M.Sc. Experimental Medicine

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NORMAL HUMAN SERUM FACTORS WHICH BLOCK PRAUSNITZ-KUSTNER SENSITIZATION WITH REAGINS

ABSTRACT

A study was made of factors in normal human sera which are able to block Prausnitz-Kustner (P-K) sensitization with ragweed reagins. DEAE-Sephadex fractions of normal human serum containing YG as the only detectable immunoglobulin had the highest activity in blocking P-K sensitization. Fractions containing YA globulin had minimal activity while YM fractions had little or no blocking activity. Heterogeneity of blocking factors in normal human sera was demonstrated by sucrose density-gradient centrifugation and Sephadex gel filtration. High molecular size fractions, with components sedimenting at 7S or greater were active, but the most active of the blocking fractions was a low molecular size fraction, sedimenting at 1-3S.

JAMES ALROY PHILLS

Short Title:

HUMAN SERUM FACTORS WHICH BLOCK

SENSITIZATION WITH REAGINS

NORMAL HUMAN SERUM FACTORS WHICH BLOCK PRAUSNITZ-KUSTNER SENSITIZATION WITH REAGINS

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TABLE OF CONTENTS

PAGE

PAGE

PAGE

PAGE

PREFATORY NOTE

The present investigations originated in close connection with the problem of the immunoglobulin nature of the skin sensitizing antibodies in human allergic serum. It is therefore appropriate to preface a consideration of reagins in allergic serum and blocking factors in normal serum with a review of some of the structural and biological properties of the immunoglobulins.

PART I: GENERAL INTRODUCTION

CHAPTER I

THE IMMUNOGLOBULINS: SOME STRUCTURAL AND BIOLOGICAL PROPERTIES

1. Heterogeneity of the Immunoglobulins

Antibodies belong to groups of serum proteins collectively known as the immunoglobulins (or γ globulins). The immunoglobulins display considerable heterogeneity in the parameters of charge, size, and antigenic determinants. Recent studies have elucidated the molecular structure of the various groups of immunoglobulins and the relationship of structure to the biological activities of these proteins.

a. Charge Heterogeneity

In 1938, Tiselius and Kabat (1) localized the antibody-active proteins of serum to those globulins that displayed the least electrophoretic mobility. On paper electrophoresis, the bulk of the γ globulins migrate as a broad, diffuse band to occupy the relatively slow, cathodic region (the "γ region") of the electropherogram (Fig. 1). Some immunoglobulins, however, have mobilities corresponding to the "β" and even "α" region of

the electropherogram. The broad and diffuse nature of the "γ" electrophoretic band is evidence of the heterogeneity of charge of the constituent immunoglobulins.

Differences between the net charges of serum protein molecules are the basic of a widely used method of separating γ globulin fractions: viz., ion-exchange chromatography. Thus, a column of DEAE-cellulose treated with a crude immunoglobulin solution and eluted successively with buffers of increasing molarity and decreasing pH can be made to yield successive peaks containing γ globulins of increasing net molecular charge (2, 3).

b. Molecular Weight Heterogeneity

In addition to charge, the γ globulins show heterogeneity of molecular size and weight (4, 5). Ultracentrifugal analysis of electrophoretically separated fractions of serum globulin shows that 7S is the characteristic sedimentation coefficient of the bulk of the γ globulin in normal and immune serum (6). This 7S fraction has a molecular weight of about 145,000, and includes both the electrophoretically slowest γ as well as γ globulins of higher mobility. Antibody activity is also present in the more rapidly sedimenting (19S) component of immune serum, of about 1,000,000 molecular weight (7). This 19S component moves electrophoretically with the faster γ

globulins.

c. Antigenic Determinants

Immunodiffusion (8) and immunoelectrophoresis (9) in agar have revealed the existence of antigenic similarities and differences between 19S and 7S γ globulins (10). For example, rabbit antiserum prepared against human 19S gave precipitin lines with both 19S and 7S on gel diffusion against whole human serum. After specific anti-7S antibody had been removed by the addition of purified 7S γ globulin and spinning out the resulting precipitate, the antiserum (absorbed in this way) still gave a precipitin line with 19S globulin. Thus, 19S and 7S γ globulins each have some specific antigenic determinant not shared with the other.

The antigenic heterogeneity of γ globulins was further revealed with the discovery of a third class, the γA globulins (11), electrophoretically faster moving than the bulk of the 7S γ globulin and antigenically distinct from both it and the 19S γ globulins (10,11).

Immunoglobulins are differentiated on the basis of antigenic specificity. All antibodies or immunoglobulins share a common antigenic structural unit, the light or L chain (12).

The heavy or H chains (13) of the immunoglobulin classes are antigenically distinct and determine the characteristics of each of the major classes of immunoglobulins, which are designated (14) as: γG, γA and γM. The antigenic determinants that confer specificity on the different classes are a property of the Fc region of the heavy chains (see below). Within the γG and γA immunoglobulin classes, there are subclasses defined by additional antigenic determinants located on the H chain (15, 16). Two antigenic types of L chains (κ and λ) have been described which are common to all immunoglobulin classes (17,18).

2. Structure of γG Globulin

a. Polypeptide Chains

As noted earlier, the γG globulins have a molecular weight of approximately 145,000. Each molecule is made up of two identical L and two identical H polypeptide chains (Fig. 2) (19, 20). Each light chain has a molecular weight of approximately 20,000 and differs markedly in primary structure (amino acid sequence) from the H chain, which has a molecular weight of approximately 53,000 (21). In addition to non-covalent bonds, the light and heavy chains are held together by

disulfide bonds, one disulfide bond holding the two H chains together and at least one disulfide bond linking one L chain to one H chain (22). The light and heavy chains can be readily dissociated by reduction with such agents as mercaptoethanol followed by alkylation with iodoacetamide (19).

b. Enzymatically Prepared Fragments

Porter (23) reported that papain digestion split rabbit YG globulin into 3 fragments. Two of the fragments were similar, each containing one antibody site capable of specific combination with antigen. The univalent fragments were therefore designated Fab (antibody fragments). The third fragment derived from rabbit YG globulin was crystallizable, and hence called the Fc fragment. Human YG globulin can be similarly digested by papain to produce equivalent fragments (24, 25), and these fragments have the same nomenclature as described above for the rabbit fragments (14). The Fab and Fc fragments have molecular weights of 52,000 and 48,000, respectively. Both have the same sedimentation co-efficient of 3.5S (26, 27). The Fab fragment can be further degraded by reduction to its constituent parts: the L chain and part of the H chain (the Fd piece).

Nisonoff et al (28) showed that pepsin degrades YG

globulin to a 5S fragment with a molecular weight of 100,000. The 5S fragment is composed essentially, but not precisely, of two Fab fragments (29), and retains bivalent antibody activity, so that it precipitates with antigen (28). This fragment is designated F $(ab)_2$. The Fc portion of the YG globulin molecule is digested into smaller fragments by pepsin (30). The $F(ab)_2$ fragment can be split into 2 identical halves by reduction of a single disulfide bond (31).

Fig. 3 shows the model of the YG antibody molecule suggested by Edelman and Galley (32). The molecule has an ellipsoid shape measuring 240 Å in length and 19Å by 57 Å in cross section. The combining sites in this model are visualized as shallow craters, each involving one L and one H chain. The N-terminal amino acids are at the antibody combining site, while the C-terminal amino acids are at the other end of the molecule on the H chains. The carbohydrate moiety is fixed to the H chains at the C-terminal end.

The sequence of degradation steps to chains and fragments of the above model of a YG globulin molecule is shown in Fig. 4 (32).

3. Structure of Other Immunoglobulins

a. YA and YM Globulins

The YA and YM globulins represent combinations of light with heavy chains. The L chains of YA and YM are reported to be the same as those of YG. Thus, antibodies against YG light chains react with both YA and YM (33).

As has already been noted, the individual classes of Y globulin rest on differences in the antigenic structure of the heavy chains of these proteins, differences readily distinguishable by means of specific antisera. The YA molecule is assumed to be a polymer of a basic group of two light chains and two heavy chains. The sedimentation co-efficient of YA globulin ranges from 8S to 14S, but most of the YA globulin in serum is of the 8S variety (34).

The molecular weight of YM globulin is approximately 800,000 to 1,000,000, or 5 to 6 times that of YG (35). Since reduction of YM globulin readily dissociates the molecule from one with a sedimentation co-efficient of 19S to one of approximately 7S (36), the YM globulin molecules appear to consist of five to six 7S units.

A further difference in the structure of the Y

globulins relates to their sugar content. YG globulin contains 2.5% by weight of carbohydrate (37). It is not known at present if the carbohydrate plays any part in the antigenicity or function of the YG molecule. YA globulin contains about 10% carbohydrate associated with its H chain (34). Like the YA globulins, YM globulins contain 10% carbohydrate by weight associated with the H chain (37).

b. Low Molecular Weight Immunoglobulins

The low molecular weight immunoglobulins of serum have antigenic structures similar to those of light chains and react with antisera against the light chains of YG globulin (38, 39). They have molecular weights of approximately 15,000 and appear to contain at least two groups of related proteins: one with sedimentation co-efficient of approximately 3S and an electrophoretic mobility of γ_1 globulin (anodic); the other, having a sedimentation co-efficient of approximately 2S and an electrophoretic mobility of a γ_2 globulin (cathodic) (40). Williams and Schmid (41) have shown that only 21% of the isolated 3Sγ_1 globulins contain antigenic structures similar to the light chains. The remainder are antigenically distinct from the conventional immunoglobulins, as well as their derivative fragments. This could be interpreted to mean that other

immunoglobulin structures which are present in the $3SY_1$ globulin and which are not related to light chains are poor antigens.

Low molecular weight Y globulins are also found in the urine (42, 43). The urinary proteins have an electrophoretic mobility between the Y_1 and Y_2 globulins at pH 8.6, an average sedimentation co-efficient of 1.73, a molecular weight of approximately 12,900 and thermosolubility properties like those of Bence-Jones proteins. These Y globulins are immunochemically related to the L chains of other immunoglobulins, but their size and the results of immunochemical studies indicate wide differences between these proteins and L chains (44). The origin of the low molecular weight Y globulins in serum and urine is controversial and it would appear that they are in part breakdown products of serum Y globulin and in part synthesized independently (39, 44).

c. Secretory YA Globulin

The body secretions (saliva, tears, colostrum, nasal and bronchial secretions, etc.) contain immunoglobulins, of which YA is present in highest concentration (45). Serum and secretory YA globulins are not structurally identical upon antigenic analyses. It appears that secretory YA globulins contain an additional structural unit (T chain) having the same

molecular weight as the L chains but, presumably, antigenically distinct from the latter. Secretory YA globulins are principally 11S molecules consisting of 4 H chains, each having a molecular weight of 64,500, 4 L chains, each of 22,000 molecular weight and 1 T chain, of 22,000 molecular weight (45). The "T" chain or "transport piece" apparently facilitates transport of these molecules into secretions.

4. Biological Properties of the Immunoglobulins

a. Antibody Activity

The YG globulins comprise at least 80% of the serum antibodies. Antibodies to gram positive pyogenic bacteria, antiviral antibodies and anti-toxins in adult human sera are found almost exclusively among the YG globulins.

The YA globulins comprise about 10% of the antibodies in human sera. Isohemagglutinins, anti-brucella, anti-diptheria and anti-insulin antibodies have been found in isolated YA fractions (46, 47). YA globulin is also found in relatively large amounts in saliva, colostrum and tears, all of which contain only a trace amount of YG globulin (45, 48).

Between 5 and 10% of the total serum antibody is found in the YM class. The YM antibodies, which usually appear first in the course of immunization (49), readily coat particulate antigens such as bacteria (50), but bind relatively poorly to soluble antigens such as toxins. Thus, most of the human serum antibodies to the lipopolysaccharide antigens are YM globulins. The heterophile and Wasserman antibodies, cold agglutinins, and antibodies to endotoxins of gram negative bacteria are found almost exclusively in the YM globulin class (51).

b. Placental Transport

The normal human infant is born with a plasma concentration of YG globulin equal to or higher than that of the mother and very little, if any YM or YA. This quantitative relationship is attributable to the fact that YG globulin readily traverses the placenta while YM and YA do not, and to the fact that the infant at birth synthesizes little or no YG or YA and very little if any YM (52). The transfer of YG globulin from mother to fetus, as well as the failure of such transfer of YA and YM has been attributed to the specific structure of the Fc portion of the YG heavy chains (52). The permeability of the placenta to YG globulin and its impermeability to YA and YM is not simply a matter of molecular size, since albumin and

transferrin (molecular weights: 65,000 and 90,000, respectively) cross the placenta far less readily than YG (53). Evidence for the presence of an active transport site on the Fc piece is further suggested by studies in rabbits which demonstrated that the Fc fragment of YG globulin passes the placental barrier much more readily than does the Fab fragment (52).

c. Complement Fixation

The complement fixation reaction is initiated by Y globulin in antigen-antibody reactions (54). Complement fixation can also be produced by aggregated Y globulin (55) or Fc fragments (54). However, the 5S pepsin fragment (which lacks the Fc region) can also fix a significant amount of complement, so that the precise localization of this function is not clear (56).

d. Fixation of Antibody to Skin

The antibodies that mediate certain hypersensitivity reactions have the property of fixing to skin and other tissues (57). The technique of passive cutaneous anaphylaxis (PCA) has been used to investigate the skin attaching properties of various antibodies in the guinea pig (58). In the PCA reaction,

intradermal injection of antiserum is followed (after a latent period of 3 or 4 hours) by intravenous injection of antigen together with a blue dye. A positive reaction is shown by diffusion of dye through a localized area of increased permeability due to release of histamine and other vasoactive mediators (58). The latent period is required presumably for antibody to become fixed to cells in situ (e.g., to mast cells) (59).

Ovary et al (57) showed that human YG antibodies are able to elicit a positive PCA test in guinea pigs whereas YM antibodies of the same specificity gave a negative response, apparently owing to an inability to fix to guinea pig tissue. Subsequent studies have shown that normal human YA and nine different YA myeloma proteins belonging to the two major antigenic groups, were all unable to sensitize guinea pig tissues (60). These studies indicate that, of the human immunoglobulins, only YG can sensitize the skin of the guinea pig. Further investigation revealed that the Fc piece of the YG globulin was the skin fixing fragment of the molecule (61).

Of the 7S antibodies of the guinea pig, only the Y_1 electrophoretic group can sensitize guinea pig skin for PCA reaction; the Y_2 group is inactive (62). Similarly, the rat, rabbit, dog and mouse can be passively sensitized for PCA by the corresponding Y_1, and not by Y_2 antibodies. Such tissue-

fixing antibodies have been termed homocytotropic (63).

The γ_2 guinea pig antibodies can sensitize the skin of other species (e.g., man and mouse) and have therefore been called heterocytotropic. Mammalian antibodies in general can be categorized as homocytotropic, (i.e., they sensitize the homologous species) and heterocytotropic (i.e., they sensitize a number of foreign species). In Table I are summarized the physico-chemical and biological properties of mammalian cytotropic antibodies.

