. The amount of radioactivity (counts per minute - C.P.M.) in each 4 drop aliquot was measured using the Packard gamma counter. The C.P.M. was plotte against each sample number. The optical density of each sample was read at 280 m and also plotted against the tube number. The results are shown in figures 4.6 and 4.7.

Discussion : The results indicate that none of the labelled 19 S macroglobulin has been affected by the 0.01M potassium borohydride. Since the labelled macroglobulin appears to be identical in all respects with the unlabelled 19 S component in the native 29 S - 19 S mixture it appears that the normal 19 S component in this mixture is also unaffected by 0.01M potassium borohydride. It seems most likely, therefore, that 0.01M potassium borohydride is selectively reducing the 29 S material while the 19 S component is unaffected.

Antigenic Relationships between the 29 S component of Normal Pig Serum and other proteins present in Normal Pig Serum.

Introduction: One of the most convincing methods for establishing the existence of similar chemical entities in two given proteins is to raise an antiserum against one of them and then determine whether this antiserum will cross react with the other. The usual method of testing this cross reactivity is by immunoelectrophoresis or simple gel diffusion. If the two proteins in question have chemical characteristics in common then antiserum directed against one of them will form a precipitate with the other by using either of these techniques This procedure has been used in this study in an attempt to establish direct chemical relationships between the 29 S component of normal pig serum and other proteins present in normal pig serum. If the 29 S component reacted with antiserum

prepared against anyone of the other serum proteins then it would be possible to conclude that the two components had common antigenic determinants and possibly sub-units in common. It was of most interest to establish the relationship, if any, between the 29 S component and normal pig IgG and normal pig IgM.

It is known that the IgG and IgM molecules from a given animal have antigenic determinants in common (associated with the light chains of the molecules) and hence an antiserum against either of these would cross react with the other. This cross reactivity would be shown by the presence of a precipitin line on immunoelectrophoresis. An antiserum raised against whole IgG would show therefore whether or not the 29 S component possessed the antigenic determinants present on light chains, heavy chains, or both. To test for a specific relationship between IgG and 29 S component it is essential therefore to raise an antiserum against the heavy (γ) chains of IgG and see if this antiserum will cross react with the 29 S component. Similarly, to test for a specific relationship between IgM and the 29 S component, an antiserum against IgM heavy (μ) chains is necessary.

Method : IgG and IgM were isolated from normal pig serum as described in Chapter II. The purity of both preparations

Figure 4.8. Antigenic Relationships between the 29 S component of Normal Pig Serum and other proteins present in Normal Pig Serum.

Immunoelectrophoresis to illustrate the specificity of antisera used in this work.

