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ISOLATION OF AN ALLERGEN FROM KENTUCKY BLUE GRASS POLLEN BY A REVERSED IMMUNOSORBENT

BY

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A Thesis

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TABLE OF CONTENTS

PAGE

PAGE

PAGE

PAGE

ABSTRACT

This study describes the isolation and partial characterization of an allergen from the retentate (R) (i.e. the non-dialysable fraction) of the aqueous extract of Kentucky Blue grass (KBG) pollen. The immunoelectrophoretic technique employing a polyspecific antiserum to R served not only to identify the presence of at least 10 different antigens in R, but also served as a source of an immunizing antigen (i.e. an immunoprecipitin band) for the production of a monospecific antiserum to one of the antigens referred to as 'A' without its prior isolation from R. Antigen A was shown by crossed-radioimmunoelectrophoresis to bind IgE antibodies present in a pool of human sera from individuals allergic to KBG pollen and is therefore referred to as allergen A.

A reversed immunosorbent, prepared by coupling the globulin fraction of a sheep antiserum to allergen A to Affi-Gel 10, was used for the immunospecific adsorption of allergen A from R. Allergen A was recovered by elution with a 2M solution of potassium iodide at pH 7.0. The immunochemical purity of allergen A was first established by the finding that only one antigen-antibody system was detected by crossed-immunoelectrophoresis (CIE) employing a polyspecific antiserum to R. Secondly, using a hyperimmune antiserum prepared by the repeated immunization of rabbits with allergen A, antibodies with specificity to allergen A only were detected by immunoelectrophoresis and CIE using R as the antigenic preparation.

The allergenic properties of the purified allergen A were demonstrated by the findings that it could: (i) elicit passive cutaneous

anaphylaxis (PCA) reactions in rats passively sensitized with murine reaginic antiserum to A; (ii) neutralize, to the extent of 94%, the PCA reactions normally elicitable with R, and (iii) inhibit in the radioallergosorbent test, to the extent of 67%, the binding of human IgE antibodies to R. On the basis of the latter two observations, it was inferred that allergen A lacks some allergenic activity present in R.

By rocket immunoelectrophoresis employing the monospecific antiserum to A, it was found that R contains allergen A to the extent of 20%. The protein content of allergen A is of the order of 56%. The electrophoresis of allergen A on polyacrylamide gels containing sodium dodecyl sulfate revealed a major component with a molecular weight corresponding to 16,500 daltons.

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LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

At the end of the 19th century, the different immunological manifestations were viewed primarily as defense mechanisms. However, at the beginning of this century, Portier and Richet (1) proposed that certain immunological mechanisms similar to those involved in protection from microbial infection could also result in harmful effects and reactions which often proved fatal. This state of increased and altered immunological reactivity of the body to a foreign substance resulting in tissue damage upon subsequent exposure to the same agent is referred to as "hypersensitivity".

During the late 19th century, some experimental studies into the nature of hypersensitivity were done by several investigators employing different animals. In 1890, Koch (2) found that the first injection of tubercle bacilli into dogs had no observable harmful effect, but a second injection resulted in inflammatory reactions. In 1894, Flexner (3) demonstrated that rabbits previously injected with dog serum died upon reinjection. Richet (4), in 1898, noted that an immediate shock-like reaction followed the administration of eel serum to a dog sensitive to the serum. In their studies on the toxicity of extracts of sea anemones, Portier and Richet (1) observed that dogs given a second injection of the extract, several weeks after the first, often became acutely ill, went into shock and died within a few minutes. Richet called this harmful response anaphylaxis (Greek, ana = against; phylaxis = protection) denoting an increase in susceptibility to a toxic substance rather than the expected

increase in resistance.

At the beginning of the 20th century, von Pirquet (5) coined the term "allergy" (Greek for "altered action") to denote an immune deviation from the original state or a "changed reactivity" of an individual. von Pirquet included all forms of altered immunologic responsiveness, encompassing reactions to toxins, bacteria, other infectious agents, and also to urticaria-producing foods. The agents causing such changes are referred to as "allergens".

One of the earliest recorded observations of allergy to plants was made in 1565 by Botallus (6) who noted that some individuals, when exposed to certain flowers, began to sneeze. Bostock (7) in 1828 was the first who used the term "hay fever" to describe the allergic diseases which was believed to be due to toxic substances in pollen. In 1831, Elliotson (8) showed a correlation between the period of strongest symptoms of hay fever and the time of pollination of flowers. Subsequently it was established by Gordon (9), Phoebus (10), Salter (11) and Wyman (12) that pollen was the causative agent for hay fever. In 1865, Blackley (13,14) performed the first diagnostic skin test for hay fever and also showed that grass pollens were able to induce the condition similar to that of hay fever in allergic individuals. Later, Dunbar (15) further confirmed that the pollen was the causative agent for hay fever after repeating the skin diagnostic experiments of Blackley. Meltzer (16), in 1910, demonstrated that an allergic individual suffering from hay fever was sensitive to an extract of the pollen and thereby launched investigations to identify the nature of those substances responsible for inducing the allergic reactions.

ALLERGENS

Allergens are defined as materials which stimulate the production of, and which react with, IgE antibodies. The diverse range of naturally occurring allergens includes pollens from grasses, trees and plants, house dust, foods, animal danders, fungi and insect venoms. Most of these allergens are substances with molecular weights ranging from 15,000 to 40,000 daltons with the exception of bee venom melittin which may have a molecular weight of 2,800 daltons (17). They may consist of proteins, glycoproteins, or carbohydrates of foreign animal or vegetable origin, or pigments.

ANTIBODIES INVOLVED IN ALLERGIC REACTIONS

In 1921, Prausnitz and Küstner (18) first recognized that the condition of immediate hypersensitivity was brought about by the production of an antibody which could be detected in the serum of allergic individuals. They demonstrated that the allergic reactivity of an atopic patient could be passively transferred with the patient's serum to the skin of a normal individual, i.e. the injection of the allergen into the sensitized skin sites resulted in an inflammatory reaction similar to that obtained on injection of the allergen into the skin of the allergic patient. This passive transfer test, referred to also as the Prausnitz-Küstner (P-K) test was the first test for the demonstration of skin sensitizing antibodies in the sera of allergic individuals. The P-K test has been widely used for evaluating the skin activity of various pollen extracts.