CHAPTER II

BIOLOGICAL ACTIVITY AND IMMUNOGLOBULIN NATURE OF HUMAN REAGINS

Clinical allergy is found in a segment of the population with an inherited predisposition to become hypersensitive to environmental substances, such as the pollens of trees, grasses, ragweed, as well as foods, mould spores, fungi and dust. Such individuals (termed atopics), on inhalation or ingestion of the appropriate allergen rapidly develop symptoms such as rhinitis, asthma and urticaria. The combination in vivo of allergen with human homocytotropic antibodies (fixed to target organ cells, such as mast cells) causes the release of pharmacological mediators such as histamine. The mediators, in turn, act on secondary tissues, mainly smooth muscle and blood vessels, and the symptom complex produced constitutes the clinical manifestations of the allergic state. The homocytotropic antibodies of man responsible for atopic hypersensitivity are called skin sensitizing or reaginic antibodies.

1. Fixation of Reagins to Skin and other Tissues

a. Systemic Anaphylaxis

The role of reaginic antibodies in systemic anaphylaxis is supported by indirect evidence. All of 18 patients with recent immediate systemic reactions to penicillin had skin sensitizing anti-pencillin antibodies in their serum demonstrable by the P-K test (64). There was a good correlation between skin sensitizing antibody titers and severity of symptoms accompanying intravenous injection of ragweed extract in a group of ragweed allergic patients (65.) In another group of patients undergoing treatment with aqueous ragweed extract or ragweed extract emulsions, skin sensitizing antibody titers were significantly higher in patients who had systemic reactions compared to control patients (66).

Blood obtained from allergic donors and transfused to normal recipients produced clinical symptoms in these recipients when they were exposed to the specific allergen. On direct skin tests with specific allergen, the transfused recipients showed positive wheal and erythema reactions, indicating that following transfusion, the reaginic antibodies had become fixed to at least this tissue (67).

b. Sensitization by Local Cutaneous Transfer

A characteristic feature of reaginic antibodies is their marked capacity to sensitize human epithelial tissue. This property forms the basis for the passive transfer or Prausnitz-Kustner (P-K) test (68) for the detection and assay of serum reagins. Aliquots of serum containing reaginic antibody are injected intradermally into the skin of normal recipients, followed, after a period of 24 - 48 hours, by challenge with antigan administered by intradermal injection or pin prick at the same site. A wheal and erythema reaction (due to local release of histamine) is produced at this site indicating the fixation of reaginic antibody to the skin of the normal individual. The P-K test continues to be a primary method of assay of reaginic antibodies (69).

Stanworth (70) confirmed earlier observations that reaginic antibodies are firmly and quickly attached to fixed tissue elements. Studies by Loveless et al (67) indicated that human reaginic antibody persists at a passively sensitized skin site for at least 4 weeks. The amount of reaginic antibody necessary for passive transfer is extremely minute: for example, Ishizaka et al (71) reported that passive sensitization could be accomplished with as little as 10^{-4} µg of antibody nitrogen.

In contrast to human YG globulin antibody, human

reaginic antibody does not appear to sensitize the guinea pig for PCA (72). However, passive transfer of sensitivity has been accomplished in the monkey (73) and other sub-human primates (74). Indeed, Rose et al (75) have demonstrated a close correspondence between the P-K titers in humans and the PCA reactions obtained in the monkey. It would appear that the skin fixing activity of human reagins is species specific.

c. Anaphylactic Reactions in Vitro

Katz and Cohen (76) first demonstrated the release of histamine from venous blood of allergic individuals following in vitro contact with specific antigen. Lichenstein and Osler (77) employed washed human leukocytes from ragweed sensitive donors in an in vitro test for the detection of reaginic antibodies and concluded that such leukocytes acquire a coating of antibody in vivo. Washed leukocytes derived from the blood of non-allergic donors can be passively sensitized in vitro with reaginic serum to release histamine on challenge with specific allergen (78).

Several human tissues, including lung (70), appendix (80), uterus and ileum (81) have been passively sensitized with human allergic serum to give in vitro Shultz-Dale reactions on challenge with appropriate allergen.

Arbesman et al (82) have demonstrated that isolated strips of Rhesus monkey ileum may be passively sensitized with human allergic serum for Shultz-Dale reactions. In a further development, Goodfriend and his associates (83, 84) have demonstrated that chopped preparations of monkey lung and skin can also be passively sensitized with allergic serum to release smooth muscle stimulators on challenge with allergen.

2. Comparison of Reaginic with Blocking Antibodies

Cooke et al (85) observed that following hyposensitization therapy with specific allergen, serum of the treated allergic patients acquired the capacity to inhibit the response of passively sensitized skin to the specific allergen. Loveless (86) found that the antibody induced in normal subjects by cutaneous injections of pollen allergen did not sensitize skin but inhibited (blocked) P-K sensitization with reagins by specifically combining with the same pollen allergen. Unlike reagins, the blocking antibody was stable after heating at 56°C for as long as 5 hours and disappeared from the inoculated cutaneous site in less than 24 hours.

Blocking antibody has been shown to sediment ultra-

centrifugally with the 7S components of serum (87) and to be eluted from DEAE cellulose with the γ_2 globulins (88).

The maternal fetal membranes in man appear to be impermeable to reaginic antibody (89), whereas the blocking antibody, a 7S γ_2 globulin, presumably directed against the same antigenic determinants, does cross these structures (90). Unlike blocking antibody, reaginic antibody appears to be incapable of fixing complement (77).

In addition to heat lability, human reaginic antibodies are inactivated by reduction with mercaptans, under conditions in which human γG antibodies retain their antigen binding capacity (91). It is not known whether the reducing agents act by damaging the tissue binding sites of reaginic antibody or whether they may affect other portions of the molecule, such as the antigen combining site. Ishizaka and Ishizaka (92) have suggested that the tissue binding site of the molecule is degraded.

3. Association of Reagins with γA Globulins

After the isolation and characterization of the γA globulin class (Chapter I), evidence accumulated that reaginic activity was associated with this class of immunoglobulins.

Augustin and Hayward (93) found that reaginic activity was mainly localized in those serum fractions obtained by DEAE-cellulose chromatography which were rich in YA globulins. Vaerman et al (94) also found reaginic activity in purified YA globulin fractions prepared from allergic sera by a zinc sulphate precipitation method. Terr and Bentz (95) fractionated four allergic sera by Sephadex G-200 gel filtration and found a close correlation between skin sensitizing activity and YA globulin fractions. The bulk of the skin sensitizing activity was eluted from the gel along with most of the YA globulin on the ascending side of the 7S peak. From the distribution it was inferred that the size of the reaginic antibodies was greater than 7S. This was confirmed in the studies of Anderson and Vanier (96) employing the technique of sucrose density-gradient centrifugation. The sucrose density-gradient fractions, containing the peak of reaginic activity, had a sedimentation coefficient of approximately 8S.

Important evidence associating reaginic antibodies with YA globulin was reported by Fireman et al (72). These workers performed immunoabsorption of reagin-rich YA globulin fractions by antisera specific to human YA myeloma globulin. Absorption of the YA globulin was accompanied by loss of reaginic activity.

Further evidence associating YA globulins and reaginic antibodies was the finding that human lacrimal and salivary secretions contain reaginic activity and YA as the only detectable immunoglobulin (97, 98).

Finally, the association of reaginic antibody with YA globulins was strongly supported by the finding by Ishizaka et al (99) that YA prepared from normal human serum by zinc sulphate precipitation blocked P-K sensitization with ragweed reagins. As this latter finding was of particular relevance to the present investigation, it will be discussed separately in the following chapter.

CHAPTER III

BLOCKING OF PRAUSNITZ-KUSTNER SENSITIZATION WITH REAGIN BY NORMAL HUMAN SERUM COMPONENTS

1. The PCA Model

As has already been noted, one of the characteristic properties of reaginic antibody is a relatively firm fixation to human epithelial tissues. A close experimental model of the biological activity of human reagin, in which tissue fixation has been thoroughly studied, is passive cutaneous anaphylaxis (PCA) in the guinea pig. Of particular relevance was the finding by Ovary (100) and others (101, 102) that the PCA reaction mediated by guinea-pig γ_1 antibody can be blocked very effectively by non-immune (normal) guinea-pig γ_1 globulin. A plausible mechanism for this effect is that the normal γ_1 globulin has a structure - particularly in the Fc region - similar to that of antibody γ_1 globulin and is able to compete with the latter for sites on the target organ cells. If this mechanism were valid for inhibition of cutaneous reactions in mammalian species in general, it would follow that the normal human serum globulin able to competitively inhibit the P-K reaction in man

would have a similar structure to the reaginic antibody.

2. Blocking of P-K Sensitization with Reagin by Normal Human YA Globulin

Based on the above general considerations, Ishizaka et al (99) studied the ability of normal human serum YG, YA and YM globulins to block P-K sensitization with ragweed reagins. The purity of the immunoglobulin fractions was established by immunodiffusion in agar with specific antisera. The reaginic sera utilized was diluted to 1:4000, sufficient to sensitize normal skin to give appreciable wheal and erythema reactions. In the blocking test procedure, the normal serum globulins were injected intradermally and 24 hours later the reaginic serum was injected into the same sites. The globulin-serum sites were challenged 24 hours later with aqueous ragweed extract. Ishizaka et al reported that 2.5 µg or more of YA globulin nitrogen effectively blocked the wheal and erythema reaction. In contrast, amounts of YG and YM globulin as high as 10 - 20 µg N were essentially without effect. From their findings, Ishizaka et al suggested that the most likely mechanism for the blocking of sensitization is that normal YA globulin has an affinity for the same tissue sites with which reagin combines. As fixation

of skin is a characteristic property of reagins, it was concluded that reagins are in fact YA globulins.

In a later study, Ishizaka et al (103) tested the separated H and L chains of normal human YA globulin for their ability to block passive sensitization with reagins. The blocking activity was found to be associated with the H chain rather than the L chain of the YA globulin molecules. Since the H chain carries the specific class antigenic determinants of the immunoglobulins, this finding was interpreted as further evidence that reaginic activity was specifically associated with the YA class of immunoglobulin.

3. Aims of the Present Investigations

Despite the accumulated experimental data associating reaginic activity with YA globulins, much evidence began to appear that cast doubt on this conclusion. Loveless (104) and others (105, 106) demonstrated that reagins were present in the sera of allergic individuals with no detectable YA globulin. Goodfriend and his associates (107-110) reported that fractions of allergic serum could be prepared by DEAE-Sephadex chromatography which were reaginically active and contained YG as the only detectable immunoglobulin. Menzel and Sherman (111) were

able to separate most of the reaginic activity of a ragweed allergic serum from almost all of the YA globulins by means of Rivanol fractionation. Terr and Bentz (112) found P-K transferable reaginic activity in the sera of non-atopic patients with serum sickness to be associated with their YG and YM fractions, while YA globulin rich fractions were inactive.

Thus, from the studies cited, evidence emerged that reaginic antibodies in both atopic and non-atopic sera might be associated with globulins other than YA. Such evidence conflicted with the finding by Ishizaka et al (99) that normal human YA globulin uniquely blocked passive sensitization with reagins of normal human skin sites. It was therefore considered of interest to re-examine the relationship of the major immunoglobulin classes YG, YA and YM, to blocking activity and to characterize the blocking factors in normal human serum. A report of some of the findings of this study has been published (113).

PART II: THE PRESENT INVESTIGATIONS

CHAPTER IV

RELATIONSHIP OF NORMAL HUMAN SERUM IMMUNOGLOBULINS TO BLOCKING OF PRAUSNITZ-KUSTNER SENSITIZATION

1. Introduction

As noted in Chapter III, Goodfriend and his associates (107-11) reported that reaginic activity was localized in DEAE-Sephadex fractions devoid of YA and containing YG as the only detectable immunoglobulin. The method of DEAE-Sephadex chromatography employed by these workers was adopted in order to establish the relationship between the major immunoglobulin classes and blocking activity in normal human serum. The DEAE-Sephadex fractions were compared for immunoglobulin content and activity in blocking P-K sensitization with ragweed allergic sera.

2. Materials and Methods

a. Sera

Six sera, four normal (A.K., A.B., T.W., J.P.) and two ragweed allergic (S.M., B.U.), were employed for this study. The sera were stored in aliquots at -10°C until used.

b. Allergen

An aqueous extract of short ragweed pollen of known nitrogen content, was purchased from Center Laboratories, Port Washington, N.Y. The extract was kept in sterile vials at 5°C.

c. DEAE-Sephadex Chromatography

DEAE-Sephadex A-50 (Pharmacia) was allowed to swell in distilled water for 72 hours and de-fined by repeated decantations. The slurry was equilibrated with the initial starting buffer before pouring into a water jacketed column (2.5 x 26 cm) thermostated at 5°C. The initial buffer was also run through the column for at least 24 hours prior to chromatography.

Aliquots of 6 ml of normal serum was applied to the

column and the proteins were eluted by Tris*-HCl buffers of pH 8.0. An initial peak was eluted with 0.1 M HCl buffer and subsequent peaks by a gradient of 0.1 to 0.5 M buffer (Method I). In separate experiments, the elution scheme of the Tris-HCl buffers, pH 8.0 was modified to include an initial step of 0.14 M followed by a gradient of 0.14 to 0.3 M and finally by a step of 0.5 M buffer (Method II). The eluates were collected in 5 ml volumes in a Spinco fraction collector refrigerated at 5°C. Eluate fractions were monitored by fluorescence in a Turner Fluorometer (Model 111) or by the absorption at 280 mμ in a Beckman DU spectrophotometer. The cuts for the various fractions were made according to the shape of the fluorescence or optical density curves. The fractions were concentrated to initial serum volume (6 ml) by ultrafiltration in Diaflo cells (Amicon Corp., Cambridge, Mass.) using UM-2 filters, and exhaustively dialysed against saline. The fractions were analysed for their immunoglobulin content (described below) and Millipore filtered (0.22 μ filters) prior to being assayed for blocking activity.

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* Tris = tris (hydroxmethyl) amino-methane, was of certified primary standard grade, purchased from Fisher Scientific Co., Montreal.

d. Gel Filtration on Sephadex G-200

Sephadex G-200 (Pharmacia) was allowed to swell in distilled water for 3 days and de-fined by repeated decantations. The slurry was equilibrated with 0.1 M Tris-HCl + 1.0 M NaCl, pH 8.0, before pouring into a water-jacketed column (2.5 x 100 cm) thermostated at 5°C. The same buffer was run through the column for at least 24 hours prior to chromatography. DEAE-Sephadex fractions of normal human serum (A.K.) were separately filtered on Sephadex G-200 and fractions were eluted with the same buffer employed for equilibration. Eluate fractions were collected in a Spinco fraction collector at 5°C. The eluates were pooled and the pooled fractions processed as described in Section c.

e. Single Radial Immunodiffusion

Normal sera and serum fractions were tested for their content of YG, YA and YM globulin by single radial immunodiffusion (114) using immunoplates of Hyland Laboratories. Three standard solutions (supplied by Hyland Laboratories) of known immunoglobulin content and three test solutions were separately deposited in the antigen wells of each immunoplate. The YA and YM immunoplates were incubated for 16 hours at room temperature

and the YG immunoplates for 4 hours at 37°C. The precipitate ring diameters formed were measured with a magnified scale. Standard curves drawn for ring diameter versus immunoglobulin concentration (mg/100 ml) were used to determine the immunoglobulin content of the sera and serum fractions. The experimental error varied from 5 to 15% and a sensitivity of 10 µg/ml was observed for YG and YA globulin and 20 µg/ml for YM globulin.

f. Blocking of P-K Sensitization

Aliquots (0.1 ml) of DEAE-Sephadex fractions of normal serum and saline controls were injected into the back of a normal (non-allergic) recipient and 24 hours later 0.05 ml of reaginic serum S.M. diluted 1:100 with saline, were injected into each of the same sites. One day later the sites were challenged with 50 PNU* of aqueous ragweed extract. The length and width of the wheals were measured and the square area of the wheal calculated. The square areas of the blocking and saline control sites were compared to determine the percentage blocking of the P-K reaction.