1. Well a : μ chains
   Well b : whole pig serum
   Trough : Rabbit anti pig IgG.

2. Well a : pig IgG
   Well b : pig IgM
   Trough : rabbit anti pig μ chains

3. Well a : whole pig serum
   Well b : pig IgM
   Trough : rabbit anti pig γ chains

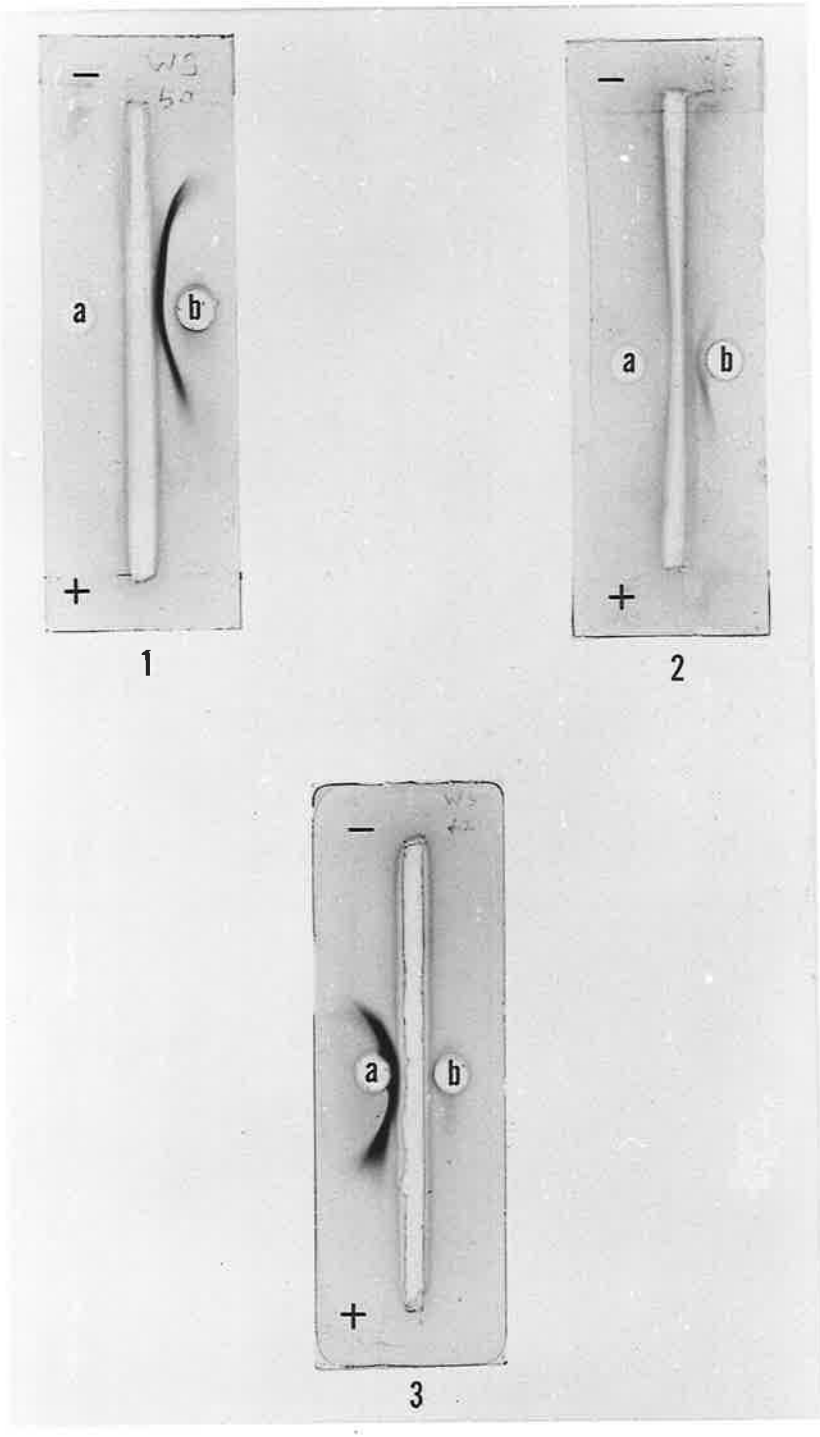

−
a
b
+
1
−
a
b
+
2
−
a
b
+
3

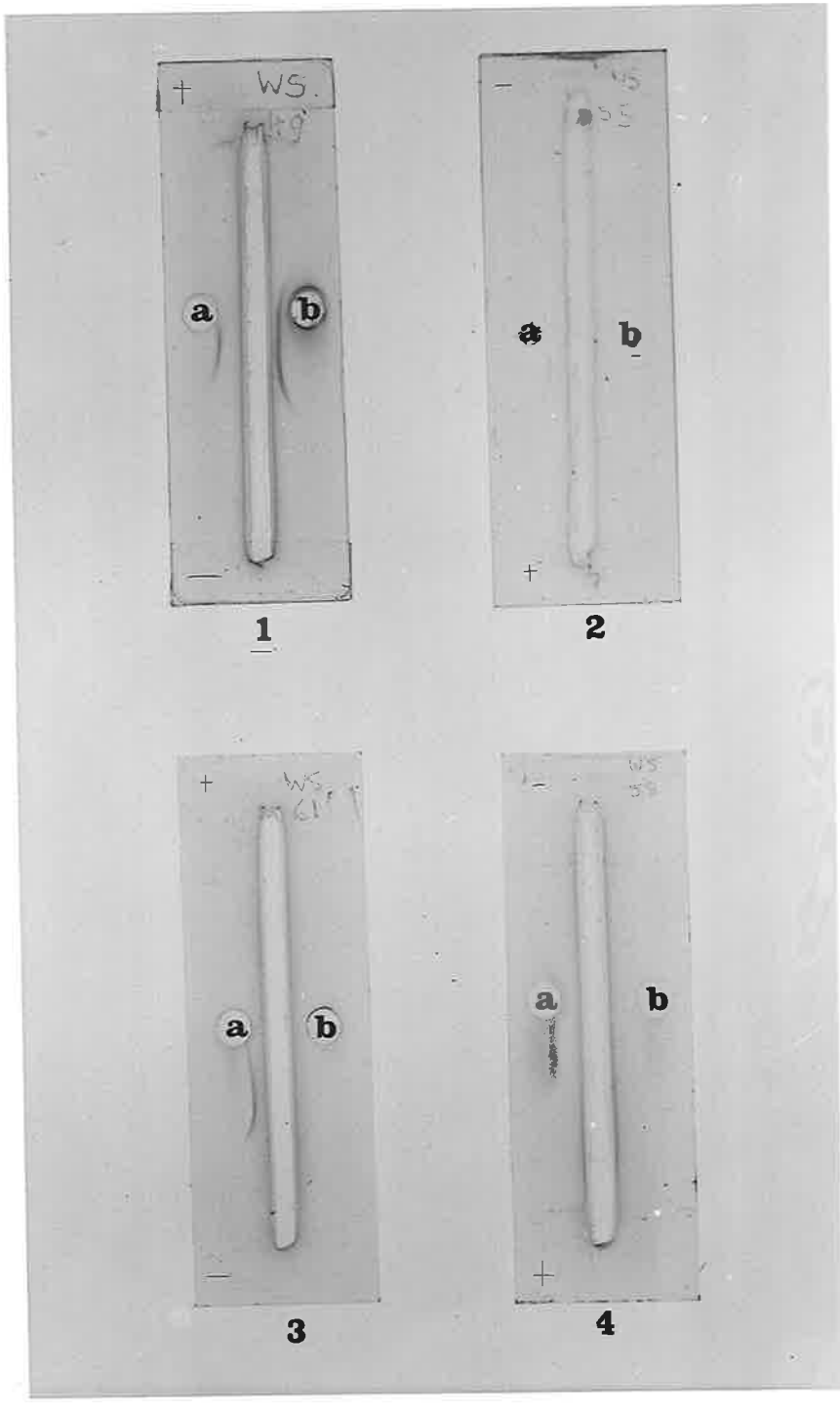
Figure 4.9. Antigenic Relationships between the 29 S component of Normal Pig Serum and other proteins present in Normal Pig serum.

1. Well a : pure 29 S
   Well b : pure IgG
   Trough : rabbit anti pig IgG

2. Well a : pure IgM
   Well b : pure 29 S
   Trough : rebbit anti pig μ chains

3. Well a : pure IgG
   Well b : pure 29 S
   Trough : rabbit anti pig γ chains

4. Well a : pure 29 S
   Well b : pure 29 S
   Trough : rabbit anti 29 S & 19 S
   (Samples of 29 S in well a and well b are of different concentrations)


+
WS.
a
b
—
1
—
a
b
+
2
+
a
b
—
3
a
b
+
4

was tested by immunoelectrophoresis against rabbit anti whole pig serum and each preparation showed only one precipitin band.

IgG heavy chains were prepared as described on page 40 checked for purity in a similar manner to the IgG and IgM preparations and then used to raise a rabbit anti pig γ chain serum. The specificity of this antiserum was confirmed by immunoelectrophoresis against whole pig serum.

IgM heavy chains were prepared from a sample of pure IgM as described on page 37 and used to raise a rabbit anti pig u chain serum. The specificity of this antiserum was tested as before.

Thus three different antisera, rabbit anti pig IgG, rabbit anti pig γ chain and rabbit anti pig u chain were prepared. Each of these antisera was extensively tested to ensure that they were completely specific and showed no cross reactivity except where expected. The 29 S component from normal pig serum was tested by immunoelectrophoresis against these antisera. The results of each of these tests are shown in figures 4.8 and 4.9. An antiserum against the partially purified 19 S and 29 S protein preparation was also raised by injection of this material into a rabbit. On immunoelectrophoresis against this antiserum, the purified 29 S material

showed one precipitin band.

Results: The 29 S component used was that isolated from Sepharose 4B as described on page 43 . This gave a blurred precipitin line on immunoelectrophoresis against rabbit anti pig 19 S and 29 S preparation. The only other antiserum with which the 29 S component formed a precipitate was rabbit anti pig IgG. Since the 29 S component did not precipitate with antiserum raised against the heavy chain of IgG, the 29 S component must possess an antigenic determinant similar or identical to an antigenic determinant on the L chains of IgG. This, therefore, excludes the possibility that the 29 S material present in normal pig serum is a simple polymer of normal pig IgG. Since the 29 S component did not react with anti u chain serum then this result also excludes the possibility that the 29 S component is a polymer of IgM.

The lack of any cross reactivity with either γ or u chains also casts doubt on the possibility of the 29 S entity being an antibody - antigen complex. It is possible, however, that in such a complex the heavy chains of the antibody involved (whether it be IgM or IgG) may be protected by the complexed antigen molecules.

CHAPTER V

DISCUSSION

Many systems have been described in which, along with the more normal 7 S and 19 S globulin components in mammalian sera, the presence of proteins of a higher molecular weight have been observed. In general no more than a passing reference has been made to these components as they only become evident during the final stages of the preparation of IgG and IgM. They are fairly easily removed during the preparation of these components, e.g. by ultracentrifugation on a standard sucrose gradient. (Page 31). Most investigatory work which has been initiated on this type of system has in general been carried out on the serum of patients suffering from macroglobulinaemia. The probable reason for this is that the material from this particular source is available in fairly large quantities. It should be remembered, however, that similar components are not only present in abnormal sera but are also present in the sera of many normal animals of various species.

It appears that in none of the work previously carried out has there been any attempts to isolate macroglobulins with sedimentation coefficients greater than 19 S from the other globulin fractions present - 19 S and 7 S. This has led

to difficulties in interpreting any accumulated data, particularly data concerning the antigenic relationships between the various components present in a particular serum. In the present study, the intention was to isolate one of these higher molecular weight components (the 29 S component of normal pig serum) and investigate its physical and chemical properties. As previously described in the text it proved virtually impossible to obtain sufficient isolated material to carry out physical and chemical tests because of the marked tendency of the material to precipitate out of solution when the concentration was increased. It was possible to obtain sufficient material for use in immuno-chemical studies and in particular as an immunogen. The use in immuno-chemical studies of the pure 29 S material was an integral step in determining the antigenic (and hence structural) relationships between this particular material and other serum components.