Reaginic antibodies in man have been identified by Ishizaka et al (19,20) as belonging to a class of unique immunoglobulins,

designated as immunoglobulins E (IgE). These antibodies are also referred to as cytotropic antibodies since they bind or fix via their Fc regions to the corresponding receptors on the surface of mast cells and basophils. Reaginic homocytotropic antibodies have been identified in man (21), monkey (22), cow (23), rabbit (24), dog (25), rat (26), mouse (27), sheep (28), pig (29), and guinea pig (30). The state of immediate hypersensitivity in man is characterized by (i) the presence of IgE antibodies which have the ability to bind to their target tissues, primarily mast cells and basophils; (ii) the rapid onset of symptoms, usually within a few minutes following exposure to the allergen; and (iii) the release of pharmacologically active agents (primarily histamine, slow-reacting substance of anaphylaxis (SRS-A), serotonin (5-hydroxytryptamine), eosinophilotactic factor of anaphylaxis (ECF-A), kinins, prostaglandins and bradykinin) from mast cells and basophils. These mediators act on blood vessels and smooth muscle tissues leading to wheal and flare skin reactions, itching, sneezing, etc.

It was first demonstrated by Ishizaka et al (31,32) that the stimulus which would trigger mast cells or basophils sensitized with IgE antibodies to release histamine had to be at least a bifunctional agent such as the divalent antibody or the $F(ab')_2$ fragment of antibodies to IgE which were able to bridge together two adjacent IgE molecules bound by their Fc portion to the cells. The univalent antibody fragment, Fab, although still capable of combining with the cell-bound IgE molecules, could not crosslink or bridge two adjacent IgE molecules and thus was not effective for mediating the release of chemical mediators from the cells.

The immunoglobulin E possesses many unique physicochemical

properties. Thus IgE migrates with the mobility of the slow moving β-globulins (33,34). It has a molecular weight of approximately 196,000 daltons and a sedimentation coefficient of 8S (35,36). IgE consists of 2 light chains (either type κ or λ) and 2 heavy chains of type ε. The heavy chain consists of one variable (V) region and four constant (C) domains, Cε1, Cε2, Cε3 and Cε4. IgE has a high carbohydrate content of 12% and comprises only 0.004% of the total serum immunoglobulins. Like IgG and IgD, IgE normally exists only in monomeric form. It does not form a precipitate with the homologous allergen in vitro which may be attributed to the fact that, although IgE antibodies are divalent and may have a high affinity for the allergen, their concentration is too low to be detected by standard tests involving immune precipitation. IgE does not cross the placenta (37,38) and is retained by the choroid plexus (39). Its activity is lost at 56°C, i.e. it is thermolabile. Its biological activity is destroyed either by digestion with papain (40) or by reduction, e.g. with 0.1M mercaptoethylamine (36,40,41).

Many workers (42,43) using various antigens have demonstrated that a two-cell system, involving the participation of B-lymphocytes and T-lymphocytes, was required for the formation of IgE antibody. In recent years, several investigators (44-46) have demonstrated that antigens modified by either physical or chemical means resulted in products with greatly reduced antigenicity. In addition, some of the modified allergens acquired the capacity to specifically induce immunological suppression of humoral antibody responses in animals which had been sensitized to the native antigens. More recently, Lee and Sehon (47,48) have suppressed the formation of IgE antibodies using allergens conjugated to polyethylene glycol. They proposed that the mechanism of immuno-

logical suppression operates at the cellular level which interferes with the normal cooperation between helper T cells and the Bε cells involved in the formation of IgE antibodies. Our understanding of the basic properties of allergens should facilitate attempts in producing more effectively modified allergens for use in the suppression of the IgE antibody response. It is clear that such studies require the availability of purified allergens.

As early as 1935, Cooke et al (49) used the procedure, referred to as hyposensitization therapy, in which allergic individuals received injections of a mixture of the relevent allergens administered initially at a low dose to avoid any local or systemic reactions. The dosages were gradually increased until the allergic individual could tolerate higher amounts of the allergenic extract without displaying serious systemic reaction. It was demonstrated that upon hyposensitization therapy an increased tolerance towards the offending allergen was observed. It was suggested that this tolerance was due to the formation of "blocking" antibody (refer to p.7) in the patient. Bernton et al (50) in 1962 also demonstrated that symptoms of allergic patients were alleviated by passive immunization with human globulins rich in blocking antibodies. It was observed that circulating IgE antibody levels increased slightly during the initial hyposensitization period and then gradually fell below pretreatment levels over a period of several years. During the hyposensitization treatment, in addition to affecting the levels of skin-sensitizing antibodies, blocking antibody of the IgG class with specificity for the injected allergen was also formed in the allergic individuals. The protective capacity of the blocking antibody has been attributed to the fact that it may competitively inhibit the binding

of that allergen with IgE antibody and thereby preclude the release of pharmacologically active agents from the target cells.

ANTIBODIES OF THE IgG CLASS

IgG has a molecular weight of about 150,000 and a sedimentation coefficient of approximately 7S (51). It has a relatively low carbohydrate content of 2% (52) and comprises the majority of the total serum immunoglobulins (80%). It is electrophoretically a slow moving γ-globulin (53) and possesses the lowest anodic mobility at pH 8.6.

IgG is the major immunoglobulin to be synthesized during the secondary response. It can cross the placenta and provides a major line of defense against infection for the first few weeks of a baby's life which may be further reinforced by the transfer of colostral IgG across the gut mucosa in the human neonate. Unlike the skin-sensitizing IgE antibody, IgG binds neither to mast cells nor to basophils and therefore cannot trigger the release of pharmacologically active agents. IgG antibodies are divalent and are capable of combining with the allergen. They are sometimes referred to as blocking antibodies and are present in both treated and non-treated allergic individuals. Repeated injection of the offending allergen in small doses into an allergic individual during hyposensitization treatment will lead to an increase in the blocking antibody level.

Unlike IgE, antibodies of the IgG class are stable to heating at 56^{o}C (53) or to reduction by mercaptoethylamine (0.1M) (40). They can form a precipitate with the homologous allergen *in vitro*. Homocytotropic antibodies of the IgG type have been identified in the guinea pig (IgG1a), in the mouse (IgG1), and in the rat (IgGa); there is increasing evidence for the existence of a similar antibody in man (54).