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* 1 PNU (protein nitrogen unit) = 10^{-5} mg.

3. Experiments and Results

a. Blocking Activity of DEAE-Sephadex Fractions Prepared by Method I

Normal human serum A.K. was chromatographed on DEAE-Sephadex A-50 (Method I) and the distribution of YG, YA and YM immunoglobulins among the eluate fractions which were obtained is shown in Fig. 5. The blocking effect of the DEAE-Sephadex fractions on the P-K reactions is given in Table II. While most of the fractions had blocking activity, those containing only YG globulin blocked most effectively. Fractions containing both YG and YA also blocked but to a lesser degree than those containing only YG globulin. On the other hand, Fraction IX containing YM, YG and YA globulins had no blocking activity.

b. Blocking Activity of DEAE-Sephadex Fractions Prepared by Method II

A better separation of the blocking factor from the YA globulin was obtained by DEAE-Sephadex chromatography using a modified system of elution buffers (Method II). The distribution of the immunoglobulins among the various fractions derived from serum A.K. is shown in Fig. 6. The activity of the fractions

in the blocking of the P-K reaction is shown in Table III. Again, preparations containing YG globulins only as well as both YG and YA globulins blocked effectively, whereas those containing YM, YA and YG had little or no blocking activity. It is noteworthy that fraction VI contained YA globulin at more than twice the concentration found for this immunoglobulin in the active fractions and yet was essentially inactive in blocking P-K sensitization.

A similar distribution of blocking activity was observed on DEAE-Sephadex chromatography (Method II) of normal human serum A.B. (Fig. 7, Table IV). In contrast to DEAE-Sephadex fraction VI of serum A.K., fraction VI of serum A.B. showed some blocking activity but of an appreciably lower order than the activity of fractions containing YG globulin alone.

Because of the marked difference in activity between fraction VI and other YA containing fractions, it was of interest to determine if a component in this fraction might be inhibiting the blocking effect. The non-blocking fraction VI and blocking fractions III and IV of serum A.K. were concentrated 2-fold and equal aliquots of the concentrated fractions were separately combined as shown in Table V. The addition of fraction VI to fractions III and IV was without effect on the activity of the latter preparations, demonstrating the absence

from fraction VI of an inhibitor of blocking activity.

c. Molecular Size Distribution of YA globulins in Blocking and Non-blocking Fractions

In view of the presence in normal serum of polymeric forms of YA globulin (115) it was possible that the failure of DEAE-Sephadex fraction VI to block P-K sensitization was due to the presence in this fraction of inactive polymeric and/or aggregated YA globulins as the predominant species of this protein. A study was therefore made to determine if the YA-containing blocking and non-blocking fractions of serum A.K. had the same molecular size distribution of YA globulins. After incorporating YG globulin to serve as a marker, a pool of the blocking fractions III, IV and V and the non-blocking DEAE-Sephadex fraction VI were separately filtered on Sephadex G-200. The elution profiles of optical density and YA globulin content are shown in Fig. 8. While the non-blocking fraction VI showed a relatively greater proportion of higher molecular weight YA globulins, the bulk of the YA globulins in both fraction VI and the pooled fraction peaked at identical positions relative to the YG globulin.

d. Time Study of Blocking Activity

A time study was carried out to determine how long the blocking factor in normal human serum retained its effect on passive sensitization (Table VI). All the blocking fractions maintained their effects for 5 days but to a lesser degree. It is noteworthy that the YG-containing fractions maintained their blocking activity for the longest period (10 days).

e. Effect of Dilution of YG-Fractions on Blocking Activity

Compared to other DEAE-Sephadex fractions, the YG globulin containing fractions I and II of both normal sera consistently showed the highest blocking activity (Tables II - VI). It was of interest to determine the blocking capacity of these fractions on dilution to lower levels of constituent YG globulin. The results shown in Table VII demonstrate that fractions I and II retained appreciable activity even after 30- and 5-fold dilution, respectively.

f. A Control Experiment

A control experiment was done to ensure that the blocking activity of fractions I and II was not due to the presence of breakdown products of the ion-exchanger having non-

specific blocking effects. For this purpose, 0.1 M Tris-HCl was allowed to pass through the column of DEAE-Sephadex in a volume equal to that required to elute serum fractions I and II (approximately 500 ml). The buffer eluate, after concentration to 6 ml, dialysis against 0.15 M saline, and millipore filtration, was found to have no activity in blocking passive sensitization with reagins.

4. Discussion

A study was made of the relationship of the YG, YA and YM globulins in DEAE-Sephadex fractions of normal human serum to the activity of these fractions in blocking P-K sensitization. The results demonstrated that YG globulin fractions as well as fractions containing both YG and YA globulins effectively blocked passive sensitization. Fractions containing YM in addition to YA and YG globulins showed either no or minimal blocking activity.

A time study demonstrated that the active DEAE-Sephadex fractions maintained their effect for 5-10 days. This would suggest a relatively firm, long-lasting fixation of the blocking factor (s) to normal human skin. It is noteworthy

that YG containing fractions were most active in this respect.

A control experiment excluded the possibility that the activity of the YG fractions was due to the presence of DEAE-Sephadex breakdown products having non-specific blocking effects.

CHAPTER V

STUDIES ON THE HETEROGENEITY OF THE BLOCKING FACTORS IN NORMAL HUMAN SERUM

1. Introduction

In the previous chapter, evidence was presented that the DEAE-Sephadex fractions of normal (non-atopic) human sera, containing YG as the only detectable immunoglobulin, could effectively block passive sensitization with reagins to ragweed. Indeed, the blocking activity of the DEAE-Sephadex fractions paralleled the YG more closely than the YA or YM globulin content of these fractions. In the studies to be described in this chapter, the most active YG globulin fraction was examined by cation exchange chromatography to determine if the blocking activity was uniquely associated with the YG globulin. To investigate the molecular size distribution of the blocking factors, the most active YG globulin fraction as well as whole normal human serum were analysed by gel filtration and sucrose density-gradient centrifugation.

2. Materials and Methods

a. Cation Exchange Chromatography

Carboxymethyl (CM-) Sephadex C-50 (Pharmacia) was allowed to swell in distilled water for 72 hours and de-fined by repeated decantations. The slurry was equilibrated with the starting buffer and this buffer was run through the column for at least 24 hours prior to chromatography.

An aliquot of 10 ml of DEAE-Sephadex fraction II (see DEAE-Sephadex chromatography, Method 1, Chapter IV) was applied to a column (2.5 x 26 cm) of CM-Sephadex and the proteins were eluted with sodium acetate-acetic acid buffers of pH 5.4. Elutions were carried out stepwise using molarities of 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35. A final step utilized 0.5 M Tris-phosphate buffer, pH 8.0.

b. Gel Filtration

Sephadex G-75 superfine (Pharmacia) was allowed to swell in distilled water for 3 days, and de-fined by repeated decantations. The slurry was equilibrated with 0.1 M Tris-HCl + 1.0 M sodium chloride, pH 8.0, before pouring. DEAE-Sephadex fraction II (10 ml) was applied to a Sephadex G-75 column

(5 x 100 cm) and proteins were eluted with the buffer employed for equilibration.

The fractions derived from both chromatographic procedures were dialysed against 0.15 M sodium chloride, concentrated by pressure filtration, filtered through millipore filters and tested for blocking activity.

c. Sucrose Density-Gradient Centrifugation

The sedimentation coefficients of blocking factors in normal serum and derivative DEAE-Sephadex fractions were determined by the method of Martin and Ames (116). Serum or serum fractions were dialysed for 16 hours at 5°C against phosphate-saline buffer (0.05 M sodium phosphate + 0.15 M sodium chloride, pH 7.0) and aliquots of 0.2 ml were overlayered onto a 5-20% sucrose density-gradient. Centrifugation was performed for 18 hours at 35,000 rpm in a Spinco Model L ultracentrifuge equipped with a swinging bucket rotor (S.W. 39) pre-cooled to 5°C. After completion of the run the tubes were pierced at the bottom and 13 drops (0.2 ml) were collected from each tube. For each serum or serum fraction three separate runs were performed so that a total of 1,8 ml of sample was applied to the sucrose density-gradient. The fractions obtained were exhaustively dialysed against saline, concentrated by pressure filtration to

1.8 ml, filtered through 1/2" Swinney millipore filters (0.22 μ) and tested for blocking activity.

A YG globulin preparation (Pentex Cohn Fraction 2) served as the standard and its distribution in the gradient was determined by measuring the optical density at 280 mμ in a Beckman DU spectrophotometer. A range of sedimentation coefficients of the effluent fractions was determined by comparing the distance of migration of the fractions with that of the YG globulin standard. A sedimentation coefficient of 6.9 S was employed for the YG globulin standard as determined in a Spinco Model E ultracentrifuge equipped with Schlieren optics.

Other Materials and Methods employed in the experiments of this Chapter were as described in Chapter IV.

3. Experiments and Results

a. Relationship of YG Globulin to Blocking of P-K Sensitization

Two normal human sera (A.K. and T.W.) were separately chromatographed on DEAE-Sephadex A-50. The fluorescence profile of the eluted fractions obtained for serum A.K. is shown in Fig. 9. A similar distribution was obtained for serum T.W. Fig. 9 also shows the immunoglobulin content and activity in

blocking P-K sensitization of DEAE-Sephadex fractions I-X. As can be seen, fractions I and II (hereafter denoted DEAE-I and DEAE-II, respectively) contained YG globulin and were most effective in blocking the P-K reaction. Fractions containing YG and YA globulins (V-X) also blocked but to a lesser extent. It is particularly noteworthy that fractions IX and X, which contained the bulk of the YM as well as the YA globulins and relatively small amounts of YG globulin, had little or no blocking activity. A similar distribution of immunoglobulins and blocking activity was obtained with serum T.W. (Table VIII).

b. CM-Sephadex Chromatography of DEAE-II

DEAE-II derived from serum A.K. was further resolved on CM-Sephadex and the results are shown on Fig. 10. The blocking activity was spread amongst the CM-Sephadex fractions and there was no apparent relationship between blocking activity and YG globulin content of the fractions. Two fractions (I and VI) contained little or no detectable YG globulin yet had appreciable activity.

c. Sucrose Density-Gradient Centrifugation

The results of CM-Sephadex chromatography of DEAE-II

indicated a possible heterogeneity of the blocking factors since blocking activity was found for fractions containing YG as well as fractions devoid of YG globulin. To further characterize the blocking factors, each DEAE-II separately prepared from two sera (A.K. and T.W.) was subjected to sucrose density-gradient centrifugation and the effluent fractions tested for blocking activity. Fig. 11 shows a typical optical density profile of the effluent fractions obtained with DEAE-II of serum A.K. A similar distribution was found for DEAE-II of serum T.W. Cuts were made according to the optical density profile covering a range of sedimentation coefficients of approximately 1-3S, 3-5S and >9S. The sucrose density-gradient fractions derived from both sera were tested for YG globulin content and blocking activity. The results obtained are given in Table IX. While sucrose density-gradient fraction II, containing the bulk of the YG globulin, had some blocking activity, fraction IV, devoid of detectable YG globulin and containing components peaking at 1-3S, was highly effective in blocking P-K sensitization.

d. Gel Filtration Chromatography of DEAE-II on Sephadex G-75

The presence of low molecular weight factor or factors with blocking activity in DEAE-II was confirmed by the results of gel filtration studies using Sephadex G-75. Fig. 12 shows

the fluorescence profile and YG globulin content in the effluent fractions obtained for DEAE-II of serum A.K. Similar results were obtained for DEAE-II of serum T.W. The effluent Sephadex G-75 for both sera were separately tested for blocking activity and the results are given in Table X. While fractions I and II of both sera, containing the bulk of the YG globulin, had significant blocking activity, the most retarded fraction (III), containing trace amounts of YG globulin, was the most active blocking fraction derived from both sera.

e. Size Heterogeneity of Blocking Factors in Normal Human Serum

The isolation of an active "low molecular weight fraction"(1-3S) in DEAE-II prompted an investigation of the molecular weight distribution and sedimentation coefficients of blocking factors in whole normal human serum. For this purpose, two normal sera (J.P. and A.K.) were separately examined by Sephadex G-200 gel filtration and by sucrose density-gradient centrifugation.

The Sephadex G-200 chromatogram for one of the sera (A.K.) is shown in Fig. 13. For both sera, some blocking activity was associated with the 19S and 7S peaks (peaks 1 and 2). The albumin peak (peak 3) was least active whereas fraction VI, eluted after the albumin peak, had the highest blocking activity.

The results of sucrose density-gradient centrifugation are shown in Table XI. For both sera, most of the blocking activity was located in the 1-3S region of the sucrose density-gradient. Appreciable activity was also found with components of sedimentation coefficients greater than 5S.

4. Discussion

The results of CM-Sephadex chromatography of DEAE-II indicated a possible heterogeneity of blocking factors since fractions devoid of YG as well as those containing YG globulin had appreciable blocking activity.

Evidence for size heterogeneity was obtained by sucrose density-gradient centrifugation of DEAE-II: 2 major blocking fractions were obtained, sedimenting in the range 1-3S and $>$5S, respectively, as well as a relatively inactive intermediate fraction of approximately 3-5S. Similar results were obtained by gel filtration of DEAE-II on Sephadex G-75. By both methods of fractionation, a low molecular weight fraction was particularly effective in blocking P-K sensitization.

Further evidence for size heterogeneity was obtained by gel filtration on Sephadex G-200 and sucrose density-gradient

centrifugation of whole normal human serum. Again, appreciable activity was found for relatively high molecular size fractions but the greatest activity was associated with low molecular weight fractions (of 1-3S by sucrose density-gradient centrifugation).

CHAPTER VI

STUDIES ON THE LOW MOLECULAR WEIGHT BLOCKING FRACTION

1. Introduction

As shown in Chapter V, gel filtration of DEAE-II on Sephadex G-75 yielded a fraction, retarded on the gel, which was highly effective in inhibiting P-K sensitization with ragweed reagins. The "low molecular weight" fraction consistently contained trace amounts of YG globulin. Studies were therefore undertaken to determine if the constituent YG globulin may have participated in or even completely accounted for the blocking activity of the fraction and to determine to what extent blocking activity was due to the low molecular weight components. For this purpose, the blocking activity of the fraction was determined before and after immunoabsorption of the YG globulin.

A control study was done to exclude the possibility that low molecular weight components were artifactually produced through the interaction of serum protein (e.g., YG globulin) with the ion-exchanger DEAE-Sephadex, and thereby to provide evidence that such components are natively present in normal human serum.

2. Materials and Methods

a. Sera

A pool of 2 normal human sera (A.K. and T.W.) was employed for this study. Also employed were goat antisera (Hyland) to human γ chain, human Fc-fragment, human κ and λ light chains, and human γG, γA and γM globulins, albumin and transferrin.

b. Immunoabsorption

The method of Avrameas and Ternynck (117) was employed to prepare insoluble antiserum to human γG globulin. Nine ml of a goat antiserum to human γG globulin (obtained from Hyland Laboratories) was lyophilized, and dissolved in 20 ml of 0.2 M acetate buffer, pH 5.0. Ethyl chloroformate (0.6 ml) was added dropwise to the solution with constant stirring. A pH meter (Beckman) was used to monitor the pH which was maintained at 5.0 by intermittent addition of 1N NaOH. The mixture was stirred for 15-20 mins., during which time a white flocculent precipitate formed (the polymerized antiserum). The polymer was allowed to stand at room temperature for 1 hour and centrifuged. After decanting the supernatant, the polymer was dispersed in 0.01 M phosphate + 0.15 M NaCl, pH 7.2 (phosphate-

buffered saline).