Of systems of this type previously examined, the depth of study and the conclusions reached have shown a great degree of variance. For example, Hersh and Benedict (1966) examined a solution of chicken anti bovine serum antibody (γ G) obtained after a single injection of antigen. It was noted that chicken γ G had a higher sedimentation coefficient in a solution of high salt concentration than would be expected from the density and viscosity of the solution used, (1.5M Na Cl) suggesting that an

aggregation of γG occurs in a high salt environment. In fact these workers showed that chicken γ G forms a polymer with an extrapolated sedimentation coefficient of approximately 14 S in a solution of 1.5 M Na Cl. Based on molecular weight determinations, this corresponds to a polymer consisting of 3 or 4 units of 7 S. Hersh and Benedict have suggested that in a high salt concentration, bivalent and univalent antibodies which have been postulated to exist in chicken antisera, (Banovitz, Singer and Canning (1960)) probably polymerize with normal γG. This is a fairly clear cut example of the formation of entities of a higher molecular weight by the aggregation of smaller serum components, namely 7 S material. This explanation as to the origin of a higher molecular weight component in sera is the first of several hypotheses (Page25) to explain the presence of 29 S material in normal pig serum. In order to test this hypothesis, an antiserum was raised against the γ (heavy) chain of a preparation of pure normal pig IgG (Page 40). This antiserum was then tested by immunoelectrophoresis against a pure preparation of the 29 S material. No precipitin band was observed on immunoelectrophoresis. This indicates that no cross reactivity exists between the 29 S material and the heavy chain from normal pig IgG, suggesting that the 29 S material is not an aggregate of this particular 7 S material. It may be however, that if it is an aggregate of normal IgG then on aggregation the antigenic determinants

on the heavy chains of the 7 S subunits have been buried within the aggregate and hence are unable to react with the specific antiserum.

The second possibility to explain the origin of the 29 S component is that it is in fact a polymer of 19 S material present in normal pig serum. In 1966 Suzuki and Deutsch observed the presence of a component in the serum of a patient suffering from macroglobulinaemia which had a sedimentation coefficient of 22 S. In "normal" patients suffering from this disease the serum usually contains a high molecular weight component sedimenting at 25 S to 29 S. This component usually comprises 3 to 8% of the isolated protein. (Muller-Eberhard and Kunkel, 1959). In the individual patient discussed by Suzuki and Deutsch, a 22 S component represented 40% of the total macroglobulins in the preparation. This protein was studied in some detail by these workers and it is of interest to compare these findings with those of the present study.

The serum proteins were first collected and purified by a process similar to the procedure utilized in this study. This resulted in the preparation of a protein solution containing three major components sedimenting in the 19 S, 22 S and 28 S ranges. Apparently no attempt was made to separate these components and all subsequent studies were carried out on protein mixtures containing these three components.

Suzuki and Deutsch first tested to see if the 22 S component was similar to the 22 S component seen in some rheumatoid arthritic sera, which is found to dissociate into 7 S and 19 S components. Attempts to carry out such a dissociation in this case proved unsuccessful. These workers were able to show however, that the 22 S component of macroglobulinaemia sera could be reversibly converted to 19 S material as a function of pH. At pH 4 it was claimed that the 22 S component almost disappeared with a corresponding increase in 19 S material. Interestingly, the 28 S component showed no apparent change over this pH range. At pH 11.0 a partial conversion of 22 S to 19 S material occurs. Suzuki and Deutsch also suggested that the concentration of 22 S material was dependent upon temperature. To investigate this proposition, sedimentation studies were conducted on samples held for 12 to 15 hours at temperatures from 0°c to 40°c. At 40°c there appeared to be a decrease in the amount of 22 S component. The 28 S and 32 S components also disappeared at this temperature but were reformed when the sample was brought to room temperature. This result supports the suggestion that these components are polymers of 19 S material, although this idea is not completely substantiated on the evidence available as to the effect of changing the pH of the solution.

Interesting, although confusing, data was obtained on

this system by carrying out dissociation and reaggregation studies. When normal IgM was reduced by L-cysteine and 2-mercaptoethanol it dissociated into 8 S subunits. These were found to aggregate upon removal of the mercaptan in a manner similarly noted for other IgM globulins. Actually reaggregation of the 8 S subunits obtained by reduction in the cysteine reduced IgM was only partial and resulted in a large quantity of 8 S material which did not reaggregate. In both cases (reaggregation of material from the IgM reduced by 2-mercaptoethanol in one case and cysteine in the other) a component sedimenting with a value of 17 S appeared. This was present in the reaggregated material in greater proportion than both the 19 S and 22 S components. No 28 S or 32 S material is apparent in the ultracentrifugal sedimentation diagrams of the reaggregated material. Hence the reaggregated system is considerably different from the original. This difference is illustrated even more clearly by subjecting the reaggregated system to changes of pH. When the reaggregated macroglobulins were brought to pH 4 and pH 10 they underwent breakdown into a series of lower molecular weight components instead of the conversion of 22 S into 19 S material as noted for the native protein. This serves to present a confused picture as to the relationship between the 28 S, 22 S and 19 S components. In an attempt to clarify this picture an investigation into the

immuno-chemical relationship between these components was carried out. A summary of the results of this work appears in the table (Table 5.1). In this table results have been extracted from the paper by Suzuki and Deutsch and compared with the results obtained in the present study. It can be seen from these results that immuno-chemical studies on the 19 S - 22 S system in no way support the suggestion that the 22 S component is a dimer of the 19 S material. A certain amount of confusion arises because no apparent attempt was made to separate the 22 S material from the other serum components, hence any interpretation of the results obtained must be basically in the area of speculation. Mention is made however, by Suzuki and Deutsch as to the "complexities of the immuno-chemical reactions" of this particular system and they attempt to explain only one of the observed complexities. This is the appearance of 2 lines when the 22 S + 19 S material is reacted with anti L chain. The suggestion is that the appearance of the second line on immunoelectrophoresis is indicative of the availability of an antigenic sight in common with an L chain antigen which is buried in the IgG globulin molecule and presumably "exposed" in this particular system. That is, the subunits of the 22 S component contain L chains (as do the IgG and 19 S components) but on formation of the 22 S component, a part of the L chain is made available for an antigen-antibody reaction which is not available on the L chain

Protein	Antiserum	Results on I.E.P.
*22 S + 19 S	anti IgG	1 line
29S	anti IgG	1 line
*22 S + 19 S	anti μ chain	1 line
29 S	anti μ chain	no line
*22 S + 19 S	anti L chain	2 lines
29 S	anti γ chain	no line
*22 S + 19 S	anti γ M	1 line
*19 S + 22 S reduced to 8.8 S	anti L chain anti μ chain anti IgM	1 line 1 line 1 line
*7.8 S alkylated to 6.7 S	anti L chain anti μ chain anti γ M	1 line 2 lines 2 lines

Table 5.1. Summary of immunochemical results obtained by Suzuki and Deutsch (1966) compared with results of immunochemical studies carried out in the present work.

* After Suzuki and Deutsch (1967)

of the 19 S component. In the present study, isolation of the 29 S material in sufficient quantities for immunological purposes has, in most cases, made the interpretation of results obtained from immunochemical studies considerably more straight forward. The fact that the 29 S material does not react with anti μ chain serum from normal pig IgM is a clear indication that the 29 S component is not a polymer of 19 S material. This result is in direct contrast with the conclusions of Suzuki and Deutsch who claim that the 22 S component in the human macroglobulinemic serum described by those workers is in fact a dimer of the 19 S material also present in the serum. It must be realized however, that it is possible that the 29 S material may consist of 19 S material but if this were the case, then the lack of cross reactivity with μ chains would mean that the antigenic determinants on the chains (if they are present) in the subunits of the 29 S components, must be "buried" within the product upon polymerisation.