HISTORICAL PERSPECTIVES AND GENERAL CONSIDERATIONS ON FRACTIONATION AND CHARACTERIZATION OF ALLERGENS IN POLLENS

I. FRACTIONATION OF ALLERGENS

Since the finding (16) that pollen extracts could elicit allergic reactions in sensitized individuals, many attempts have been made during the past sixty years to isolate and characterize pollen allergens. The fact that pollens are highly complex and heterogeneous mixtures of allergenic proteins and glycoproteins, containing also pigments, carbohydrates, and low molecular weight substances (55,56), which share very similar physicochemical properties, has hindered attempts at the isolation and characterization of allergens in pollens. It was not until the late 1950's, with the introduction of ion-exchange cellulose and cross-linked dextrans that the separation of proteins according to their electrical charge and molecular size could be carried out.

(a) Grass Allergens

Since the ether-soluble lipid components of grass pollens have been found to be allergenically inactive in most allergic individuals (57,58) and since they tend to hinder subsequent purification, the first step in pollen fractionation involves removal of the ether-soluble lipid components by extracting the pollen with peroxide-free dry diehtylether using the Soxhlet extraction apparatus.

(i) Cocksfoot grass pollen

A highly purified fraction designated as 'A Antigen' was

isolated from cocksfoot pollen by O'Sullivan (59) who employed a combination of salt precipitation, gel-filtration on Sephadex G-25, ion-exchange chromatography on DEAE-cellolose and gel-filtration on Sephadex G-100. 'A Antigen' had a molecular weight of the order of 56,000 daltons. However, it should be noted that the 'A Antigen' had trace impurities which could not be separated from this allergenic preparation.

Recently, a cocksfoot pollen extract has been fractionated by isoelectrofocusing in polyacrylamide gel in a pH gradient from 3-10 (60). Although this procedure yielded fractions which appeared to differ from one another in terms of their allergenicity, it was obvious that a pure allergen was not obtained by this method.

(ii) Timothy grass pollen

Malley and his associates (61-65) isolated two allergens, Ag A and Ag B, and a low molecular weight hapten-like fraction, Ag D, from timothy grass pollen. The isolation of these fractions was achieved by conventional means, using a phosphate buffer at high pH (10.4) for the extraction and subsequent fractionation procedures. Ag A was found to be impure. Ag B appeared to be antigenically pure as tested by immuno-diffusion analysis. It had a molecular weight of 10,500 daltons (65).

(iii) Rye grass pollen

Marsh and his associates (66-73) isolated three highly purified allergens, Groups I, II, and III, from rye grass pollen by conventional means using a combination of dialysis, ion-exchange chromatography on DEAE-cellolose and gel-filtration on Sephadex G-75 and G-100. The

molecular weights for the three rye Groups I, II and III were 27,000, 11,000 and 11,000 respectively. These three rye pollen fractions were found to be antigenically and allergenically distinct from one another (70,71,73,74). However, they were resolved into multiple bands on polyacrylamide gel electrophoresis, a situation which was termed isoallergenic variation by Marsh, and it was not clear if all the components were indeed allergenically active.

(iv) Kentucky Blue grass pollen

A low molecular weight allergen designated as C-I-2d was isolated from the dialysate of the aqueous extract of Kentucky Blue grass (KBG) pollen (75) by gel-filtration on Sephadex G-25 and G-50 and ion-exchange chromatography on CM-Sephadex. C-I-2d was found to be glycoprotein containing all the amino acids except cysteine. The carbohydrate moiety was composed predominantly of galactose and glucose. C-I-2d had a molecular weight of 14,500 daltons and possessed all of the allergenic determinants present in the retentate (R) of KBG extracts. C-I-2d revealed the presence of 4 components with a pI range of 3.2-5.1 when analysed by isoelectrofocussing on polyacrylamide gel.

Another allergen, designated as allergen C, was isolated from R of KBG aqueous extracts by preparative isoelectrofocusing on a pH gradient of 3-10 and on Bio-Gel P60 (76). Allergen C had a molecular weight of 11,000 daltons and a pI value of 10. It consisted of one antigenic determinant as analysed by crossed-immunoelectrophoresis using sheep anti-R serum as the developing antiserum. Allergen C contained about 50% of protein.

(b) Ragweed Allergens

Several allergens have been isolated from ragweed pollen and subsequently well characterized. By a combination of ammonimum sulfate precipitation, ion-exchange chromatography on DEAE cellulose and gel-filtration on Sephadex G-75, King and Norman (77) isolated a highly purified allergenic protein, designated as antigen E. Antigen E appears to be the major allergen in ragweed pollen. It has a molecular weight of 37,000 and constitutes about 6% of the total protein content of the pollen extract (78). Antigen E may exist in one of four forms which differ in their net charge but have identical molecular weight and amino acid composition. Antigen E is composed almost entirely of protein (99.9%) and contains only traces of carbohydrate (arabinose).

The second most active allergen in ragweed pollen is designated as antigen K (79). Antigen K has a molecular weight of 38,200 daltons. It shares partial immunological identity with antigen E.

Another allergenically active protein, antigen Ra.3, was isolated by Underdown and Goodfriend (80) from the aqueous extract of short ragweed pollen. Ra.3 was shown to have a molecular weight of 15,000 daltons, a total hexose and pentose content of 12.4%, and an amino acid composition distinct from the major allergen of ragweed pollen, antigen E.

Later, Lapkoff and Goodfriend (81,82) isolated an allergenically active basic protein, antigen Ra.5, from the aqueous extract of short ragweed pollen by gel filtration and cation exchange chromatography. Ra.5 is a basic protein devoid of carbohydrate and has a molecular weight of the order of 5,100 daltons. Ra.5 is antigenically distinct from ragweed pollen antigens E and Ra.3. The fact that Ra.5 is

allergenic only in a restricted number of ragweed atopic patients has led to studies designed to elucidate the genetic basis of human IgE antibody responses to an antigen whose primary structure is known (83). Moreover, the relatively small size of Ra.5 lends itself to the elucidation of the nature of the allergenic determinant(s) and other structural features of protein allergens.