To remove excess ethyl chloroformate and any non-polymerized γ globulin, the precipitate was washed with 300 ml of phosphate-buffered saline, 100 ml of 0.1% sodium carbonate and 100 ml of 0.1 M. glycine-HCl, pH 2.2. The final polymer-buffer mixture was centrifuged at 10,000 rpm and the optical density (O.D.) of supernatant was determined in a DU spectrophotometer (Beckman) at 280 mu for the presence of protein. Washing was continued until the O.D. reading for the supernatant equaled that of the buffer. The polymer was washed with phosphate-buffered saline until the suspension and supernates reached pH 7.2.

The polymer was isolated by suction filtration through Whatman No. 2 filter paper and added to 9 ml of the serum fraction. The mixture was stirred overnight at 4°C and centrifuged to isolate the supernatant.

c. Immunodiffusion in Agar

Immunodiffusion tests in agar were performed on microscope slides by the method of Ouchterlony (8). The development of precipitin lines was allowed to proceed for 24-72 hours at room temperature in a humid atmosphere.

d. Polyacrylamide Gel Disc Electrophoresis

Disc electrophoresis was performed at pH 9.5 using the discontinuous buffer system of Ornstein and Davis (118, 119) in conjunction with a 6% running gel. Samples (1-2 ml) were layered on top of the gel in 3% sucrose. Electrophoresis was carried out in tubes (0.7 mm x 12 cm) for 2 - 3 hours. All samples were run until the marker dye had travelled a fixed distance (7.0 - 8.0 cm). Gels were stained with 0.5% amido black in 7% acetic acid and destained electrically.

Other Materials and Methods employed in the experiments of this Chapter were as described in Chapter IV.

3. Experiments and Results

a. Immunoabsorption of Low Molecular Weight Blocking Fraction

A low molecular weight fraction (final volume: 9 ml) was prepared by Sephadex G-75 gel filtration of DEAE-II from 60 ml of pooled normal human sera A.K. and T.W. The fraction was absorbed with insoluble goat antiserum to human YG globulin. The unabsorbed and absorbed fractions were tested by

immunodiffusion with specific goat antisera. As shown in Fig. 14, the unabsorbed fraction gave precipitin arcs with antisera to YG globulin and to the Fc fragment. A faint line was observed with antiserum to Y chain (not readily seen in the figure). The lines obtained with antiserum to YG and to Fc fragment were bent toward the wells containing antiserum to the light chains. No reaction was observed with antisera to human YA and YM globulins, albumin and transferrin. In contrast, following immunoabsorption with anti-YG (Fig. 14), no precipitin lines were obtained with any of the specific antisera, suggesting that the absorption of YG globulin had been complete.

The absorbed fraction (G-75-3A) was further analyzed by disc electrophoresis using normal serum as a reference standard. Prior to analysis, fraction G-75-3A was concentrated 10-fold. It can be seen from Fig. 15 that fraction G-75-3A gave only one band which migrated towards the anode slightly faster than albumin.

Both fractions G-75-3 and G-75-3A were tested for their ability to block passive sensitization with ragweed reagins. The results shown in Table XII demonstrate that both fractions had essentially identical activity in blocking passive sensitization.

b. Blocking Activity of Sephadex G-75-3 Fractions with and without Low Molecular Weight Components

Experiments were done to determine if the low molecular weight components in Fraction G-75-3 might have arisen by interaction of serum protein, (e.g., YG globulin), with the ion-exchanger DEAE-Sephadex. For this purpose, a 7S globulin fraction was prepared by gel filtration of normal human serum A.K. (6 ml) on a column (2.5 x 100 cm) of Sephadex G-200 (Fig. 16A). The middle peak of the chromatogram, containing primarily 7S components, was rechomatographed on Sephadex G-200 to obtain further removal of any low molecular weight components (Fig. 16B).

The 7S globulin preparation and an aliquot of serum A.K. containing approximately the same level of YG globulin (as determined by immunoplate assay) were separately chromatographed on 2 columns (each 2.5 x 26 cm) of DEAE-Sephadex prepared from the same slurry. The chromatograms obtained in both cases were essentially identical (Fig. 17). Identical cuts were made to give the corresponding DEAE-II fractions and the latter were separately filtered on Sephadex G-75.

Fig. 18A shows the chromatogram obtained for the DEAE-II fraction derived from whole serum. A peak of fluorescence was obtained in the region corresponding to the G-75-3

fraction. No similar peak was observed for the DEAE-II fraction derived from the 7S globulin preparation (Fig. 18B).

The two G-75-3 regions were separately pooled as shown in Fig. 18, concentrated by ultrafiltration to identical volumes (3 ml) and simultaneously tested for blocking activity. Only the G-75-3 fraction derived from the DEAE-II fraction of whole serum was effective in inhibiting P-K sensitization (Table XIII)

4. Discussion

The immunoabsorption technique of Avrameas and Ternynk (117) has been found to be efficacious for the specific absorption of YG globulin from serum fractions (120). Application of this technique to the G-75-3 fraction derived from DEAE-II of normal human serum removed detectable YG globulin from this fraction. Only one relatively fast-moving band was found for the absorbed fraction on disc electrophoresis at pH 9.5, indicating a high degree of purification. The absence of a similar band in a comparable region of the disc electropherogram of normal human serum is likely due to its presence in low concentration in serum. The essentially unimpaired blocking activity found for the G-75-3 fraction after absorption strongly suggests

that the YG globulin present in the unabsorbed fraction was not the active factor and that blocking activity was mediated by lower molecular weight components.

The control experiment provided evidence that the blocking activity of the low molecular weight fraction (G-75-3) was not due to the presence of extraneous materials derived from interaction of serum protein with the DEAE-Sephadex ion-exchanger. It was inferred from this result that low molecular weight blocking components of 1-3S are natively present in normal human serum.

CHAPTER VII: GENERAL DISCUSSION

1. Immunoglobulin Nature of Blocking Factors in Normal Human Serum

This study was undertaken to determine the nature of the factors in normal human serum able to block passive sensitization of normal human skin with reagins and thereby help clarify the relationship between reagins and serum immunoglobulins. As previously discussed, there was considerable evidence associating the reagins with YA globulins, but data had also accumulated which questioned this association. Studies by Goodfriend and his associates (107-110) and others (104-106) provided evidence that reaginic antibody was present in allergic serum fractions devoid of detectable YA globulin, suggesting that reagins might be associated with immunoglobulins other than YA globulin.

Since important evidence associating reaginic activity with YA globulin was the blocking effect of normal YA globulin preparations on passive sensitization with ragweed reagins (99, 103) and since this finding conflicted with the studies cited above, it was considered of interest to re-investigate

the distribution of blocking activity among the DEAE-Sephadex fractions derived from normal human serum.

The initial studies (Chapter IV; see also Ref. (113)) employed DEAE-Sephadex chromatography of normal human sera and demonstrated that DEAE-fractions containing YG as the only detectable immunoglobulin effectively blocked passive sensitization with ragweed reagins. While fractions containing both YG and YA globulins also blocked, it is noteworthy that fractions containing YG, YA and YM immunoglobulins had no blocking activity. In subsequent studies (Chapter V), an even more pronounced separation of blocking activity from YA globulin was achieved. These studies provided clear evidence that YG-containing fractions were the most active while fractions containing both YG and YA globulins blocked much less effectively. The fact that YM globulin-containing fractions were consistently inactive (113) was confirmed in these studies.

The finding that YA globulin fractions were only minimally active in blocking passive sensitization was at variance with studies by Ishizaka and his associates in which YA globulin was implicated as the unique carrier of blocking activity in normal human serum. As discussed in the General Introduction, YA globulins are heterogeneous in molecular size (34). It was therefore considered that the divergence in results obtained by

Ishizaka et al and those of the present investigation might be due to molecular size differences in the YA population. However, the elution profiles obtained by gel filtration of YA globulin on Sephadex G-200 indicated that the YA globulins in the blocking and non-blocking fractions had essentially identical molecular size distributions. Analysis of similar fractions by sucrose density-gradient centrifugation demonstrated that the bulk of the YA globulins in the blocking and non-blocking fractions were of the monomeric, 8S variety. Thus the divergence in data cannot be explained by molecular size differences in the YA globulin population.

As noted in the General Introduction, the YA globulins are heterogeneous with respect to electrophoretic charge (115). Recently, electrophoretically distinct sub-classes of human YA globulins have been described which differ in antigenic determinants on the H chains (16). Such sub-classes may also differ in the skin-fixing activity of their respective Fc fragment regions. It is possible that the disparity in the findings of Ishizaka et al and of the present study is related to electrophoretic differences in the YA population. In Chapter IV it was shown that the more anodic DEAE-Sephadex fractions of normal human serum, containing the bulk of the YA globulin, had little or no blocking activity. In contrast, fractions containing the

more cathodic of the YA globulins were active. Any blocking activity displayed by YA globulin would be due to this electrophoretic group.

The results of the present study demonstrated that DEAE-Sephadex fractions containing YG as the only detectable immunoglobulin were the most active in blocking passive sensitization with reagins. A time study showed that the YG-containing fractions were the most effective in maintaining a firm, long-lasting inhibition of P-K sensitization. Some of the more anodic YG-containing fractions were inactive, however. Reid et al (120) confirmed the findings of the present study (113) that the more electrophoretically cathodic YG globulin preparations were active in blocking P-K sensitization, while the more anodic showed little or no activity. As in the case of YA globulin, it is possible that particular, electrophoretically distinct subclasses of YG globulin carry skin-fixing sites. In this connection, it is worth noting that electrophoretically distinct subclasses of human YG globulin have been described (see General Introduction) associated with differences in antigenic determinants on their H chains. Only certain of the sub-classes of YG globulin have been shown to mediate PCA in the guinea pig, whereas others do not (15). It has not yet been excluded that some sub-classes of YG globulin also fix to the same epithelial

cell sites as do reaginic antibodies and thereby effectively compete with these antibodies. The existence of such blocking sub-groups of YG globulin could explain the finding by Ishizaka et al (99) that YG was relatively ineffective compared to YA globulin in blocking P-K sensitization, since the blocking activity of a particular YG globulin preparation would depend on the extent to which the preparation contained active sub-groups.

2. Low Molecular Weight Blocking Factors in Normal Human Serum

An alternative explanation for the blocking activity of YG globulin observed in the present studies and in those of Reid et al (121) is that the YG globulin preparations were contaminated with as yet uncharacterized immunoglobulins and/or other serum components. In order to further clarify the nature of the blocking factor or factors in the active DEAE-Sephadex fractions, studies were done on one of the most active and least complex of these fractions, DEAE-II. Chromatography of DEAE-II on the cation-exchanger CM-Sephadex demonstrated that subfractions of DEAE-II which were devoid of YG globulin were able

to inhibit P-K sensitization. Furthermore, by sucrose density-gradient centrifugation and gel filtration on Sephadex G-75, two molecular size blocking fractions were found in DEAE-II: one, associated with YG globulin, the other (G-75-3) with low molecular size components in the 1-3S range, containing relatively trace amounts of YG globulin.

The latter finding prompted an investigation of the molecular size distribution of blocking factors in whole normal sera. Two normal sera were separately filtered on Sephadex G-200 and, in addition, were separately fractionated by sucrose density-gradient centrifugation. The results obtained by both techniques demonstrated the presence in whole serum of the two active molecular size fractions: one, associated mainly with the 7S region, the other, with the low molecular weight 1-3S region.

As has already been mentioned, the active low molecular weight fraction (G-75-3) obtained by gel filtration of DEAE-II on Sephadex G-75, contained relatively trace amounts of YG globulin. Removal of the YG globulin by immunoabsorption with specific antiserum did not diminish the ability of fraction G-75-3 to block passive sensitization, indicating that the blocking activity was due to the low molecular weight components. That a high degree of purification had been achieved was

indicated by the single band observed on disc electrophoresis of the absorbed fraction. In preliminary tests, the absorbed fraction was found to be ninhydrin positive and to contain 1-2% carbohydrate (based on dry wright).

Two control experiments provided evidence that the low molecular weight fraction G-75-3 was not artifactually produced by the chromatographic techniques employed. One control excluded the possibility that passage of eluate buffer through the DEAE-Sephadex column was accompanied by "leaching" of low molecular weight columnar materials with non-specific blocking activity, into the eluate fractions. Another control excluded the possibility that interaction of serum protein with DEAE-Sephadex and with Sephadex G-75 produced low molecular weight contaminants with biological activity.

The identity of the blocking factors in the low molecular weight fraction G-75-3 remains to be determined. Immunodiffusion analysis of G-75-3 revealed no antigenic identity with human YG globulin or its Fc piece. Similarly, no antigenic relationship was observed with human YA and YM globulins, albumin and transferrin. Cross-reaction with the serum proteins could not be completely ruled out since the level of concentration of the active components in G-75-3 may not have been sufficiently high for detection by the immunodiffusion test. No

attempt was made to determine the antigenic relationship of the G-75-3 components with the recently discovered YD globulin (122) so that the possibility cannot yet be excluded that the low molecular size blocking components are fragments of the heavy chains of YD globulin.

While this study was in progress Ishizaka et al (123) described a new immunoglobulin, YE, which carries much of the reaginic activity in allergic serum. It is possible that the low molecular weight blocking fraction contains fragments of YE globulin in normal sera, fragments carrying the skin-fixing sites and thus able to compete with the YE reaginic antibody for tissue sites. Johansson et al (124) have recently reported on their discovery of a myeloma protein (YND) with heavy chain antigenic determinants distinct from those of the known immunoglobulin classes. The YND immunoglobulin appears to be similar or related to YE (125). Stanworth et al (125) have reported that YND blocked passive sensitization with reagins. This would suggest that the "high molecular weight" blocking factors in normal human serum are YND type globulins, at least in part, while the low molecular weight factors may be fragments of YND globulins. The blocking activity of normal serum fractions containing YND globulin has not yet been reported, however.

It was pointed out in the General Introduction that

normal human serum contains low molecular weight γ globulins, of which $3S\gamma_1$ and $2S\gamma_2$ types have thus far been described (40). Williams and Schmidt (41) have recently reported that some 20% of the $3S\gamma_1$ globulins contain κ and λ light chains, while the remaining 80% may be associated with as yet unknown immunoglobulin types. It seems reasonable to suggest on the basis of molecular size and antigenic properties, that the blocking components in G-75-3 may be related or identical to the latter type of $3S\gamma_1$ globulins and to the $2S\gamma_2$ variety.

The biological significance of the blocking factors, particularly of the low molecular weight components, remains to be determined. It is tempting to speculate that they bear some relationship to the biosynthesis and degradation of the immunoglobulin prototype of reaginic antibodies. Furthermore, their levels in the circulation may determine the degree of clinical hypersensitivity in the atopic patient. Further physicochemical and immunological characterization of these blocking factors may lead to a better understanding of the pathogenetic mechanisms of allergic disease.

SUMMARY

A study was made of the factors in normal human serum able to block Prausnitz-Kustner (P-K) sensitization with ragweed reagins.