The third hypothesis to explain the nature of the 29 S component is that it may in fact be a separate entity and the relationship between it and 19 S or 7 S is similar to the relationship between 19 S and 7 S. That is to say, although the 29 S is made up of 7 S subunits, these subunits are not identical to the 7 S IgG material present in the serum. Neither are they identical with the 7 S subunits which make up the normal 19 S

material. It appears, from the data that the 29 S component has similar light chains to the other serum components but the heavy chains are antigenically and therefore chemically different from both the IgG and IgM chains. On the basis of the results obtained from testing the 29 S material by immuno-electrophoresis against antisera against light chains and heavy chains from IgG and IgM it appears that this proposition is a distinct possibility. The existence of one precipitin band when anti IgG is tested against 29 S suggests that there is an antigenic similarity between IgG and 29 S. The absence of a precipitin band when anti γ chain serum is reacted with 29 S suggests that the antigenic similarity resides, as is to be expected, in the L chain. As previously noted, no cross reactivity exists between the heavy (μ) chains from IgM and the 29 S component. All this evidence strongly supports the hypothesis that the 29 S material is not a polymer of IgG or IgM but is in fact a separate entity. If it is a polymer of any of these components then presumably any antigenic sites present on the heavy chains of the subunits are buried when they polymerise to form the 29 S material. The suggestion that the 29 S material is a polymer of 7 S material is further supported by results obtained from the degradation of the 29 S component by 0.01M potassium borohydride. From the results obtained using radioactively labelled 19 S material it would appear that when treated with potassium borohydride, the 29 S material breaks down directly to 7 S units without any

apparent 19 S intermediate stage. This suggests that it is extremely unlikely that the 29 S material contains 19 S sub-units.

Similarly, from the results, it seems unlikely that the 29 S component is an antigen-antibody complex (such as that found in some rheumatoid arthritic sera for instance) although the presence of a 7 S antibody in the material may be disguised by the complexing antigen molecules if such a complex did in fact exist.

The possibility exists for the further study of this system along several different lines. The most interesting avenue to be explored is the distinct likelihood that it may be possible to ascribe some biological activity to the 29 S component. The most obvious biological role to consider is that of the 29 S component having some degree of antibody activity. To assign the role of an antibody to a particular protein involves the consideration of several criteria. Perhaps the most explicit criteria which unambiguously define an unknown protein as an antibody are the following suggested by Metzger (1967).

(1) The proteins should resemble known immunoglobulins or subunits thereof. Demonstration of disulphide linked polypeptide chains of molecular weights comparable to

"light" and "heavy" chains as well as shared antigenic determinants are most convincing.

(2) The interaction of the protein with its "antigen" should express a clearly defined specificity.

(3) The combining ratio of "antigen" to "antibody" should be well defined and limited. A ratio of one site per heavy - light polypeptide chain pair is more persuasive.

(4) Antigen binding activity should be demonstrable by those structural components equivalent to the active fragments (Fab) of known antibodies.

(5) The activity must be stoichiometrically related to all the protein in the preparation. Contamination of an otherwise inert protein with small amounts of bona fide antibody must be ruled out.

In the course of this work no attempt has been made to attribute any biological activity. Work on this particular aspect of the system has been carried out by Turner (unpublished and preliminary results tend to support the suggestion that the presence of 29 S material may have some biological significance, and in particular may be involved in aiding phagocytosis of foreign particles. As this work is still in its preliminary stages it would be premature to suggest at this time that the

material has any real biological significance. It does appear, however, that this possibility is well worth exploring and it is intended to undertake further work in the future in an attempt to elucidate the role of the 29 S component. The work of Turner (unpublished) has utilised two systems in attributing biological activity to 29 S. The first system was phage neutralization in early immuno rabbit sera, identifying 29 S by that activity which is labile to reduction with potassium borohydride. Assaying serum activities before and after reduction under these circumstances gave, firstly, total macroglobulin activity and, secondly, 29 S as opposed to 19 S activity. Turner also examined Swiss white mice immunized in the conventional manner with S. typhimurium M206, testing early antisera for 2-mercapto-ethanol and potassium borohydride lability. Both approaches showed definite 29 S antibody activity to be present very early in the immune response and suggested that it preceded the 19 S activity. These preliminary results are sufficiently encouraging to warrant further investigation into the biological aspects of the 29 S component. Similarly the information obtained in this present study suggests further ways of examining the physical and chemical properties of the 29 S material. However, it is quite clear that before any projected work of a worthwhile nature can be carried out it will be necessary to overcome the very real problem of isolating significant quantities of the pure 29 S component in a soluble

form. Should these difficulties be overcome, then once the 29 S component has been prepared in a pure form then it should be a fairly simple task to obtain a preparation of its peptide chains. By comparing these on gel electrophoresis with well defined immunoglobulin chains and also by testing the antigenic relationships existing between them it should be possible to confidently define the chemical nature of the 29 S component.

ACKNOWLEDGEMENTS

I am very pleased to acknowledge the advice, criticism and guidance of Dr. K.J. Turner during the course of this study. I am also indebted to Dr. I. Kotlarski for guidance in the preparation of this manuscript.

BIBLIOGRAPHY

ABRAMS, A., COHEN, P.P., and MEYER, O.O. (1949). The physical properties of cryoglobulin obtained from lymph nodes and serum of a case of lymphosarcoma. J. Biol. Chem. 181 : 237

ADA, G.L., NOSSAL, G.J.V. and AUSTIN, L.M. (1965). In J. Sterzl Ed. Molecular and Cellular basis of Antibody formation. Czechoslovac Academy of Sciences, Prague.

BANOVITZ, J., SINGER, S.J., and WOLFE, H.R. (1959). Precipitin Production in Chickens. J. Immunol., 82 : 481.

BELLANTI, J.A., RUSS, S.B., HOLMES, and BUESCHER, E.L. (1965). The nature of antibodies following experimental arbovirus infection in guinea pigs. J. Immunol. 94 : 1-11.

CEBRA, J.J. (1967) Cited by Porter R.R. in Essays in Biochemistry Vol.3.

CHAPLIN, H., COHEN, S., and PRESS, E.M. (1965) Preparation and properties of the peptide chains of normal human 19 S - globulin (IgM). Biochem. J. 95 : 256.

CHASE, M.W. (1967) Methods in Immunology and Immunochemistry, ed. by Curtis A. Williams and Merrill W. Chase. Academic Press (New York).

COHEN, S. (1963) Properties of the peptide chains of normal and pathalogical human γ - globulins. Biochem. J. 89 : 334.

COHEN, S., and PORTER, R.R. (1964). Structure and biological activity of immunoglobulins. Adv. Immunol. 4 : 287.

DEUTSCH, H.F., and MORTON, J.I. (1957) Dissociation of human serum macroglobulins. Science 125 : 600.

DEUTSCH, H.E., and MORTON, J.I. (1958) Human serum macroglobulins and dissociation units. I. Physiochemical properties. J. Biol. Chem. 231 : 1107.

DEUTSCH, H.F. (1963) Molecular transformations of a γ - globulin of human serum. J. Molec. Biol. 7 : 662

EDELMAN, M. and POULIK, M.D. (1961) Studies on structural units of the globulins. J. Exp. Med. 113 : 861

EISEN, H.M., LITTLE, J.R., OSTERLAND, C.K., and SIMMS, E.S. (1967) A myeloma protein with antibody activity. Cold Spring Harbour Laboratory Symposia on Quantitative Biology. Vol. XXXII. Cold Spring Harbour, L.I., New york.