THE USE OF IMMUNOSORBENTS FOR THE ISOLATION OF SPECIFIC ANTIGENS OR ANTIBODIES

Conventional procedures which are employed for the isolation of pollen allergens generally make use of the differences in the physicochemical properties of the proteins in the pollen. These methods are frequently laborious and the separations are incomplete. Another technique which has a great potential for use in the isolation of allergens is immunoadsorption chromatography (also known as affinity chromatography) (84,85). The selective isolation and purification of allergens by immunoadsorption chromatography exploits the unique immunological property of the antigens or antibodies to bind specifically and reversibly to their appropriate ligands (antibodies or antigens) (86-88). The basic requirement is that one of the components of the system (i.e. either the antibody or the antigen) should be firmly attached to an insoluble matrix, whilst at the same time retaining the specific antigenic or antibody binding activity.

A solution containing the mixture of components is passed through a column where the insolubilized ligand has affinity for the substances to be isolated. Materials not possessing appreciable affinity for the ligand will pass unretarded through the column, whereas those

which recognize the ligand will be bound in proportion to the affinity existing under the experimental conditions employed. The specifically adsorbed substances (i.e. antigens or antibodies) can be eluted after dissociation with appropriate solvents having high ionic strength or low pH.

The supporting matrix to which the ligands are attached must be physically and chemically stable under the experimental conditions employed and columns packed with the matrix must have satisfactory flow properties. Since the use of immobilized ligands depends on specific adsorption, the matrix must be free from non-specific adsorption effects. Peterson et al (89) have found that cellulose is a good supporting matrix for the preparation of immunosorbents because of its large surface area and its hydrophilic nature but its small pore structure is distinctly disadvantageous for use in columnar form (90,91). The open pore structure of Affi-Gel 10 (Bio-Rad, Richmond, California) allows it to be used for immunoadsorption chromatography of proteins with molecular weight as large as 15×16^6 daltons. Affi-Gel 10 is prepared from agarose beads (Bio-Gel A-15 m) that optimize rigidity, porosity, flow rate and resolution. The matrix is chemically cross-linked to provide stability to chaotropic solutions which are used for elution of the adsorbed materials. Crosslinking enhances stability of the gel without reducing its porosity or hydrophilic character. Aliphatic spacer arms (10 Å long) are anchored to the agarose matrix by extremely stable ether linkages which minimize ligand leakage. The gel is supplied with the spacers in the activated form which couples specifically to proteins with free alkyl or aryl amino groups without side reactions. Affi-Gel 10 is stable to acid and base (pH 2-12), salt

solutions and to organic solvents such as alcohols, dimethyl formamide and dioxane. Since Affi-Gel 10 displays essentially all the desirable features of a matrix for the immobilization of biologically active materials (86), therefore it is a good choice for the preparation of immunosorbents.

A method for coupling ligands to the matrix was proposed by Axén et al (92) using cyanogen bromide as the activating reagent. By this method almost any molecule containing amino groups may be coupled to the matrix (agarose). However, it was found recently that the cyanogen bromide activation procedure has a few disadvantages:- (i) a substantial leakage of ligand occurs from the cyanogen bromide coupled ligand-gels (93-97); (ii) cyanogen bromide is toxic and forms explosive compounds upon prolonged storage at room temperature (98); (iii) filtrates from coupling mixtures contain high levels of cyanide ion and must be kept basic to prevent liberation of hydrogen cyanide gas.

II. CHARACTERIZATION OF ALLERGENS

Although one can characterize allergens according to their physical and chemical properties, none of these has been successfully employed to evaluate allergenicity. This stems from the fact that allergenic activity has not been ascribed to certain well-defined physical or chemical parameters which would reflect the allergenic activity. To evaluate the property of allergenicity, it is customary to use either biological tests (e.g. passive cutaneous anaphylaxis (see p.58) or P-K test) which elicit the release of pharmacologically-active mediators or radioimmunoassays which reveal the extent to which the allergen will bind to the appropriate allergosorbent. In either

case, the allergenic activity is determined in relation to the degree to which the allergen binds to the homologous IgE antibody. The bio- or immuno- assays for allergenic activity, however, are incapable of resolving allergens with closely related physical or chemical properties (e.g. allergens which differ slightly from one another in isoelectric point (pI), or they may differ in their carbohydrate moieties (99) qualitatively and/or quantitatively). Therefore, it is clear that in order to assess the overall purity of an allergen, a combination of assay systems based on different physicochemical and immunochemical methods must be used.

(a) Physical analysis:

Physical analysis falls into 2 categories: molecular size and electrical charge. The molecular size of allergens may be established by (i) sedimentation equilibrium ultracentrifugal analysis (100); (ii) sedimentation diffusion analysis (101); (iii) gel-filtration (102,103) and (iv) sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (104). Various methods have also been used for analysing allergens according to their electrical charge: (i) electrofocusing (on acrylamide or Sephadex gel) (105,106,107); (ii) acrylamide disc electrophoresis (108,109); (iii) starch gel electrophoresis (110) and (iv) agarose gel electrophoresis (111).

(b) Chemical analysis:

Chemical analysis of allergens may include (i) amino acid composition as well as the sequence of amino acids in the allergenic molecule (112) and (ii) the qualitative and quantitative analysis of

carbohydrate moiety (113,114) if present.

(c) Immunological analysis:

Immunological analysis of the allergens can be analysed according to their (I) Antigenic[a] and (II) Allergenic[b] properties.

(I) ANTIGENIC ANALYSIS:

Antigenic analysis can be accomplished through the interaction with antibodies of known specificity. Some of the immunochemical methods which have been used in this study for antigenic assays are outlined briefly below.

(a) Immunodiffusion

One of the simplest and most convenient methods for demonstration of immunochemical relationships between soluble antigens and antibodies is the double diffusion (in two dimensions) method using gel on plates as described by Ouchterlony (115,116). In this method, solutions of antigen and antibody are placed in separate wells in agar gel. The reactants diffuse into the gel and precipitate in an area in which

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[a]Antigenic - the ability of a substance to combine *in vitro* with the homologous antibodies (e.g. IgG precipitating antibodies). In this thesis, the term antigenic will be used to denote the property of combining with antibodies exclusive of those of the IgE class of immunoglobulins. These definitions do not imply that an antigenic determinant is necessarily distinct or different from an allergenic determinant. They serve to distinguish the combination of IgE antibodies with their ligands from the combinations involving antibodies of classes other than IgE.