1. DEAE-Sephadex fractions containing only detectable YG globulin had the highest activity in blocking P-K sensitization. Fractions containing YG and YA globulins had only minimal blocking activity, while a fraction containing YM as well as YG and YA globulins had little or no blocking activity.
2. Sucrose density-gradient centrifugation and Sephadex G-75 gel filtration of a YG-containing fraction (DEAE-II) showed the presence of a high molecular size fraction with components sedimenting at greater than 5S and a low molecular size fraction (G-75-3) with components sedimenting at 1-3S, the latter being the most active.
3. Sucrose density-gradient centrifugation and Sephadex G-200 gel filtration of two whole normal sera confirmed the existence of a heterogeneous group of blocking factors, as blocking activity was found in the high molecular size fractions of 7S or greater, as well as in the lower molecular size fractions with components sedimenting at 1-3S. This

latter fraction was the most active.

4. Removal by immunoabsorption of trace amounts of YG globulin in the low molecular weight fraction G-75-3 was without effect on the blocking activity, indicating that the low molecular weight components were responsible for the activity of the fraction. Disc electrophoresis of the absorbed fraction showed a single component which migrated slightly faster than albumin.
5. A control experiment excluded the possibility that passage of eluate buffer through the DEAE-Sephadex columns was accompanied by "leaching" of low molecular weight columnar materials into the eluate fractions with non-specific blocking activity. A second control experiment excluded the possibility that the interaction of serum protein with DEAE-Sephadex and with Sephadex G-75 produced low molecular contaminants with biological activity.
6. Immunodiffusion with specific antisera provided evidence that the low molecular weight components did not carry the antigenic determinants of the YG, YA or YM immunoglobulins.
7. The relationship between the immunoglobulin classes, reaginic antibodies and the normal human serum blocking factors was discussed.

CLAIMS TO ORIGINALITY

1. Utilizing the technique of DEAE-Sephadex chromatography, it was shown that blocking activity in normal human serum was not uniquely related to YA globulin.
2. The YG-containing DEAE-Sephadex fractions were shown to have the highest blocking activity on P-K sensitization with ragweed reagins.
3. Heterogeneity of blocking factors in normal human sera was demonstrated using the techniques of sucrose density-gradient centrifugation and Sephadex gel filtration. High molecular size fractions, with components sedimenting at 7S or greater were active, but the most active of the blocking fractions was a low molecular size fraction with components sedimenting at 1-3S.

TABLE I

PROPERTIES OF CYTOTROPIC ANTIBODIES

Properties	Human		Guinea Pig	
	Homo	Hetero	Homo	Hetero
Immunoglobulin type	?	Probably YG	Y1	Y2
Sedimentation coefficient	7.4-11S	7S	7S	7S
Complement fixation	0	+	0	+
Heat lability	+	0	0	0
Mercaptan lability	?0	0	±	0
Passive sensitization of skin in:				
Same species	+	0	+	0
Other species	monkey	rabbit, guinea pig	rat	rat, human
Persistence in passively sensitized skin sites	Long	Short	Short	Short
Transmission across placenta	0	+	+	+

(Continued next page)

TABLE I (Continued)

PROPERTIES OF CYTOTROPIC ANTIBODIES

Properties	Rat		Mouse	
	Homo	Hetero	Homo	Hetero
Immunoglobulin type	Fast Y	Y2	Y1	Y2a
Sedimentation coefficient	7-19S	7S	7S	7S
Complement fixation	0	+	0	+
Heat lability	+	0	0	0
Mercaptan lability	±	0	±	0
Passive sensitization of skin in:				
Same species	+	0	+	0
Other species	0	Guinea pig	0	Rat, guinea pig, chicken
Persistence in passively sensitized skin sites	Long	Short	Short	Short
Transmission across placenta	?	?	?	?

(Continued next page)

TABLE I (Continued)

PROPERTIES OF CYTOTROPIC ANTIBODIES

Properties	Rabbit		Dog	
	Homo	Hetero	Homo	Hetero
Immunoglobulin type	Fast Y	YG	Fast Y	Y2
Sedimentation coefficient	7-19S	7S	7-19S	?
Complement fixation	0	+	0	+
Heat lability	+	0	+	0
Mercaptan lability	0	?+	+	0
Passive sensitization of skin in:				
Same species	+	0	+	0
Other species	0	Guinea pig, etc.	0	Guinea pig
Persistence in passively sensitized skin sites	Long	Short	Long	Short
Transmission across placenta	?	?	?	+

Reproduced from Ref. (63).

TABLE II

BLOCKING OF THE P-K REACTION BY DEAE-SEPHADEX FRACTIONS* OF NORMAL SERUM A.K.

	Immunoglobulin (µg)** YG	YA	YM	Wheal size (mm^2)	% Blocking
Saline	--	--	--	144	0
Fractions					
I***	250	0	0	56	60
II	200	0	0	72	50
III	50	0	0	72	50
IV	25	0	0	90	35
V	35	10	0	100	30
VI	30	25	0	90	35
VII	30	35	0	90	35
VIII	20	20	0	120	15
IX	25	15	40	140	0

* Prepared by chromatographic Method I (see text).

** Amount of immunoglobin in the aliquot of each fraction injected.

*** Fraction I (7.5 mg YG/ml) was diluted 1 : 3 with saline for the blocking assay.

TABLE III

BLOCKING OF THE P-K REACTION BY DEAE-SEPHADEX FRACTIONS* OF NORMAL SERUM A.K.

	Immunoglobulin (µg)**			Wheal size (mm^2) test subjects		% Blocking test subjects	
	YG	YA	YM	1	2	1	2
Saline	--	--	--	108	120	0	0
Fractions							
I***	250	0	0	35	0	70	100
II	100	0	0	36	0	70	100
III	25	15	0	25	0	80	100
IV	30	20	0	30	0	70	100
V	20	20	0	80	100	26	20
VI	35	50	60	110	110	0	0

* Prepared by chromatographic Method II (see text).

** Amount of immunoglobulin in the aliquot of each fraction injected.

*** Diluted 1 : 3 (Table I).

TABLE IV

BLOCKING OF THE P-K REACTION BY DEAE-SEPHADEX FRACTIONS* OF NORMAL SERUM A.B.

	Immunoglobulin (µg)** YG	YG	YM	Wheal size (mm^2)	% Blocking
Saline	--	--	--	146	0
Fractions					
I***	250	0	0	56	61
II	250	0	0	72	50
III	26	13	0	110	23
IV	13	28	0	100	31
V	11	21	0	88	40
VI	12	45	55	120	18

* Prepared by chromatographic Method I (see text).

** Amount of immunoglobulin in the aliquot of each fraction injected.

*** Diluted 1 : 3 (Table I).

TABLE V

BLOCKING ACTIVITY OF MIXTURES OF DEAE-SEPHADEX FRACTIONS* OF NORMAL HUMAN SERUM A.K.

	Immunoglobulin (µg)** YG	YA	YM	Wheal size (mm^2)	% Blocking
Saline	--	--	--	120	0
Fractions					
III	25	15	0	0	100
IV	30	20	0	0	100
VI	35	50	60	110	8
III + VI	30	35	60	0	100
IV + VI	30	35	60	0	100

* Prepared by chromatographic Method II (see text).

** Amount of immunoglobulin in the aliquot of each fraction injected.

TABLE VI

TIME STUDY ON BLOCKING OF P-K REACTION BY DEAE-SEPHADEX FRACTIONS* OF NORMAL SERUM A.K.

	Immunoglobulin (µg)**			% Blocking on Day***		
	YG	YA	YM	1	5	10
Fractions						
I****	250	0	0	100	41	33
II	100	0	0	100	36	20
III	25	15	0	100	30	0
IV	30	20	0	100	35	0

* Prepared by chromatographic Method II (see text).

** Amount of immunoglobulin in the aliquot of each fraction injected.

*** Time of incubation of DEAE-Sephadex fractions in skin site before P-K sensitization.

**** Diluted 1 : 3 (Table I).

TABLE VII

THE EFFECT OF DILUTION OF YG-CONTAINING DEAE-SEPHADEX FRACTIONS ON BLOCKING ACTIVITY

Fraction	Dilution	YG-Globulin Injected (µg)	% Blocking
I (Serum A.K.)*	1 : 3	250	61
	1 : 15	50	61
	1 : 30	25	37
I (Serum A.B.)**	1 : 3	250	60
	1 : 7.5	100	60
	1 : 15	50	52
II (Serum A.B.)**	1 : 1	250	50
	1 : 2.5	100	30
	1 : 5	50	38

* Prepared by chromatographic Method I (see text).

** Prepared by chromatographic Method II (see text).

TABLE VIII

BLOCKING OF THE P-K REACTION BY DEAE-SEPHADEX FRACTIONS* OF NORMAL SERUM T.W.

	Immunoglobulin (µg)** YG	YA	YM	Wheal size (mm^2)	% Blocking
Saline	--	--	--	132	
Fractions					
I***	100			75	43
IA	25			85	36
II	230			61	53
IIA	50			85	36
III	40			93	21
IV	29			110	16
V	21			115	12
VI	18	24		115	12
VII	40	52	18	105	20
VIII	12	40	40	110	16
IX	--	22	20	118	10
X	--	--	--	115	12

* Prepared by chromatographic Method I (see text, chapter IV).

** Amount of immunoglobulin in the aliquot of each fraction injected.

*** Fraction I (4 mgm YG/ml) was diluted 1 : 4 with saline for the blocking assay.

TABLE IX

BLOCKING OF P-K REACTION BY SUCROSE DENSITY-GRADIENT FRACTIONS OF DEAE-II

Fractions	Range of S^{o}_{20}	YG Globulin Content (µg)* A.K.	J.P.	% Blocking A.K.	J.P.
1	8	10	10	22	33
2	5-8	180	240	28	41
3	3-5	10	10	18	20
4	1-3	10	10	100	63

* Amount of YG globulin in the aliquot of each fraction injected.

TABLE X

BLOCKING OF P-K REACTION BY
SEPHADEX G-75 FRACTIONS OF DEAE-II

	YG globulin Content (µg)*		% Blocking	
Fractions	T.W.	A.K.	T.W.	A.K.
I	230	300	45	55
II	300	400	38	31
III	10	10	55	65

* Amount of YG globulin in the aliquot of each fraction injected.

TABLE XI

BLOCKING OF P-K REACTION BY SUCROSE DENSITY-GRADIENT FRACTIONS OF NORMAL SERA

Fractions	Range of $S^{o}20$	YG Globulin Content (µg)* A.K.	J.P.	% Blocking A.K.	J.P.
1	8	10	10	45	30
2	5-8	70	90	48	36
3	3-5	10	10	21	15
4	1-3	10	10	63	45

* Amount of YG globulin in the aliquot of each fraction injected.

TABLE XII

COMPARISON OF BLOCKING OF P-K REACTION BY FRACTION G-75-3 BEFORE AND AFTER ABSORPTION OF YG-GLOBULIN

Fractions	YG content (µg)*	Wheal Area (mm^2) 1**	2**	% Blocking 1**	2**
Saline	0	90	80	0	0
G-75-3	10	45	35	50	56
G-75-3***	0	40	30	55	60

* Amount of YG-globulin in the aliquot of each fraction injected.

1** Reaginic serum (B.U.) diluted to 1 : 300.

2** Reaginic serum (B.U.) diluted to 1 : 500.

*** Represents fraction G-75-3 after removal of YG-globulin.

TABLE XIII

BLOCKING OF P-K REACTION BY TWO PREPARATIONS OF FRACTION G-75-3

Fractions	YG Globulin Content (µg)*	Wheal Area (mm^2) 1**	2**	% Blocking 1	2
Saline	0	80	70	0	0
G-75-3A***	10	35	25	56	64
G-75-3B***	10	80	58	0	17

* Amount of YG-globulin in the aliquot of each fraction injected.

** 1 = Reaginic serum (B.U.) diluted to 1 : 300.

2 - Reaginic serum (B.U.) diluted to 1 : 500.

*** A = Sephadex G-75 fraction obtained from DEAE-II of whole serum.

B = Sephadex G-75 fraction obtained from DEAE-II of a 7S globulin preparation (see text).

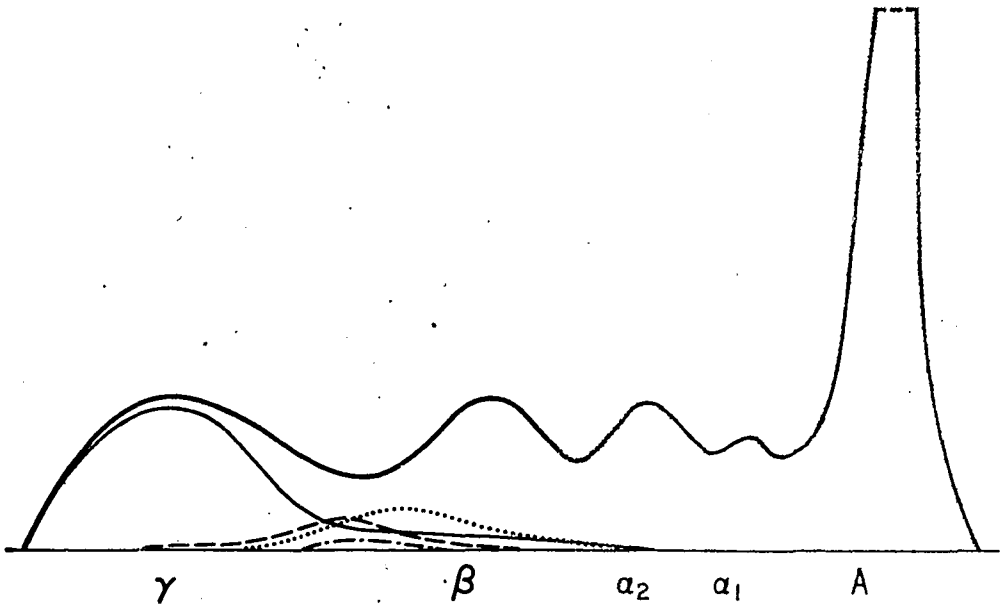


Fig. 1. Schematic diagram of an electrophoretic pattern of human serum in pH 8.6 Veronal buffer obtained by paper electrophoresis. The direction of migration is from left to right. The spread of the "γ-globulins" is denoted by the solid curve running from the "γ" to the "α" region. The remaining curves represent other immunoglobulin types. A = albumin.