FAHEY, J.L. (1965) Antibodies and Immunoglobulins. 1. Structure and Function. Journal of the American Medical Association. 194 : 71.

FEINSTEIN, A. (1966) Use of charged thiol reagents in interpreting patterns of immune globulin chains and fragments. Nature 210 : 135.

FREEMAN, M.J., and STAVITSKY, A.B. (1965) Radio immuno-electrophoresis study of rabbit antiprotein antibodies during the primary response. J. Immunol. 95 : 981.

GREENBURY, C.L., MOORE, D.H., and NUNN, L.A.C. (1963) Reaction of 7 S and 19 S components of immune rabbit antisera with human group A and AB red cells. Immunology. 6 : 421.

GREY, H.M. (1964) Studies on changes in the quality of rabbit-bovine serum albumin antibody following immunization. Immunology 7 : 82.

GREY, H.M. and KUNKEL, H.G. (1964) H chain subgroups of myeloma proteins and normal 7 S - globulins. J. Exp. Med. 120 : 253.

HEREMANS, J.F., VAERMAN, J.P., CARBONARA, A.O., RODHAIN, J.A., and HEREMANS, M.T. Protides of the Biological Fluids, ed. by H. Peeters, vol.10, p.108 Elsevier Pub. Co., Amsterdam, 1963.

HEREMANS, J.F., VAERMAN, J.P., and VAERMAN, C. (1963). Studies of the immune globulins of human serum.I. J. Immunol. 91 : 11

HEREMANS, J.F., VAERMAN, J.P., and VAERMAN, C. (1963) Studies of the immune globulins of human serum.II.J. Immunol. 91 : 11

HERSH, R.T., and BENEDICT, A.A. (1966) Aggregation of chicken G immunoglobulin in 1.5 M sodium chloride solution. Biochem. Biophys. Acta. 115 : 242.

HONG, R., and NISONOFF, A. (1965) Relative labilities of two types of interchain disulphide bond. Chem. 240 : 3883

HUNTER, W.M., and GREENWOOD, F.C. (1962) Preparation of iodine - 131 labelled human growth hormone of high specific activity. Nature 94 : 495

ISHIZAKA, K., ISHIZAKA, T., and HORNBROOK, M.M. (1966) Physico-chemical properties of human reaginic antibody. J. Immunol. 97 : 75.

JACOT-GUILLARMOD, H., and ISLIKER, H. (1965) Scission reversible des isoagglutinines 19 S : Etude de fixation des subunites. Vox Sang. 9 : 31-35.

JERNE, N.K., NORDIN, A.A., and HENRY, C. (1963) The agar plaque technique for recognizing antibody-producing cells, p109-125. In B. Amos and H. Koprowski ed. Cell bound antibodies. The Wistar Institute Press, Philadelphia.

KAWAHARA, K., and TANFORD, C. (1966) Viscosity and Density of Aqueous Solutions of Urea and Guanidine Hydrochloride. J. Biol. Chem. 241 : 3228,

KILLANDER, J., and FLODIN, P. (1962). The fractionation of serum proteins by gel filtration. Vox. Sang. 7 : 113.

KLINMAN, N.R., ROCKEY, J.H., FRAUENBERGER, G., and KARUSH, F. (1966) Equine anti hapten antibody. III. The comparative properties of γG and γA antibodies. J. Immunol. 96: 587.

KUNKEL, H.G. (1960) The plasma proteins, ed. by Putnam, F.W. Academic Press, N.Y. p.278.

KUNKEL, H.G., and ROCKEY, J.H. (1963) β_2A and other immunoglobulins in isolated and anti-A antibodies. Proc. Soc. Exptl. Biol. Med. 113 : 278.

LAMM, M.E., and SMALL, P.A. (1966) Polypeptide chain structure of rabbit immunoglobulin. II. γ M - Immunoglobulins. Biochemistry. 5 : 267.

LANDY, M., SANDERSON, R.P., and JACKSON, A.L. (1965). Humoral and cellular aspects of the immune response to the somatic antigen of Salmonella enteritidis. J. Exp. Med. 122 : 483.

LINDQUIST, K., and BAUER, D.C. (1966) Precipitin activity of rabbit macroglobulin antibody. Immunochem. 3 : 373.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951) Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193 : 265.

MARKHAM, R. (1960) A Graphic Method for the Rapid Determination of Sedimentation Coefficients. Biochem. J. 77 : 516.

MERLER, E., KARLIN, L., and MATSUMOTO, S. (1968) The valency of human γM immunoglobulin antibody. J. Biol. Chem. 243 : 386.

METZGER, H. (1967) Characterization of a human macroglobulin. V. A Waldenstrom macroglobulin with antibody activity. Proc. Nat. Acad. Sci. U.S.A. 57 : 1490.

MILLER, F., and METZGER, H. (1965) Characterization of a human macroglobulin. J. Biol. Chem. 240 :

MILLER, F., and METZGER, H. (1966). Characterization of a human macroglobulin III. The products of trypsin digestion. J. Biol. Chem. 241 : 1732.

MULLER-EBERHARD, H.J., and KUNKEL, H.G. (1959). Ultracentrifugal characteristics and carbohydrate content of macromolecular γ -globulins. Clin. Chem. Acta 4 : 252.

ONOUE, K., YAGI, Y., and PRESSMAN, D. (1964) Multiplicity of antibody proteins in rabbit anti p-azobenzenarsonate sera. J. Immunol. 92 : 173.

ONOUE, K., YAGI, Y., GROSSBERG, A.L., and PRESSMAN, D. (1965) Number of binding sites of rabbit macroglobulin antibody and its subunits. Immunochemistry 2 : 401 - 415.

OSTLER, A.G., MULLIGAN, J.J., and RODRIQUEZ, E. (1966) Weight estimates of rabbit anti human serum albumin based on antigen-binding and precipitin analysis : specific haemogglutinating activities of 7 S and 19 S components. J. Immunol. 96 : 334.

PETERMANN, M.L., and BRAUNSTEINER, H. (1954) A cryoglobulin of high sedimentation rate (macroglobulin) from human serum. Arch. Biochem. Biophys. 53 : 491.

PETERSON, E.A., and SOBER, H.A. (1960) Chromatography of proteins. I. Cellulose ion-exchange adsorbants. J. Amer. Chem. Soc. 78 : 751.

PIKE, R.M., and SCHULZE, M.L. (1964) Production of 7 S and 19 S antibodies to the somatic antigens of Salmonella typhosa in rabbits. Proc. Soc. Exptl. Biol. Med. 115 : 829.

PIKE, R.M. (1967) Antibody Heterogeneity and Serological Reactions. Bact. Review. 31 : 157.

POTTER, M., APPELLA, E., and GEISSER, S. (1965) Variations in the heavy polypeptide chain structure of gamma myeloma immunoglobulins from an inbred strain of mice and an hypothesis as to their origin. J. Mol. Biol. 14 : 361 - 372.

PUTNAM, F.W., (1959) Abnormal Serum Globulins. I. The Non identity of macroglobulins. Arch. Biochem. Biophys. 79 : 67.

REDDIN, J.L., ANDERSON, R.K., JENNESS, R., and SPINK, W.W. (1965) Significance of 7 S and macroglobulin brucella agglutinins in human brucellosis. New Eng. J. Med. 272 : 1263.