[b]Allergenic - the ability of a substance to combine *in vivo* or *in vitro* with the homologous reaginic IgE antibodies.

neither reactant was originally present. Precipitin bands are formed at equivalence where optimal concentrations of antigen and antibody are present (i.e. antigen and antibody are maximally precipitated at equivalence). The number of precipitin bands should indicate the minimum number of individual antigen-antibody systems present.

Precipitates formed by Ouchterlony's double diffusion technique are generally identified by the use of a reference antigen in the same diffusion plate. Three basic precipitin patterns underlying the antibody-antigen interactions are: reactions of identity, non-identity and partial identity.

For example, Underdown and Goodfriend (62) have used this immunodiffusion technique to evaluate the antigenic purity and uniqueness of antigen Ra.3 and antigen E from short ragweed pollen using rabbit antiserum to water-soluble components in ragweed. They demonstrated that each of the two antigenic preparations gave a single precipitin arc in reactions of non-identity, and concluded that the preparations were antigenically pure and did not possess any cross-reactivity with each other. Later, Lapkoff and Goodfriend (64) demonstrated the antigenic purity of ragweed antigen Ra.5 using an antiserum to the water soluble ragweed antigens and anti-Ra.5 antiserum by immunodiffusion analysis.

(b) Immunoelectrophoresis

The analytical immunoelectrophoretic method for the study of antigens and antibodies is based upon their characteristic mobility in an electric field. Each antigen-antibody system will form one precipitin band which can be stabilized in a suitable matrix.

There are many applications for the immunoelectrophoretic

technique. These include: (i) identification of the minimum number of antigenic components present in a mixture; (ii) determination of the purity of a fractionated material by comparing it with the immunoprecipitated bands obtained with the unfractionated material; (iii) as an initial step for purification. In this manner, Kisil et al (117) have used the immunoelectrophoretic technique to demonstrate that various fractions obtained from the non-dialyzable aqueous extract of ragweed pollen by zone electrophoresis on Sephadex G-25 were heterogeneous. In addition they showed that none of the electrophoretic fractions was immunochemically pure, some fractions contained at least three immunoelectrophoretically distinct components.

Several factors should be considered when using this technique. The antigen well should generally be small to minimize diffusion effects during electrophoresis. Increasing the distance between the antigen well and the antiserum trough has two consequences: (i) precipitates of major reactions are sharper with less severe effects of antigen or antibody excesses; (ii) minor reaction systems take longer to develop and may never appear sufficiently dense for adequate study. Therefore, a relatively short distance will facilitate the development of shaper precipitin bands. The supporting medium should be pure in order to give a good resolution. Equivalence relationships between the concentrations of antigen and antiserum should be used since: (i) reactions where antibody is in excess are forced toward the center line of the plate and become progressively diffuse with increasing antibody excess; (ii) reactions where antigen is in excess are forced toward the antiserum trough, and in extreme antigen excess, the antigen will migrate out of the field. At low ionic strength of the buffer a constant current will increase

the voltage gradient and thus increase the migration rates. However, this also increases electro-osmosis (i.e. the migration of the liquid phase carries the dissolved antigen towards the cathode). Choosing a pH nearer the isoelectric points of two proteins that migrate close together at pH 8.6 may maximize the effect of any charge difference between them and leads to their separation. Maintaining low temperatures during electrophoresis is also important since higher temperatures could cause proteins to denature and result in lesser number of precipitin bands forming. A relatively high voltage (4 to 6 volts/cm) for a short period of time should be used to minimize diffusion effects ; on the other hand, prolonged electrophoresis may be required to effect a greater separation of components.

(c). Crossed-Immunoelectrophoresis (CIE) (or Two-dimensional Immuno-electrophoresis)

The classical immunoelectrophoretic method of Grabar and Williams (118,119) as already described had a pioneering value and constituted a major step in the evolution of quantitative immuno-diffusion methods. However, the method is slow due to the fact that a period of at least 1-2 days is required for the antibodies and antigens to diffuse towards each other and to form precipitin bands. Also the bands obtained at best provide only semi-quantitative answers. In 1960, Ressler (120) showed that antigens could be forced, by an electric current, into an agar gel bed containing antiserum. The quantitation of the protein in a single antigen-antibody system is given by the rocket-shaped precipitate formed. However, the precipitates resulting from more than one antigen-antibody system were seen as multiple rocket-

shaped curves superimposed upon each other. Thus the precipitin arcs formed were not distinct from one another.

In 1965, Laurell (121) improved the resolution of this method by first separating the protein antigens electrophoretically in agarose gel, followed by a second electrophoresis perpendicular to the direction of the first electrophoresis into antibody-containing agarose. The antigens were initially well separated electrophoretically in the first dimension and thus the precipitin arcs formed on electrophoresis into the antibody-containing gel were more distinct. The higher resolution obtained by this technique makes it superior to the Grabar-Williams classical immunoelectrophoretic technique which is essentially non-quantitative and only detects the minimum number of antigenic components present in a mixture.

The application of CIE is restricted to those antigens whose electrophoretic mobility differs significantly from that of the antibody under the conditions used for the second stage electrophoresis at right angles to the first stage.

Under suitable conditions (e.g. at pH 8.6), the antibody molecules will remain stationary in the gel, while antigen molecules will move into the antibody-containing gel during the electrophoresis and will continue to migrate until they are all precipitated by the antibodies. The end of the electrophoresis is reached when there is no more free antigen to migrate. The area enclosed by the precipitate formed is proportional to the concentration of antigen applied and inversely proportional to the concentration of antibody used.

Qualitatively, three basic types of precipitin patterns may be obtained: (i) identity, (ii) partial identity and (iii) nonidentity.

These are described in Figure 1.