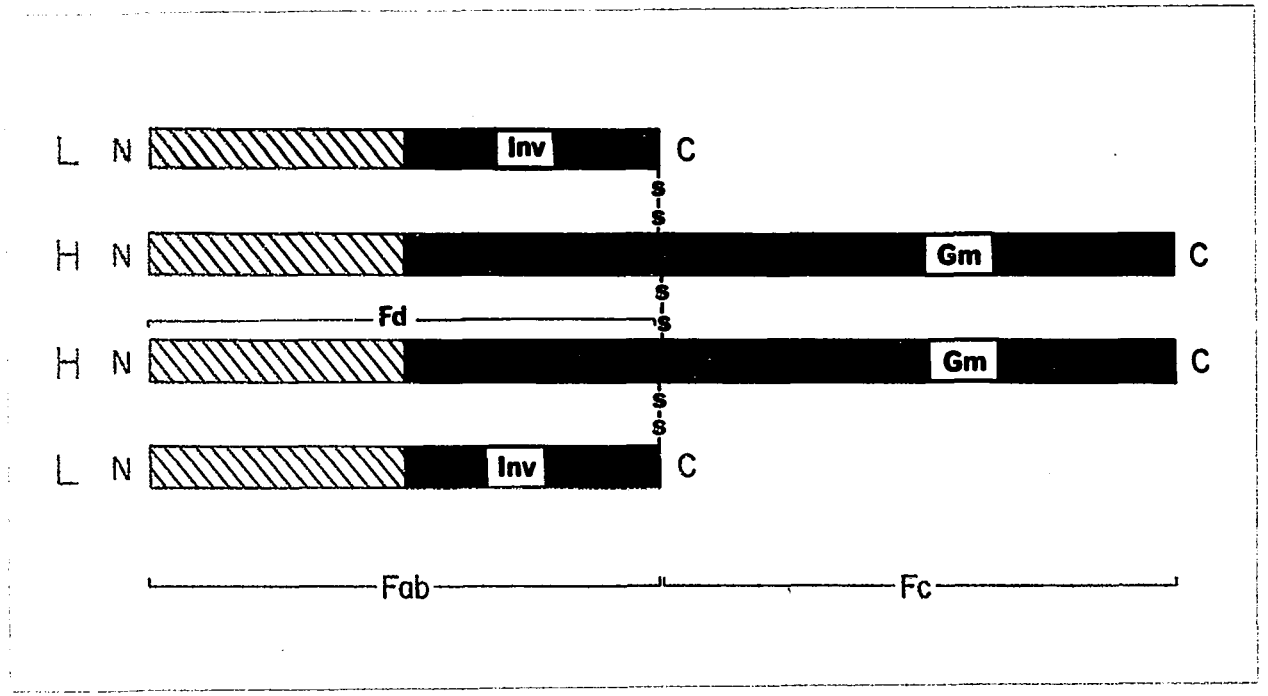


Fig. 2. Schematic diagram of YG-globulin. The heavy chains consist of the Fc and Fd fragments. C = carboxy terminal end; N = amino terminal end; S-S = disulfide bond. Gm and Inv refer to genetically determined factors on the heavy (H) and light (L) chains.

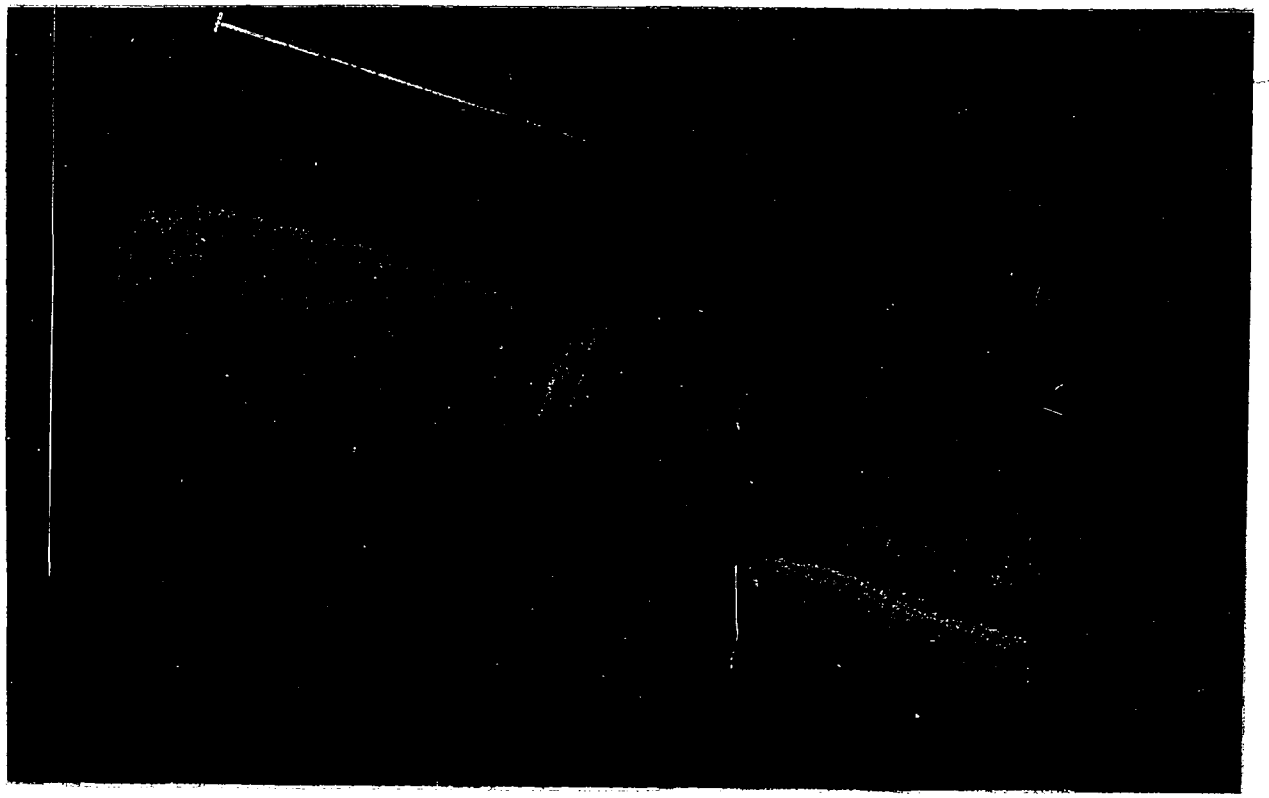


Fig. 3. Three-dimensional model of structure of YG-globulin molecule. (From Ref. 32).

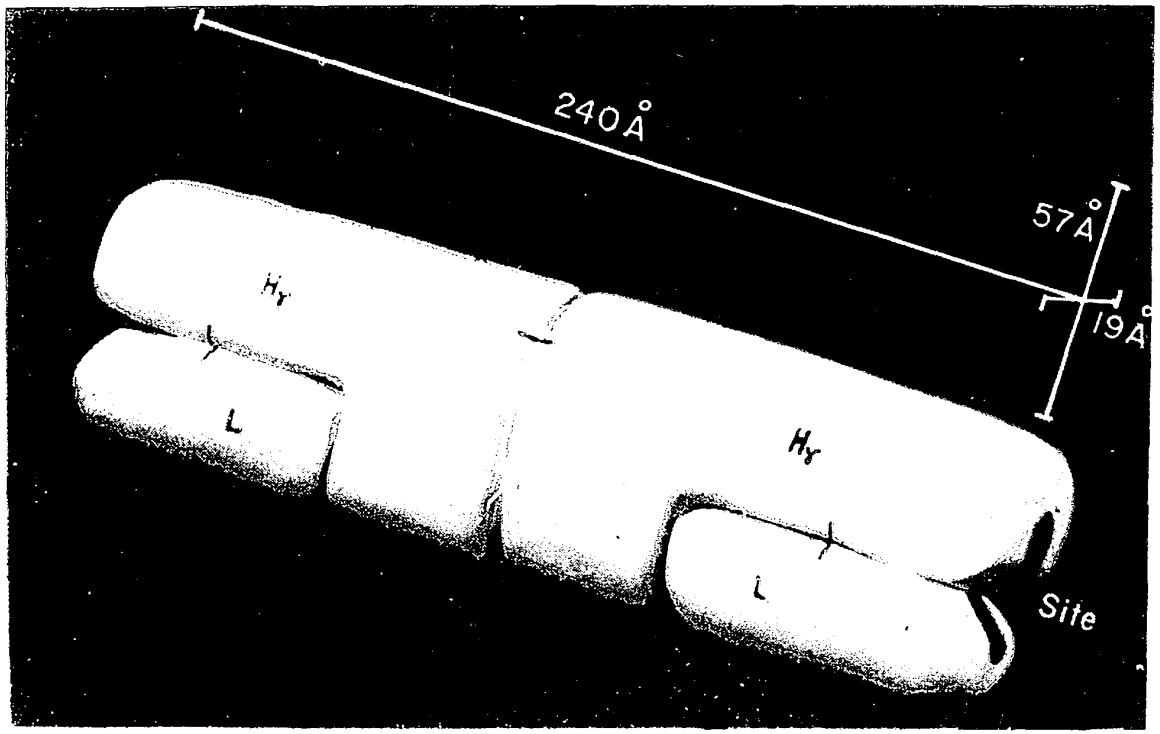


Fig. 1 [illegible] molecule. (From ref. 32).

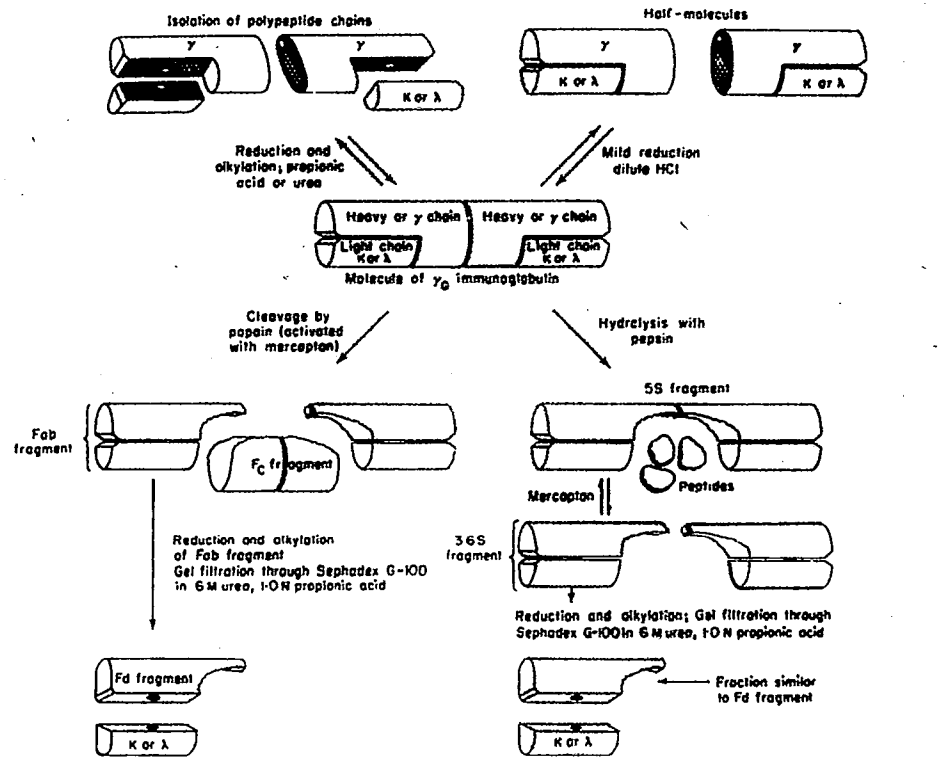


Fig. 4. Degradation of YG-globulin molecule to chains and fragments.

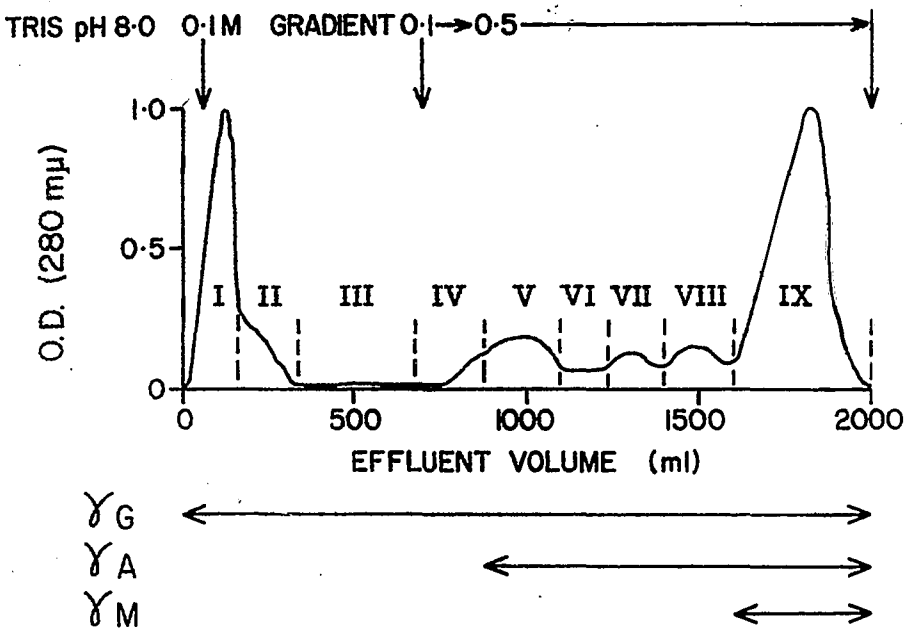


Fig. 5. DEAE-Sephadex chromatography of normal human serum A.K. by Method I (see text).

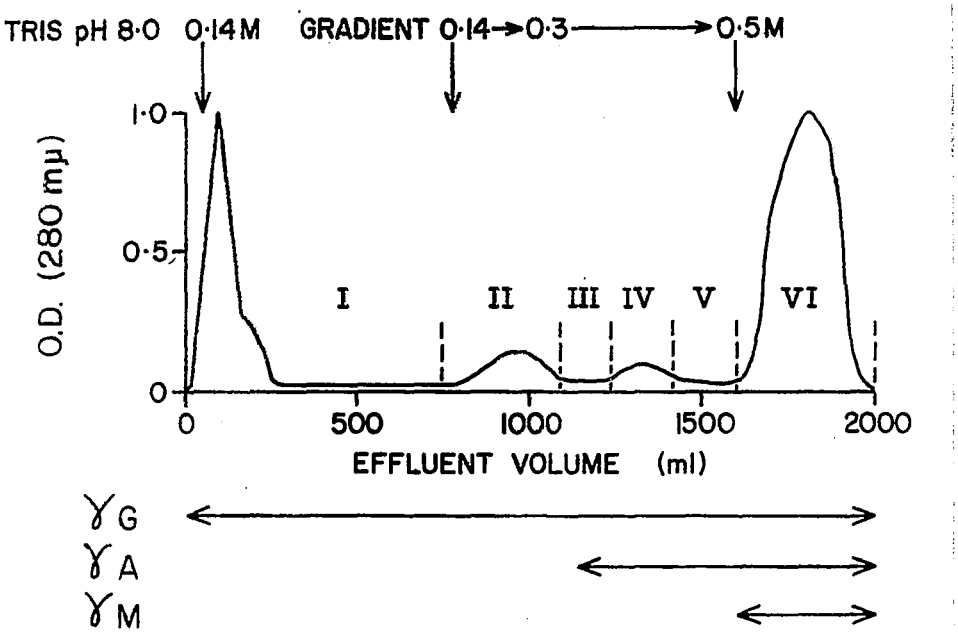


Fig. 6. DEAE-Sephadex chromatography of normal human serum A.K. by Method II (see text).