ROSSEN, R.D., DOUGLAS, R.G., CATE, T.R., COUGH, R.B., and BUTLER, W.T. (1966) The sedimentation behaviour of rhinovirus neutralizing activity in nasal secretion and serum following the rhinovirus common cold. J. Immunol. 97 : 532.

ROTHMAN, U.S.E. (1965) Isolation of guinea pig immuno-globulin A (IgA). Acta. Soc. Med. Upsalien. 70 : 241.

SCHACHMAN, H.K. (1959) In "Ultracentrifugation in Biochemistry Academic Press (New York and London).

SCHEIDEGGER, J.J. (1955) Une micro-methode de l'immuno-electrophorese. Int. Arch. Allergy. 7 : 103.

SJOQUIST, J. (1966) Heterogeneity of heavy (γ) chain preparations from human γG - immunoglobulins. Nature 210 : 1182.

SMITHIES, O. (1959) An improved procedure for starch-gel electrophoresis : further variations in the serum proteins of normal individuals. Biochem. J. 71 : 585.

SOUTH, M.A., COOPER, M.D. WOLLHEIM, F.A., HONG, R., and GOOD, R.A. (1966) The IgA system. I. Studies of the transport and immunochemistry of IgA in the saliva. J. Exp. Med. 123: 615.

STELOS, P., and TALMAGE, D.W. (1957) The separation by starch electrophoresis of two antibodies to sheep red cells differing in hemolytic efficiency. J. Infect. Diseases. 100 : 126.

SUZUKI, T., and DEUTSCH, H.F. (1966) Occurrence and properties of a macroglobulin dimer in some hypoglobulinemic sera. J. Exp. Med. 124 : 819.

SUZUKI, T., and DEUTSCH, H.F. (1967) Dissociation, reaggregation, and subunit structure studies of some human γM-globulins. J. Biol. Chem. 242 : 2725.

SVEHAG, S., and MANDEL, B. (1962) The production and properties of poliovirus neutralizing antibody of rabbit origin. Virology 18 : 508.

SVEDBERG, T., and PEDERSON, K.O. (1940) In "The Ultra-centrifuge". Oxford Univ. Press (London and New York).

TOMASI, T.B., Jr., TAN, E.M., SOLOMON, A., and PENDERGAST, R.A. (1965). Characteristics of an immune system common to certain external secretations. J. Exp. Med. 121 : 101.

TURNER, K.J., and ROWLEY, D. (1963) Opsonins in pig serum and their purification. Aust. J. of Exp. Biol. and Med. Sci. 41 : 595.

WEIDANZ, W.P., JACKSON, A.L., and LANDY, M. (1964) Some aspects of the antibody response of rabbits to immunization with enterobacterial somatic antigens. Proc. Soc. Exptl. Biol. Med. 116 : 832.

WIGZELL, H., MOLLER, G., and ANDERSON, B. (1966) Studies at the cellular level of the 19 S immune response. Acta. Pathol. Microbiol. Scand. 66 : 530.

WILKINSON, P.C. (1966) Immunoglobulin patterns of antibodies against Brucella in man and animals. J. Immunol. 96 : 457.

WINTER, A.J. (1965) Characterization of the antibody for Vibrio fetus endotoxin in sera of normal and V. fetus infected cattle. J. Immunol. 95 : 1002.

YOON BERM KIM, BRADLEY, S.G., and WATSON, W.D. (1966) Ontogeny of the Immune Response. II. Characterization of 19 S G - and 7 S G - Immunoglobulins in Piglets. J. Immunol. 97 : 189.

ADDENDUM

Most of the more recent developments in the field of antibody structure and function have focussed on the sequence of amino acids in the constituent polypeptide chains and subsequent speculation on the gentic system responsible for the unusual features of these sequences.

The main classes of immunoglobulins have been previously outlined and defined on the basis of their general properties and the class of their heavy chains (page 3). Within a given class of heavy chains, subclasses have been distinguished by their antigenic determinants. For example, there are four subclasses of human IgG immunoglobulin, IgG 1, IgG 2, IgG 3 and IgG 4. Differences in the subclasses reflect variations in the amino acid sequences of the carboxyl-terminal parts of γ chains. In contrast to the heavy chain classes, the two major classes of light chains, κ and λ are found in all immunoglobulins.

Although they share the same overall structure, immunoglobulins within a single class are a heterogeneous mixture of chemically different molecules. Even purified antibodies directed against a simple hapten antigen are a population of diverse molecules (Haber, 1968). For this

reason it is not possible to purify such a complex mixture of proteins in order to determine an unequivocal amino acid sequence. Hence the use of myeloma proteins in this type of work. (Although myeloma proteins have not been shown to be antibodies in the sense that they have been electively induced by stimulation with known antigens, they have the same basic structure as normal immunoglobulins, and each myeloma protein appears to be a unique example of one of the many different immunoglobulin molecules in the normal heterogeneous population.

A provisional picture of the entire covalent structure of the IgG 1 myeloma protein is now available (Edelman et al, 1969). The structure is essentially that outlined earlier (page 4). The IgG 1 molecule consists of two identical light chains and two identical heavy chains (Fleishman et al, 1963). Each heavy chain and the adjacent light chain interact via noncovalent forces and a single disulphide bond. Two light chain-heavy chain pairs of half molecules (Palmer, 1964) are linked together by non covalent interactions and by two neighbouring disulphide bonds between the heavy chains There is some indication that the molecule is sharply differentiated in terms of function: the amino terminal portions of the chains for antigenic binding, and the carboxyl terminal portions for physiological functions within the

organism. More detailed studies indicate that the molecule has been structurally differentiated into variable regions having diverse amino acid sequences and constant regions having nearly invariant sequences of amino acids (Wilkinson, Press and Porter, 1966).

The complete amino acid sequence of a human IgG immunoglobulin has been determined (Edelman et al, 1969) and the arrangement of all the disulphide bonds has been established. That light chains have an unusual primary structure has been shown by many studies (Cunningham et al, 1968; Hilschmann et al, 1965). Each κ and λ chain may be divided into an amino-terminal variable or V region and a carboxyl-terminal constant or C region (Hilschmann et al, 1965). V regions of different proteins have diverse amino acid sequences; C regions differ in only a few residues. The diversity of the amino acid sequences of V regions is primarily a result of the substitution of one amino acid for another at certain positions in the linear sequence, although small changes in the length of the chain may occur. Three subgroups of K chains have been proposed on the basis of differences in amino acid sequence (Milstein, 1967). On the average, members of the same subgroup differ in about ten of the first one hundred and eight residues, while

members of different subgroups differ in about 40% of the first one hundred and eight residues. The sequences of proteins in different subgroups differ extensively suggesting that they are specified by more than a single gene. (Edelman and Gally, 1969). For this reason the diversity of V regions of these polypeptide chains represents a major genetic problem.

It has now been shown that heavy chains also contain V and C regions (Gottlieb et al, 1968; Press, 1967). Results obtained by Gottlieb et al (1968), suggest that the nature of sequence variation in heavy chains (from human IgG-globulin) follows rules of variation similar to those governing the structure of light chains. These workers were able to show that a given light chain is similar to its analagous heavy chain, but is no more closely related to it than to other light chains. Turner and Cebra (1971) have reported that sequences of fragments of heavy chains from guinea pig IgG were closely homologous with those from corresponding sections of IgG from other species. They also showed that although the proline-rich hinge regions from the guinea pig, rabbit and human proteins were nearly identical in sequence, the adjacent sections N terminal to the hinge region all varied markedly in amino acid sequence and number of residue present.