Caldwell et al (122) have used the CIE technique to demonstrate the antigenic heterogeneity between Chlamydia trachomatis and Chlamydia psittaci. They showed that only one common antigen was detected between C. trachomatis and C. psittaci which have 19 and 16 distinct antigens respectively as identified by CIE. They also used the CIE technique to study the precipitins formed with hyperimmune rabbit serum to chlamydiae antigens and with serum from patients with chlamydial infections (123) . They demonstrated that CIE could be used as a technique for preparation of monospecific antiserum by immunization of animals with precipitins excised from developed CIE plates. They also showed that CIE could be a powerful analytical tool for the detection of those antigens to which humans made antibodies during the course of infection.

Of relevance to the study reported in this thesis, one may cite the recent findings of Aukrust et al (124) . They have used the CIE technique to show that the parvalbumin fraction of codfish white muscle extract contained 17 antigens. By means of crossed-radioimmunoelectrophoresis (CRIE) it was found that at least 7 of these antigens had also the ability to bind human IgE antibodies.

(II) ALLERGENIC ANALYSIS:-

Immunochemical and immunobiological methods employed in this study for the qualitative and quantitative analysis of allergenicity of pollen extracts are discussed briefly below.

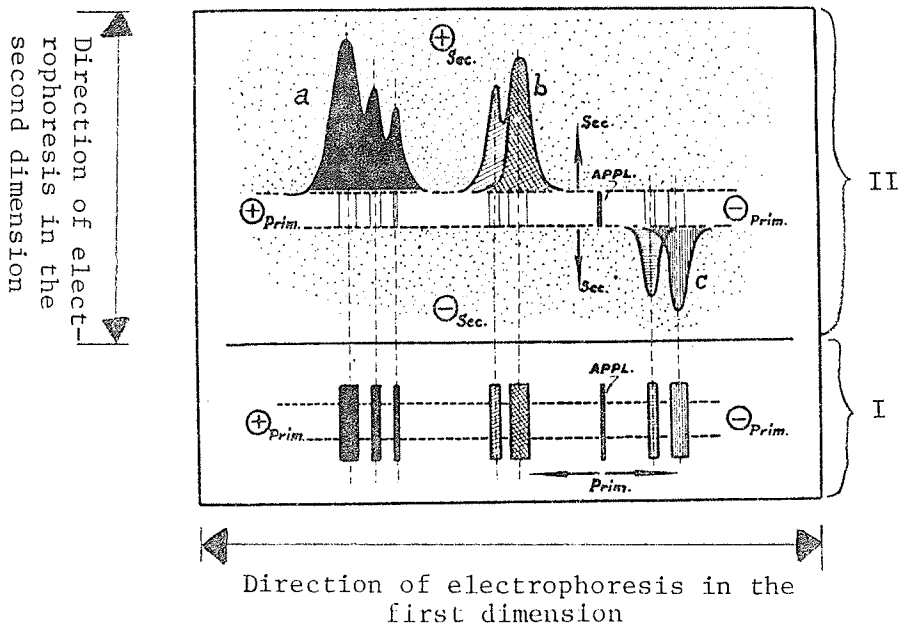


Figure 1: Schematic representation of crossed-immunoelectrophoresis according to the version of Laurell (121).

(a) Identical antigens of anodic mobility.

(b) Partially identical antigens of anodic mobility.

(c) Nonidentical antigens of cathodic mobility.

Prim. = Primary (first dimension)

Sec. = Secondary (second dimension)

Appl. = Sample application

(+) = Anode

(-) = Cathode

(I) = Gel slab from first dimension

(II) = Crossed-immunoelectrophoretic pattern

(a) Crossed-Radioimmunoelectrophoresis (CRIE)

CRIE can be employed as an immunochemical method for qualitative and quantitative analysis of allergens in vitro. It is a valuable tool in facilitating the identification of those antigens which possess also allergenic activity.

Several conditions are necessary for the success of the CRIE technique: (i) precipitating antibodies to the relevant antigens (allergens) are present in the first developing antiserum; (ii) the allergenic determinants should still be present in sufficient concentration following formation of immune complexes; and (iii) the human IgE antibodies of the relevant specificities are present in the second developing antiserum and can be bound to the allergens even though these allergens are precipitated in the form of an immune-complex.

At this point it may be relevant to question the relationship between allergenicity and antigenicity, i.e. are these two properties a function of identical, similar, or different determinants? According to Løwenstein et al (125) there is no chemical basis which distinguishes allergens as being different from antigens. Moreover components with allergenic activity differ in structure just as much as antigens do. During CIE, the allergens are precipitated by antibodies to what are referred to in this thesis as antigenic determinants. It is conceivable that if the antigenic and allergenic determinants are of similar or identical specificities, the precipitating (e.g. IgG) antibodies may block the subsequent binding of reaginic antibodies with their homologous allergenic determinants. If the allergenic specificity to which the IgE antibody specificity is directed is different from antigenic specificity to which the IgG antibody specificity is directed, there should be no

competition for combination for the respective determinants by the reaginic and precipitating antibodies. In addition, steric hindrance resulting from the close proximity of different determinants will also prevent the combination of reaginic antibodies with their homologous allergenic determinants due to prior exposure to precipitating antibodies. As a consequence, the incorporation of radioactive anti-IgE antibodies will be blocked and radiolabelled immunoprecipitin peaks will not be visualized on the radioautograph.

Aukrust and Aas (126) have developed a reference system for CRIE which took into account the fact that the quality and activity of the ^{125}I-labelled anti-IgE would vary from one preparation to another. They used a system of CRIE which employed crude codfish extract as the antigen, rabbit antiserum against a codfish albumin as the developing antiserum and serum from a patient allergic to codfish as IgE reference serum. The reference system permitted a direct comparison of the degree of autoradiographic staining obtained from one experiment to another.

Aukrust et al (127) found that CRIE was superior to RAST and P-K tests regarding its ability to identify distinct allergens. They used this technique to discriminate the major allergen (DS 22) from a minor codfish allergen present also in the crude extract. They also found close correlation between the results obtained from CRIE, RAST and P-K test for the demonstration of allergenicity in codfish extract.

Aukrust et al (124) used the CRIE technique to identify the major allergens of codfish white muscle. They demonstrated by means of CRIE with a pool of sera from 8 allergic patients that at least 7 out of 17 antigens present in the crude extract CIE preparations had

also the ability to bind human IgE antibodies. The major codfish allergenic fraction, DS 22, was found to contain two antigenic components which were able to bind human IgE antibodies.