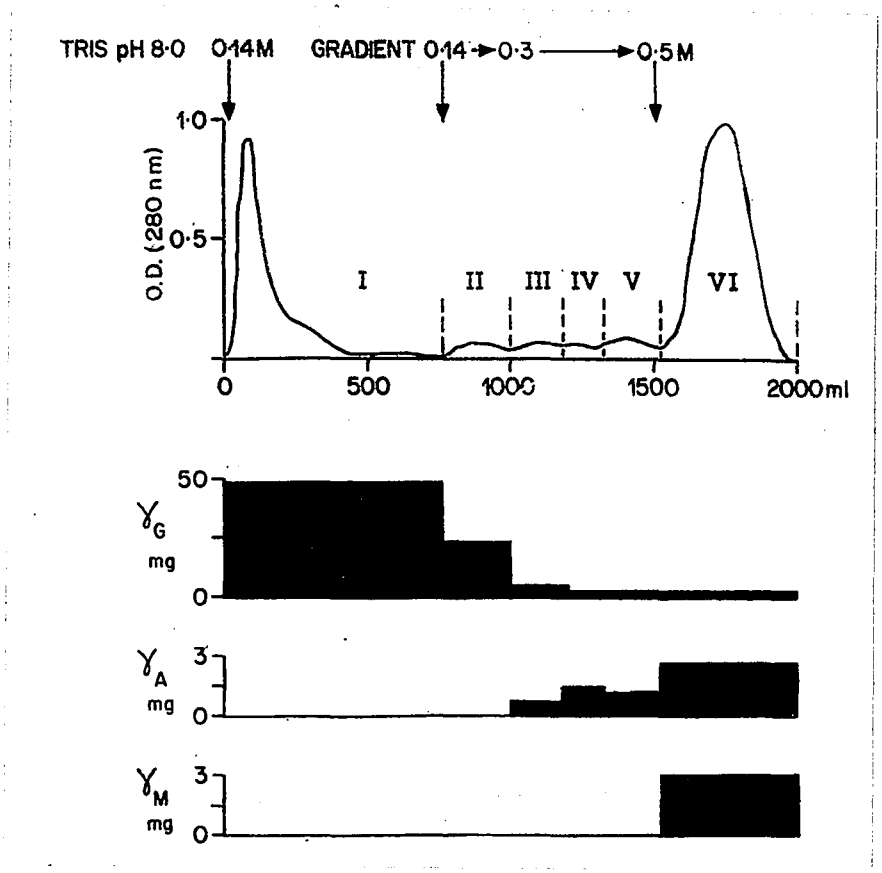


Fig. 7. DEAE-Sephadex chromatography of normal human serum A.B. by Method II (see text).

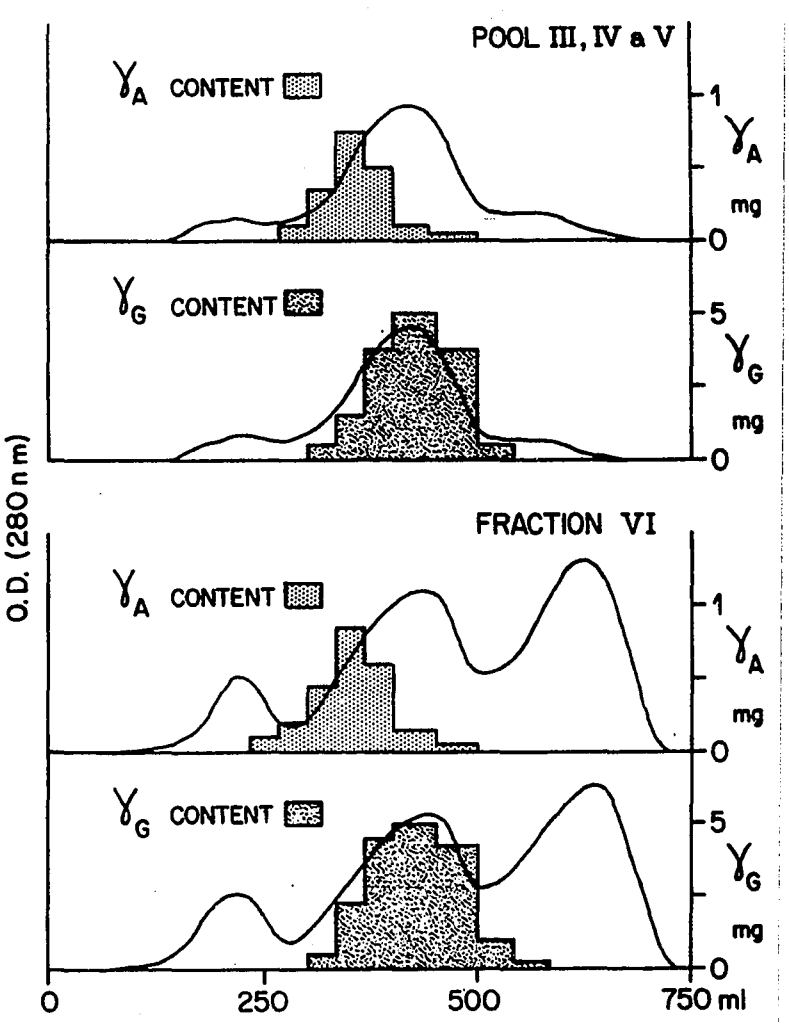


Fig. 8. Gel filtration on Sephadex G-200 of pool III - V and fraction VI of serum A.K. Prior to chromatography, 2 ml aliquots of fraction I from serum A.K. (containing 7.5 mg YG globulin/ml) were separately added to 4 ml each of the pooled fraction (containing 0.55 mg YA globulin/ml and fraction VI (containing 0.50 mg YA globulin/ml).

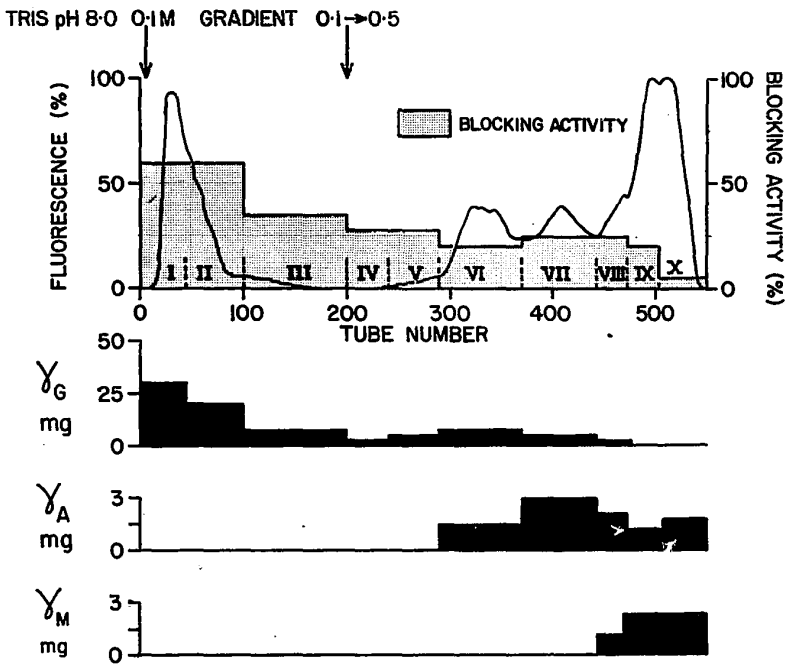


Fig. 9. DEAE-Sephadex chromatography of normal human serum A.K. (Method I).

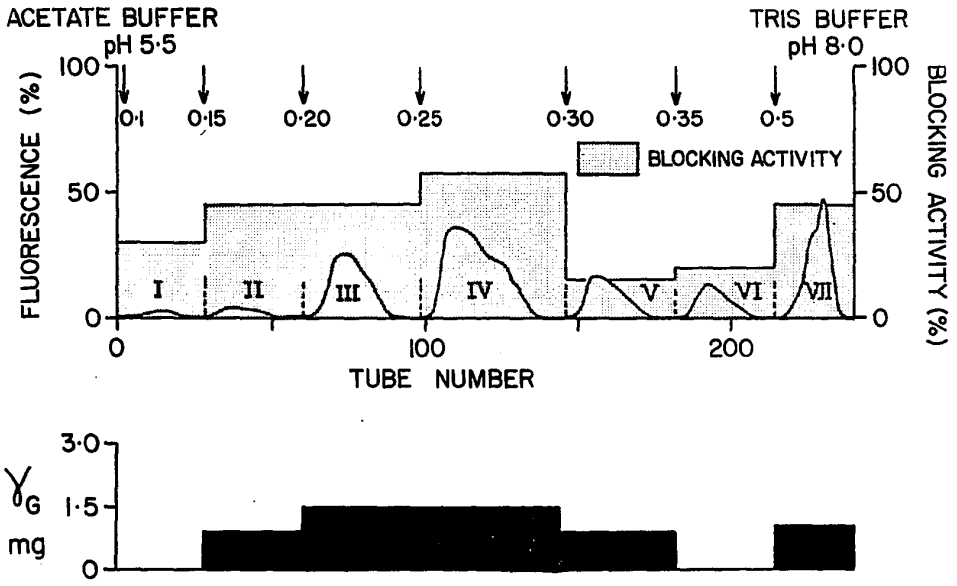


Fig. 10. CM-Sephadex chromatography of DEAE-Sephadex fraction II of normal human serum A.K.

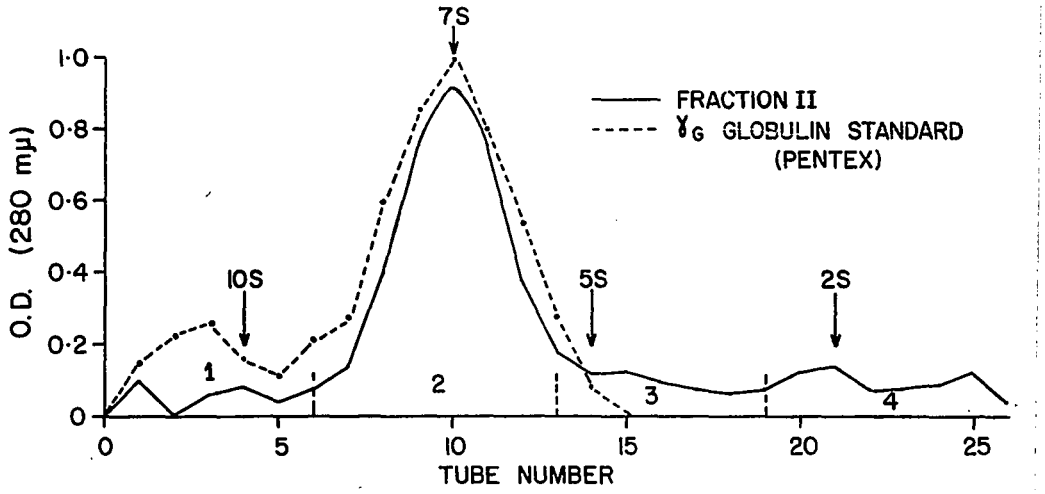


Fig. 11. Sucrose density-gradient centrifugation of DEAE-Sephadex fraction II of serum A.K.

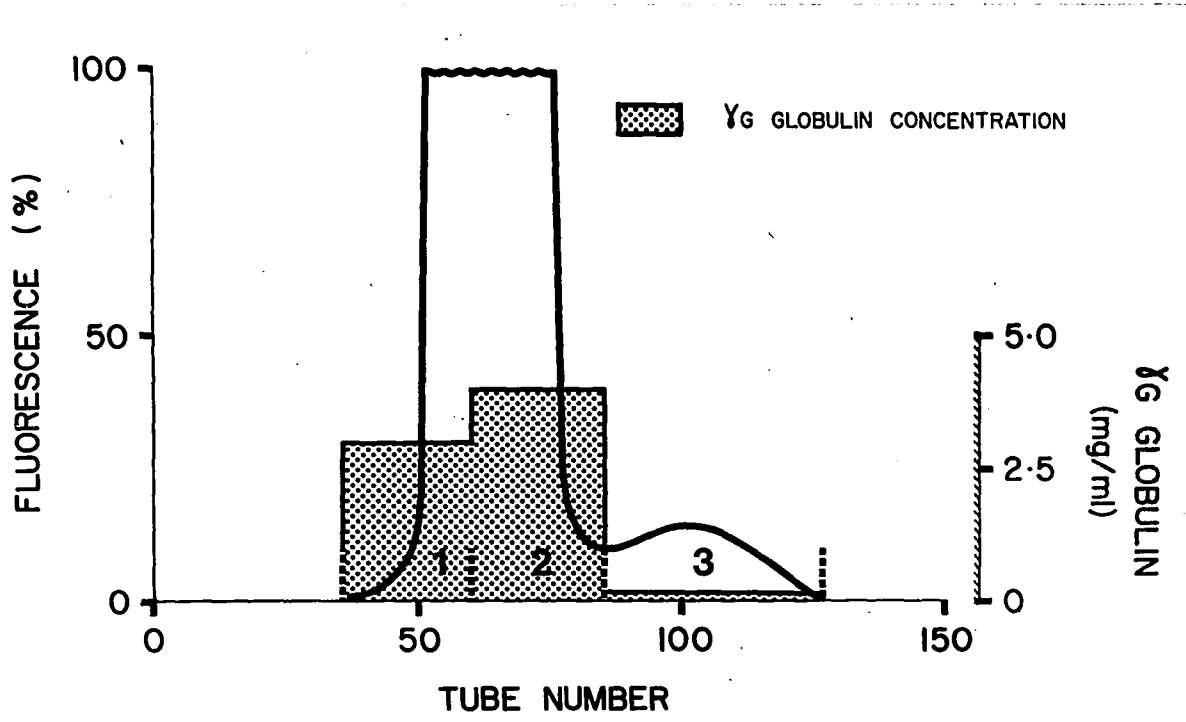


Fig. 12. Gel filtration on Sephadex G-75 (superfine) of DEAE-Sephadex fraction II of serum A.K.

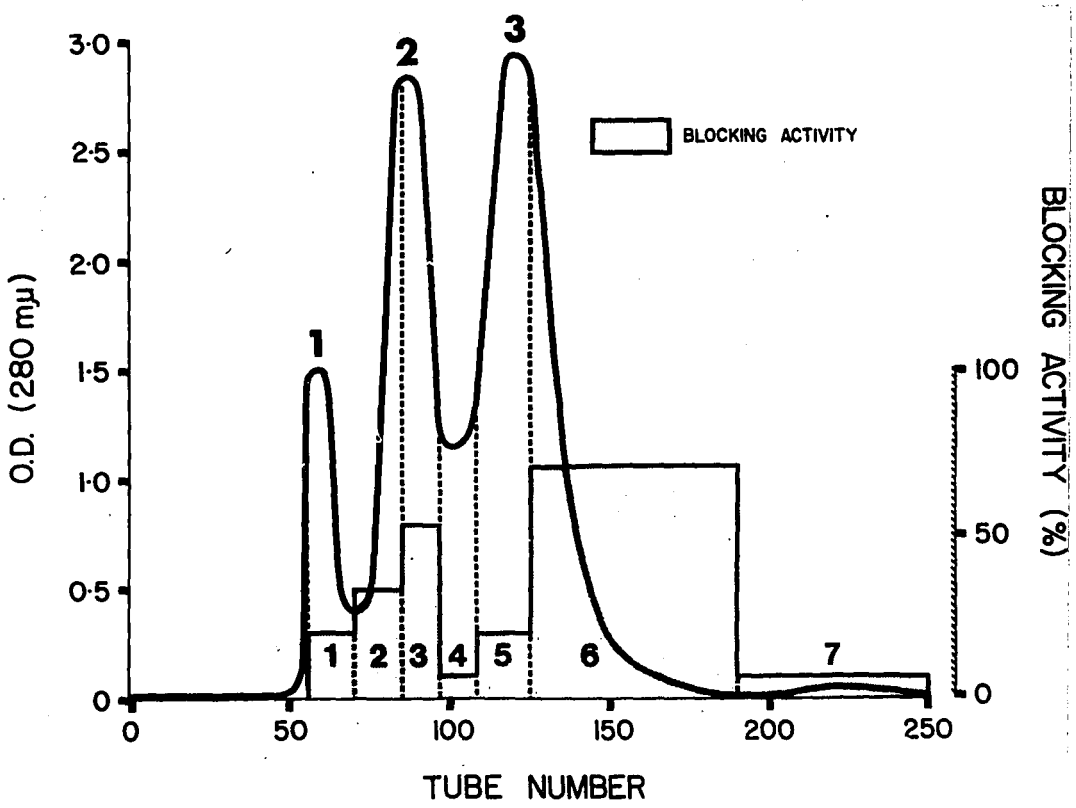


Fig. 13. Gel filtration on Sephadex G-200 of normal human serum A.K. Fractions 1-7 were each concentrated to the initial serum volume and tested for blocking activity.