It has been shown (Wilkinson, Press and Porter, 1966) that the N terminal amino acid of rabbit heavy chain is PCA (pyrrolid-2-one-5-carboxylic acid) and that this is followed by a mixed sequence; an N terminal fragment of molecular weight about 4000, can be prepared in 30% yield from Fd fragments by cleavage with cyanogen bromide (Press, Givol, Piggot, Porter and Wilkinson, 1966). This fragment has at least one of the N terminal sequences of heavy chain. Wilkinson (1969) compared the extent of similarity between the N terminal sequence of a rabbit IgG heavy chain and the N terminal sequence of a human IgG 1 heavy chain. He found the extent of homology to be about of the order of 60% with stricking similarities in several particular segments.

Gottlieb et al (1968) also demonstrated homology betwee two heavy chains from different immunoglobulin sources and numbers of both x and λ classes of light chains. In order to maximise the homology between a heavy chain and any of the light chains it was necessary to leave a gap in part of the light chain sequence, suggesting that some time during the evolutionary divergence of heavy and light chains (assuming they are evolutionarily related), an insertion may have occurred in the gene for the heavy chain (or a deletion in the gene specifying light chain).

Comparisons of heavy chains and Fc fragments suggest that much of the variability of different myeloma proteins, and presumably antibodies, resides in the Fc fragment (Frangione, 1965). The accumulated evidence indicates that heavy and light chains have similar structures. The variable regions are approximately the same size, but the constant region of the heavy chains is about three times as long as that of the light chain. The data obtained by Gottlieb et al (1968) suggest that the variable regions are evolutionarily related and that it is possible that they hav similar conformations. This evidence is somewhat contradic ory to that of Edelman et al (1969) who compared the sequenc of chains from a human IgG immunoglobulin with another myeloma protein. The results suggest that the variable regions of heavy and light chains are homologous and similar in length. The constant portion of the heavy chain contains three "homology regions" each of which is similar in size and homologous to the constant region of the light chai

Increased information concerning the amino acid sequences of polypeptide chains in immunoglobulins has also resulted in elucidation of the position of disulphide linkages. For example it has shown that there are two inter-heavy chain disulphide bonds in IgG 1 proteins; the

half cystines which form these bonds lie within four residues of each other. The amino acid sequence in the vicinity of these bonds is identical in three human IgG 1 heavy chain (Gally et al, 1968); Frangione and Milstein, 1967); similar amino acid sequences occur in IgG 3 and IgG 4 proteins. Frangione (1968) and Milstein (1967) have suggested that in contrast to other subclasses IgG 3 immunoglobulins probably have five inter-heavy chain disulphide bonds.

Human IgG immunoglobulins contain about 3% carbohydrate, and most of the carbohydrate is attached to the heavy chain at one position in the Fc portion of the constant region. Glycopeptides with similar sequences have been obtained from IgG immunoglobulins of several mammalian specie (Howell, et al, 1967). It has been shown that in about 35% of rabbit IgG immunoglobulins, a single residue of galactosamine is attached glycosidically to the hydroxyl group of a threonyl residue just preceding the half cystine which forms an inter-heavy chain disulphide bond (Smyth and Utsumi, 1967) Moroz and Uhr (1967) have shown that the carbohydrate is attached to the IgG molecule after the synthesis and assembly of the polypeptide chains but before the molecule is secreted from the cell. There is no evidence that the carbohydrate moiety is concerned with antibody specificity.

The second major class of immunoglobulins, IgM immunoglobulins has been previously described (page 13). This class contains the first antibodies to appear in the serum in certain mammalian primary immune responses (Uhr, 1964). The basic structure of the IgM molecules has been referred to (page 14). More recently optical rotatory dispersion studies suggest that the five units of IgM immunoglobulins interact only weakly (Dorrington and Tanford, 1968). Electron micrographs of normal and pathological IgM immunoglobulins have shown spider-like figures with five legs of varying lengths, often connected by a circle or a semicircle in the centre of the molecule. The dimensions of the legs suggest that they might represent IgG-like subunits of the IgM molecule (Svehag et al, 1967). The amino acid sequences of μ chains from four pathological IgM immunoglobulins have been reported (Bennett, 1968). The amino acid terminal residue appears to be PCA in all four proteins, and the amino terminal sequences are similar to the corresponding sequences in human μ chains.

Kishimoto et al,(1971) have reconfirmed that the IgM antibody molecule has ten detectable combining sights and that one half of the total combining sites in a given preparation had a much higher affinity for the specific hapten

than the other half. They were able to show that equal numbers of high and low-affinity sites appeared to be present on a single molecule of antibody. The $F(ab^1)_2$ fragment prepared by peptic digestion of the IgM antibody showed similar hapten-binding properties to those of the native molecule, indicating that the observed difference in the affinity of the combining sites was not related to the state of assembly of the subunits of the native molecule.

The third major class of immunoglobulins is the serum IgA immunoglobulins (page 11). IgA immunoglobulins exist both as monomeric proteins, containing two light chains and two α chains, and as polymers of two or more of the monomeric units linked probably through disulphide bonds between the α chains (Abel et al, 1968). Light chains - heavy chain disulphide bonds appear to be absent in certain mouse IgA myeloma proteins (Abel et al, 1968) and also in the minor IgA 2 subclass of human immunoglobulins (Grey and Kunkel, 1968). In these molecules, the light chains are present as monomers or as disulphide-bonded light chain dimers non-covalently attached to the heavy chains (Abel et al, 1968). It has been suggested (Edelman and Gally, 1969) that these findings may be related to the observation that isolated chains can exist as dimers held together

either by non covalent interactions or by a disulphide bond (Gally and Edelman, 1964). The work of Abel et al, (1968) suggests that mouse α chains may be considerably shorter than other mammalian α chains, and that the absence of a light chain-heavy chain disulphide bond could therefore be explained by deletion of the region of the chain containing the half-cystine which forms part of the interchain bonds in the other proteins.

Secretary IgA is mainly an immunoglobulin dimer containing a glycoprotein usually called secretory piece (SP). The dimer is apparently synthesized by cells (called immunocytes) adjacent to secretary epithelial cells, and the majority of current evidence indicates that its conjugation with SP takes place subsequently (Brandtzaeg et al, 1970). It has been claimed by some workers (Rossen et al, 1969) that the immunoglobulin moiety and the SP are produced by the same cell but the majority of workers have reported the presence of SP only in the epithelium (Tomasi et al, 1965; Cebra, 1969; Tourville and Tomasi, 1969). Immunofluorescence with a highly specific antiserum demonstrated free SP in the cytoplasm of secretary epithelial cells, and also associated with their cell membranes - perhaps intercellularly (Brandtzaeg, 1969). It is therefore difficult to

decide at present whether IgA is complexed with SP inter-, intra- or extracellularly. Moreover, very little is known about the conjugation mechanism. Structural studies of human secretary IgA have indicated that the process primarily induces inter-unit disulphide bonds, and that the quaternary structure thereafter is noncovalently stabilized (Brandtzaeg, 1970). It has been suggested that this protei conjugation may be of biological significance by rendering secretory IgA antibodies relatively resistant to degredation (Brown et al, 1970).

The most popular theory as to the gentic determination of the peculiar structure of antibodies is that outlined by Gally and Edelman (1970). The most significant feature of the antibody molecule is the organisation of each polypeptid chain into variable and constant regions. The variation in the amino acid sequences of the constituent chains is the basis of antibody diversity.