(b) Passive Cutaneous Anaphylaxis

Passive Cutaneous Anaphylaxis (PCA) is a useful immunobiologic tool for detecting as little as 0.1 µg antibody protein. This technique was summarized by Ovary (128) in 1964 who utilized the local anaphylatic reaction in the skin as a highly sensitive test for the presence of homocytotropic antibody.

Passive Cutaneous Anaphylaxis Neutralization is a variation of the PCA technique which can be used to compare the allergenic activity of different extracts. The allergenic activity (or potency) of different fractions is evaluated in terms of their ability to neutralize the reaginic antibodies produced against the original whole extract. The degree to which the PCA reactions normally elicitable with the whole extract are inhibited due to neutralization of reaginic antibodies by the allergenic fraction being tested, provides an indication of the level of allergenic activity.

(c) Radioallergosorbent Test (RAST)

RAST has become widely accepted as an *in vitro* method for detecting serum IgE antibodies directed to specific allergens (129,130)

Adolphson et al(131) have demonstrated the usefulness of the RAST as a specific and sensitive tool for immunochemical studies of allergens. They found highly significant correlations between the reactivities of allergens (antigen E, antigen K, and Ra.3 from short

ragweed pollen) as determined on one hand by the leukocyte histamine release method and the levels of serum IgE antibody as determined by RAST on the other.

Aukrust et al (127) suggested that RAST was the most convenient technique for the identification of fractions containing allergenic activity. They used this in vitro immunological method to identify allergens present in codfish extract. They also demonstrated that a close correlation exists between RAST, CRIE, and P-K test. On the basis of these correlative results, the techniques of RAST and CRIE (amongst others) were employed with confidence as methods to evaluate allergenic activity in the experiments reported in this thesis.

(d) Inhibition of RAST

The importance of standardizing allergens on the basis of their potency (i.e. allergenicity) is emphasized by the findings that the potency of commercial allergenic extracts may vary over a thousand fold. In addition, the ability to examine for potency is important in evaluating the stability of allergenic solutions to different agents and storage conditions. On the basis of such evaluations, a rational selection of the most appropriate allergens for diagnostic or therapeutic purposes can be made.

Over the past sixty years, many methods have been used to express the biologic activity of allergenic extracts, on the basis of e.g. (i) the weight/volume measurement (132), (ii) the total nitrogen content (133), and (iii) the protein nitrogen unit (134). Arbesman and Eagle (135) and Yunginger et al (136) found that none of the above methods correlated with the biologic activity of allergenic extracts as measured

by direct skin testing or by neutralization of P-K tests. Attempts to use the inhibition of RAST technique to standardize various allergenic extracts (136,137,138) have been successful.

It was recently found by Arbesman et al (138) that the allergenic potency of 7 different ragweed extracts established by RAST inhibition correlated with the antigen E content of the extracts. On the other hand, there was a wide discrepancy between the potency of different extracts when they were evaluated according to the amount of protein nitrogen unit, total protein or dilution (weight/volume) of the different extracts required to cause 50% RAST inhibition. Antigen E was previously found by King et al (77,78) to be the major antigen which possesses also the major allergenic activity in ragweed extract.

Yunginger et al (136) also evaluated the potency of allergenicity by RAST inhibition and by direct RAST assay and found that they strongly correlated with the potency of the commercial Alternaria extracts as measured by skin testing. These findings suggest that RAST inhibition might provide a method for standardization of allergenic extracts which would obviate the need for and eliminate the hazards associated with in vivo tests.

The results obtained by the direct RAST and RAST inhibition are reliable and reproducible. Data obtained from quadruplet samples done at the same time are in close agreement. However, there are two drawbacks for the RAST techniques in general: (i) it is necessary to have purified human IgE and to obtain the specific anti-human IgE antiserum, and (ii) the decay of radioactive label on the anti-human IgE antibody which necessitates frequent preparation of freshly labelled reagent.

SCOPE OF THE PRESENT STUDY

Over the past sixty years, attempts have been made to isolate pure allergens from the highly complex mixture of pollen allergens. The rationale for this quest for pure pollen allergens is based on the following considerations:-

(1) Airborne pollens from trees, plants and grasses are known to be the causative agents for hay fever allergies in both man and animals. Hyposensitization treatment involves the injections of low doses of the offending agent into the patient. Noon (132) succeeded in desensitizing patients suffering from hay fever by a series of injections, of water-soluble extracts of the offending pollen, given at various time intervals. He interpreted that the success of desensitization or hyposensitization as being due to the formation of neutralizing antibodies in response to the injection of the pollen extracts. However, some investigators (139, 140) demonstrated that injection of the crude extract of the pollen which would contain virtually all of the allergens resulted in increased sensitivity of the allergic individuals. It was proposed that these treated individuals become sensitized to other allergenic components of the pollen to which they were not previously sensitive. At present, almost all the commercial allergenic extracts for clinical use are heterogeneous mixtures and may contain materials which are not allergenic or, even worse, they may be toxic to the patients (141). Therefore, in order to eliminate the possibility of inducing formation of additional reagins with different specificities, it is of prime importance to purify and characterize those components to which the allergic individuals become sensitive and to select for therapy only those allergens which had been

originally responsible for eliciting the formation of IgE antibodies in each patient.

(2) The relative potencies of allergenic preparations have been compared with one another on the basis of concentration by weight, protein content or protein nitrogen units, but none of these has proven to be satisfactory for measuring the allergenic potency of a given preparation, which would be a true reflection of the allergenic content. Consequently, there is a pressing need for the isolation of purified allergens which are essential for the standardization of allergenic extracts on the basis of their potency. Such purified allergens would be used for diagnostic purposes as well as for immunotherapy.

(3) Purified allergens are essential for the studies on the immunogenetic basis of the immune recognition of different allergenic determinants.

One objective of the present study was to isolate and characterize an allergen from the retentate (R) of Kentucky Blue grass (KBG) pollen by immunochemical methods.

The choice of KBG pollen for this study was predicated on the fact that this pollen represents one of the major allergens responsible for hay fever and upper respiratory tract allergies in the Prairies and also in other parts of North America and Europe.