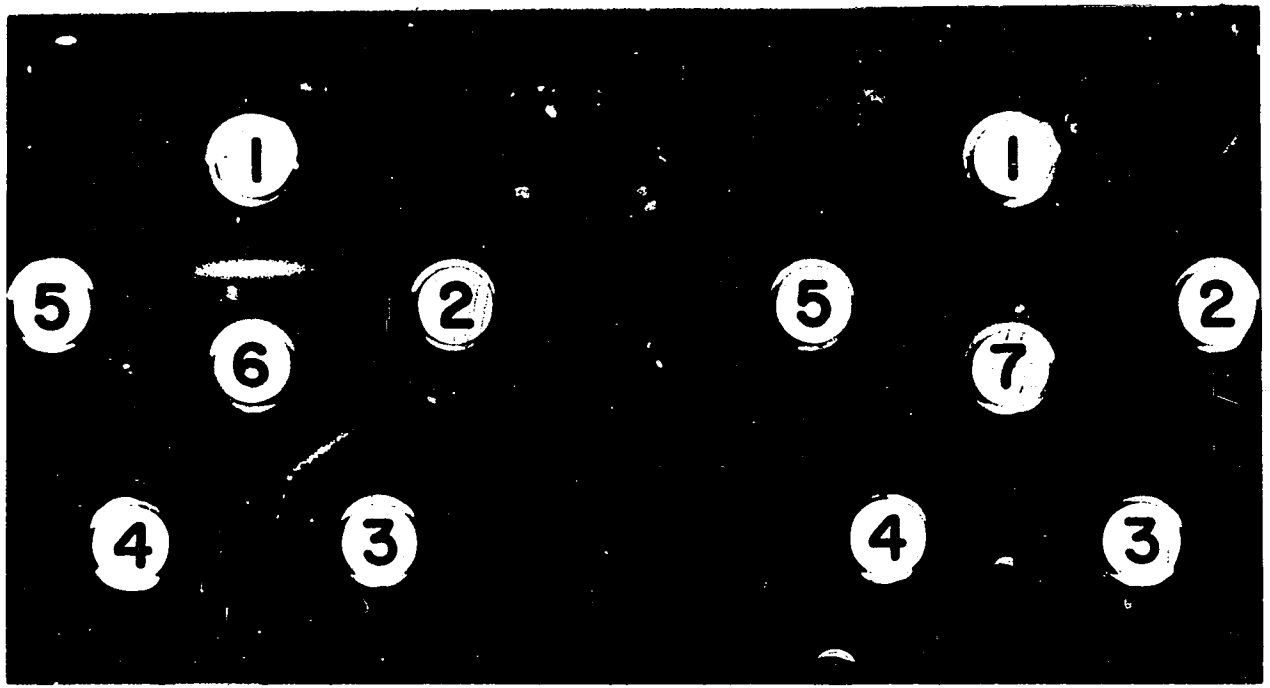
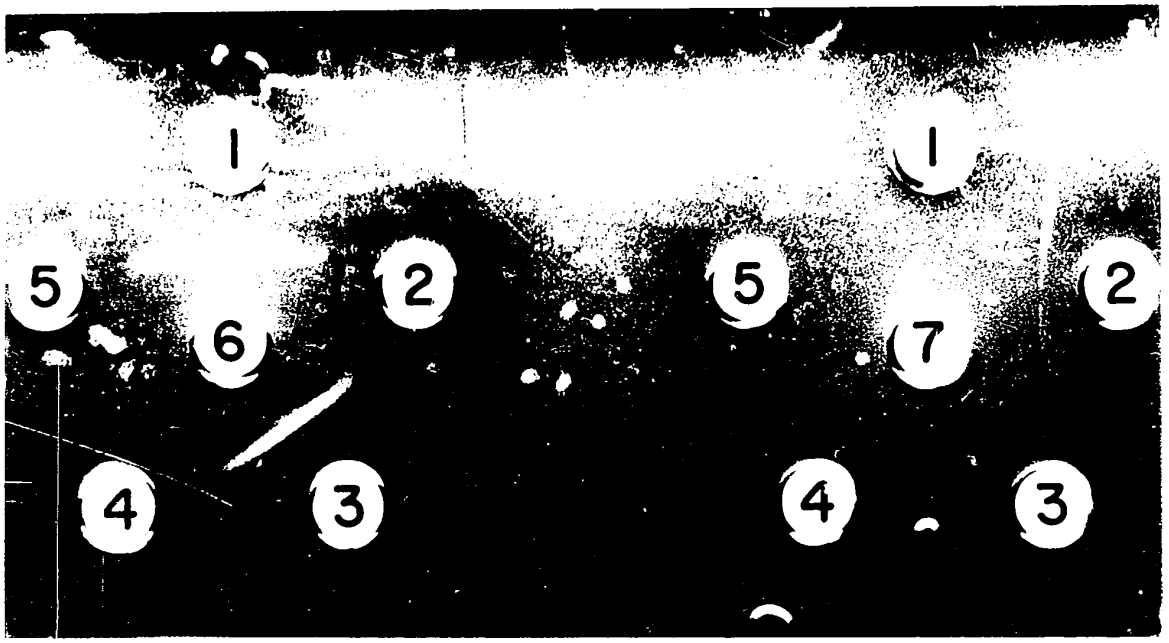


Fig. 14. Immunodiffusion in agar of fraction G-75-3 before (well 6) and after (well 7) absorption of YG-globulin. Wells 1, 2, 3, 4 and 5 contained goat antisera to human YG-globulin, γ chain, Fc fragment, κ light chain and λ light chain, respectively.



[illegible] immunoelectrophoresis of fraction 6-7b-3 before (well 6) and after (well 7) [illegible] wells 1, 2, 3, 4 and 5 contained goat antisera to [illegible] fragment, κ light chain and λ light chain [illegible]

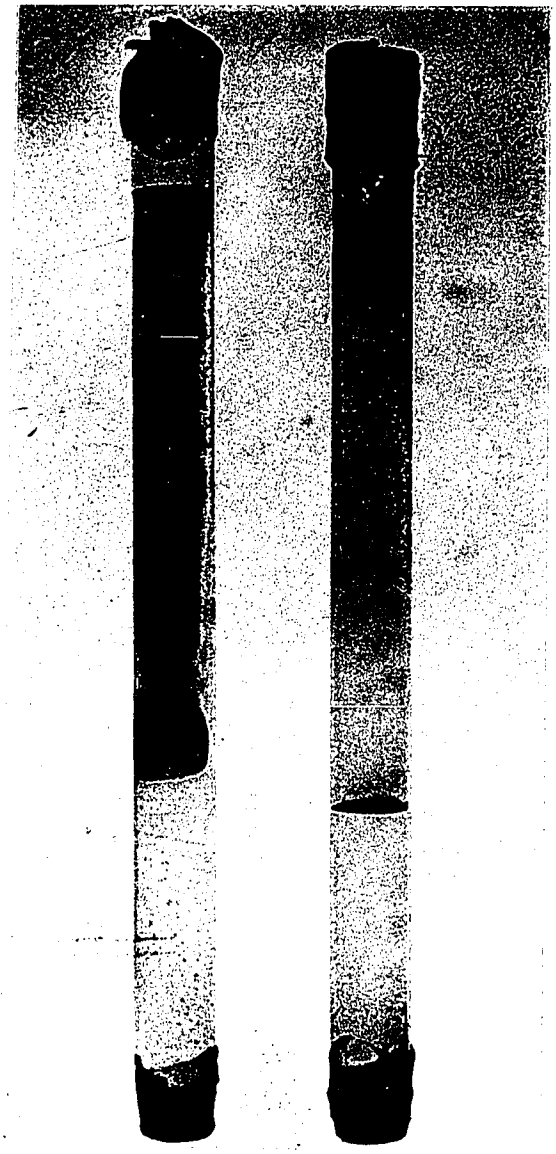


Fig. 15. Polyacrylamide gel disc electrophoresis at pH 9.5 of normal human serum (left) and G-75-3 after absorption of YG-globulin (right).

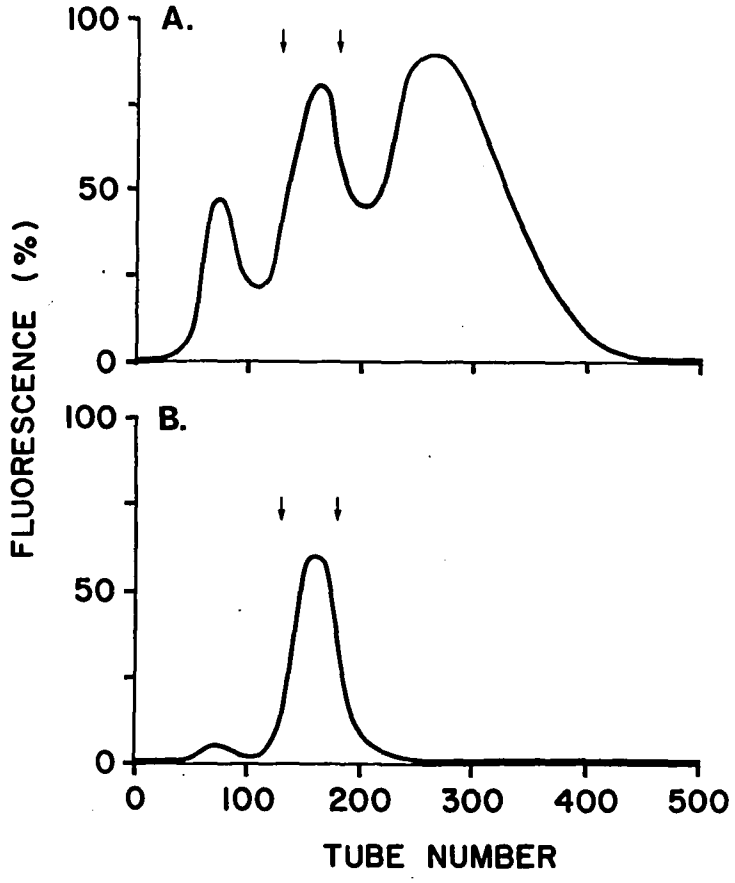


Fig. 16. Gel filtration on Sephadex G-200 of normal human serum A.K. (A) The arrows denote the cut made to obtain a 7S globulin fraction. The latter was re-chromatographed on Sephadex G-200 (B) and a cut made (arrows) to obtain a purified 7S globulin preparation.

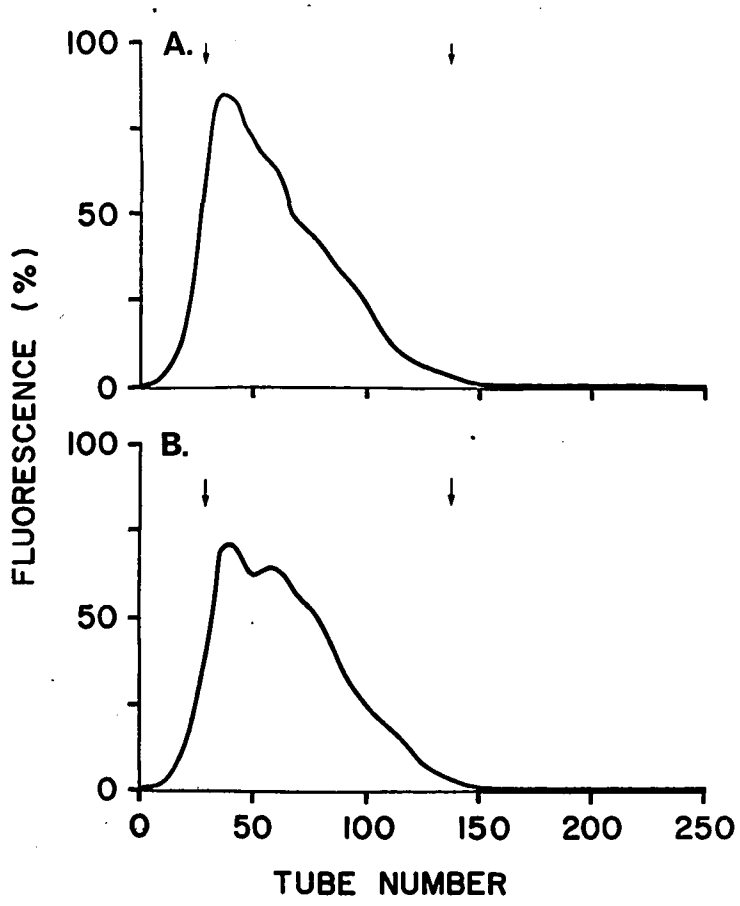


Fig. 17. Preparation of DEAE-II from whole normal human serum A.K. (A) and from the corresponding 7S globulin preparation (B). In each case, the fraction (arrow to arrow) was eluted from DEAE-Sephadex with 0.1 M Tris-HCl, pH 8.0.

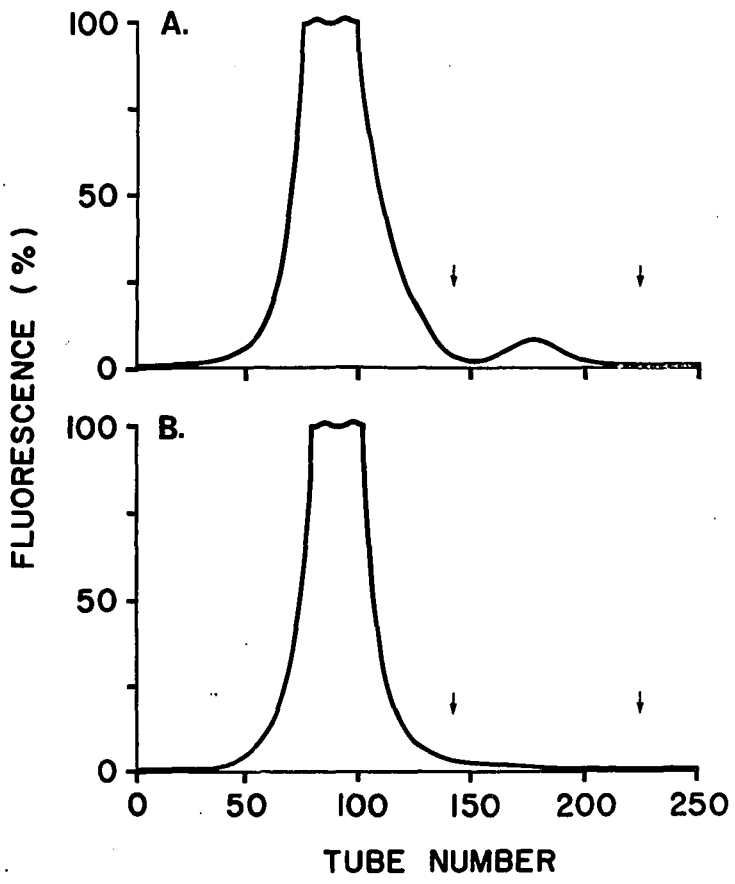


Fig. 18. Gel filtration on Sephadex G-75 (superfine) of DEAE-II derived from normal human serum A.K. (A) and the corresponding 7S globulin preparation (B). The arrows denote cuts made to obtain the two preparations of G-75-3.

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