It has been suggested that IgG molecules are different from other proteins in that each polypeptide chain appears to be specified by two genes rather than one. The suggesti is that there are separate genes for V and C regions. Since studies of immunoglobulin synthesis strongly suggest that the chains are made in one piece from a single messenger RNA

(Fleishman, 1967), it follows that in the precursors of antibody-forming cells a mechanism must exist to join information from separate V and C genes at the DNA level. The joining process has been named translocation.

Edelman and Gally (1970) have postulated that each major kind of immunoglobulin chain is specified by a cluster of linked genes which they have called a translocon. V and C genes within a translocon function in a coordinated way to construct a nucleotide sequence specifying the entire immunoglobulin polypeptide chain. The molecular mechanisms of translocation is presently a matter for speculation, however these workers claim that the translocation hypothesis can account for a number of singular phenomena in the cells of immunity. For instance, the apparent ability of cells which have been synthesizing IgM immunoglobulin to switch over to the production of IgG antibodies directed towards the same antigen. The switch can be explained as a result of a translocation of a V gene from a position next to the C gene that specifies the μ chain to a position next to the C gene for the γ chain. It has also been suggested that translocation mechanisms may be important in influencing the differentiation of antibody - forming cells. First, translocation would restrict the number of different antibod

chains that a lymphoid cell can make. This is significant because the clonal selection theory requires that each cell be restricted to make antibodies of only one specificity. Secondly, the translocation event suggests an hypothesis to explain the origin of antibody diversity. It is not known conclusively whether the diversity of V regions arises solely during evolution of animal species. Edelman and Gally (1970) support the theory that special mechanisms exist to alter the V region gene sequences during mitotic proliferation of the precursons of antibody forming cells. It is claimed that such a somatic mechanism would help explain the enormous repertoire of different antibody sequences.

BIBLIOGRAPHY - ADDENDUM

ABEL, C.A., and GREY, H.M. (1968) Studies on the Structure of Mouse IgA Myeloma Proteins. Biochemistry. 7 : 2682

BENNETT, J.C. (1968) The Amino Terminal Sequence of the Heavy Chain of Human Immunoglobulin M. Biochemistry. 7 : 3340

BRANDTZAEG, P. (1969) A new antigenic determinant of secretory immunoglobulin A. Acta path. microbial. Scand. 77 : 334

BRANDTZAEG, P., FJELLANGER, I. and GJERULDSEN, S.T. (1970) Salivary secretions from individuals with normal or low levels of serum immunoglobulins. Scand. J. Haemat. 12 : 83

BROWN, W.R., NEWCOMB, R.W. and ISHIZAKA, K. (1970). Proteolytic degredation of exocrine and serum immunoglobulins. J. clin. Invest. 49 : 1374

CEBRA, J.J. (1969) Immunoglobulins and immunocytes. Bact. Rev. 33 : 159.

CEBRA, J.J., and SMALL, P.A., Jr. (1967) Polypeptide Chain Structure of Rabbit Immunoglobulins.III. Secretory IgA - Immunoglobulin from Colostrum. Biochemistry. 6 : 503.

DORRINGTON, K.N., and TANFORD, C. (1968) The Optical Rotatory Dispersion of Human IgM - immunoglobulins and their Subunits. J. Biol. Chem. 243 : 4745.

EDELMAN, G.M., CUNNINGHAM, B.A., EINARGALL, W., GOTTLIEB, P. RUTISHAUSER, U., and WAXDAL, M.J. (1969) The covalent structure of an entire IgG Immunoglobulin molecule. J. Proc. Soc. U.S. Nat. Acad. Sc. 63 : 78.

FLEISCHMAN, J.B. (1967) Synthesis of IgG Heavy Chain in Rabbit Lymph Node Cells. Biochemistry. 6 : 1311.

FRANGIONE, B., and FRANKLIN, E.C. (1965) Structural Studies of human immunoglobulins. Differences in the Fd fragments of the heavy chains of myeloma proteins. J. Exptl. Med. 122 : 1.

FRANGIONE, B., and MILSTEIN, C. (1967) Disulphide Bridges of Immunoglobulin G1 Heavy Chains. Nature. 216 : 939.

GALLY, J.A., and EDELMAN, G.M. (1971) Somatic Translocation of Antibody Genes. Nature. 227 : 341

GALLY, J.A., and EDELMAN, G.M. (1964) Protein - Protein interactions among L Polypeptide Chains of Bence - Jones Proteins and Human IgG immunoglobulin J. Exptl. Med. 119 : 817.

GOTTLIEB, P.D., CUNNINGHAM, B.A., WAXDAL, M.J., KONIGSBERG, W.H. and EDELMAN, G.M. (1968) Variable Regions of Heavy and Light Polypeptide Chains of the same IgG - Immunoglobulin Molecule. Biochemistry 61 : 168.

GRUBB, R. (1956) Agglutination of Erythrocytes coated with "incomplete" Anti-RH by certain Rheumatoid Arthritic Sera and some other sera. Acta Path. Microbiol. Scand. 39 : 195.

HABER, E. (1968) Immunochemistry. Ann. Rev. Biochem. 37 : 497.

HILSCHMANN, N., and CRAIG, L.C. (1965) Amino Acid Sequence Studies with Bence - Jones Proteins. Proc. Natl. Acad. Sc. U.S. 53 : 1403.

HOWELL, J.W., HOOD, L., and SANDERS, B.G. (1967) Comparative analysis of the IgG Heavy Chain Carbohydrate Peptide. J. Mol. Biol. 30 : 555.

KISHIMOTO, TADAMITSU and KAORU ONOUE. (1971) Properties of the combining sites of rabbit IgM antibodies. (1971) J. Immunol. 106 (2) : 341.

MOROZ, C., and UHR, J.W. (1967) Cold Spring Harbor Symp. Quant. Biol. 32 : 263.

PRAHL, J.W., MANDY, W.J. and TODD, C.W. (1969) The Molecular Determinants of the A11 and A12 Allotypic Specificities in Rabbit Immunoglobulin. Biochemistry. 8 : 4953.

PRAHL, J.W., and PORTER, R.R. (1968) Allotype-Related Sequence Variation of the Heavy Chain of Rabbit Immunoglobulin G. Biochem. J. 107 : 753.

PRESS, E.M., GIVOL, D., PIGGOT, P.J., PORTER, R.R. and WILKINSON, J.M. (1966). Proc. Koy. Soc. B. 166 : 150.

ROSSEN, R.D., SOUTH, M.A., and BUTLER, W.T. (1969) Localization of external secretary IgA piece by immunofluorescence in tissues from patients with agammaglobulinemia. Prot. biol. Fluids. 16 : 325.

SMYTH, D.S., and UTSUMI, S. (1967) Structure at the Hinge Region in Rabbit Immunoglobulin - G. Nature. 216 : 332.

SVEHAG, S.E., CHESEBRO, B., and BLOTH, B. (1967) Electron microscopy of virus - IgM antibody' complexes and free IgM immunoglobulins. Nobel Symp. 3 : 269.

TURNER, K.J., and CEBRA, J.J. (1971) Structure of heavy chain from strain 13 guinea pig immunoglobulin - G (2) : II Amino acid sequence of the carboxyl-terminal and hinge region cyanogen bromide fragments. Biochemistry. 10 (1) : 9.

WILKINSON, J.M. (1969) Variation in the N-Terminal Sequence of Heavy Chains of Immunoglobulin G from Rabbits of Different Allotype. Biochem. J. 112 : 173.

WILKINSON, J.M., PRESS, E.M., and PORTER, R.R. (1966) The N-Terminal Sequence of the Heavy Chain of Rabbit Immunoglobulin IgG. Biochem. J. 100 : 303.