The immunoelectrophoretic technique was used as an initial step to identify an antigenic component 'A' present in R. A monospecific antiserum to 'A' was produced in a sheep by immunization with immunoprecipitin bands 'A' excised from immunoelectropherograms, i.e. the antiserum was prepared without prior isolation of the antigenic component 'A'. By CRIE, component 'A' was found to possess allergenic activity. A

reversed immunosorbent was employed in this study for the isolation of this allergen (designated as allergen A) from R using the sheep anti-A (S597) serum. The purity of allergen A isolated by this immunospecific method was established by crossed-immunoelectrophoresis employing hyperimmune antisera to the crude pollen extract, and by the production of a monospecific antiserum in rabbits using allergen A as the immunizing antigen. The allergenicity of the purified material was established by inhibition of radioallergosorbent test (RAST), passive cutaneous anaphylaxis (PCA), and neutralization of PCA.

ISOLATION AND CHARACTERIZATION OF A NONDIALYZABLE ALLERGEN OF KENTUCKY BLUE GRASS POLLEN

MATERIALS AND METHODS

Preparation of the Nondialyzable Fraction of Kentucky Blue Grass Pollen

An aqueous extract of Kentucky Blue grass (KBG) pollen (Hollister-Stier Laboratory, Mississauga, Ontario, Canada) was prepared according to the procedures described by Ekramoddoullah and Sehon (142) and is illustrated in Figure 2. The KBG pollen was defatted with diethyl ether in a Soxhlet extraction apparatus at room temperature for 24-32 hours, until all the ether soluble pigmented materials were removed. Traces of ether were subsequently evaporated off at reduced pressure in a desiccator or more conveniently by keeping the pollen residue overnight in a fumehood. The defatted pollen was suspended in distilled water (500 ml water per 100 g of pollen) and extracted with constant stirring for 4 hours at room temperature. It was then filtered by suction through a Buchner funnel using filter paper, Whatman No.3. The pollen residue was washed with a small volume of water and filtered in the same way The combined filtrate, a brown colored aqueous extract, was lyophilized.

The nondialyzable fraction was obtained from KBG aqueous extract according to the procedures established by Ekramoddoullah et al (143). Briefly, the KBG aqueous extract was dialyzed through Visking tubing, No.20 (Union Carbide Canada Limited, Lindsay, Ontario, Canada) against several changes of distilled water for 48 hours at 4^{o}C. The nondialyzable components, referred to as the retentate or R, were

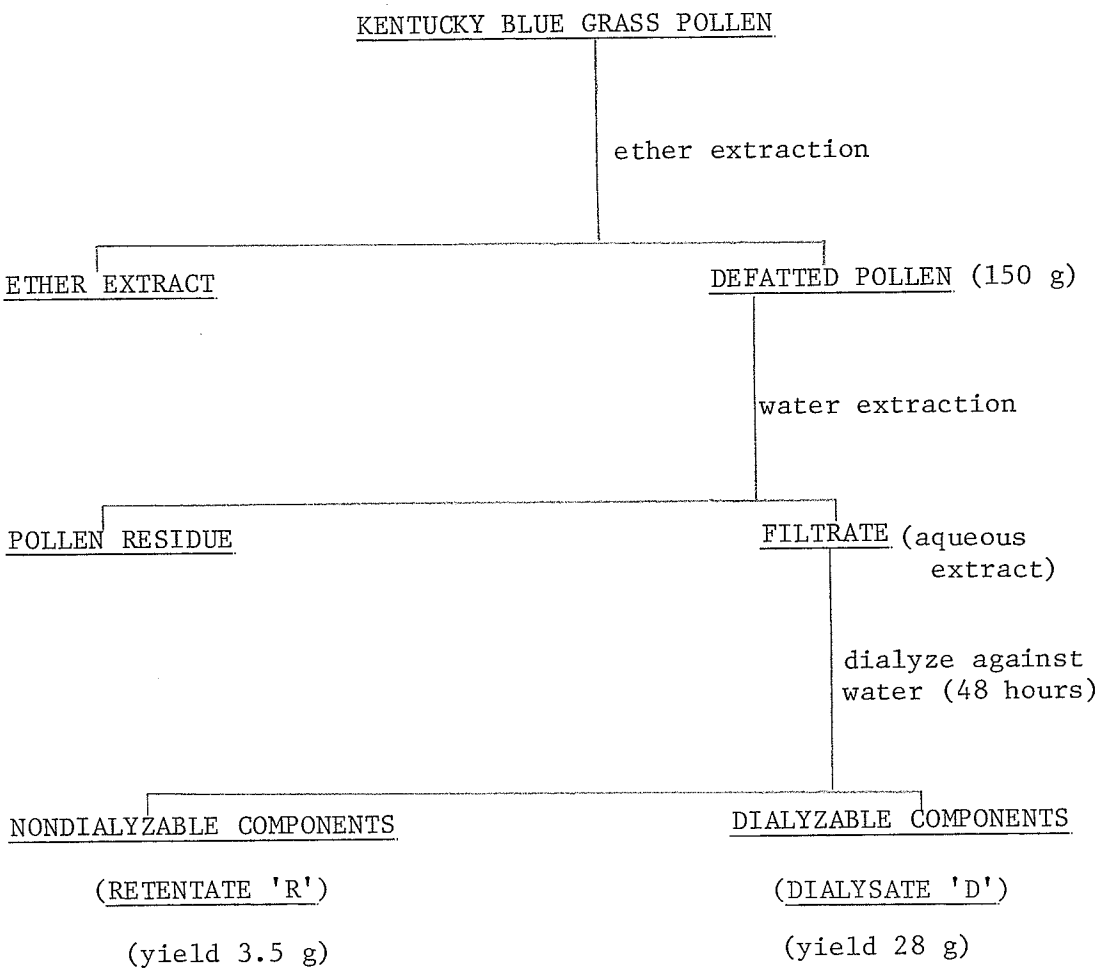


Figure 2: Flow Diagram for the Preparation of the Nondialyzable Fraction, Retentate (R), of Kentucky Blue Grass (KBG) Pollen.

lyophilized and stored in the dry state.

Preparation of Antisera to R

1. Murine reaginic antisera:

The formation of anti-R reaginic antibodies was induced in A/HeJ mice (6-7 weeks of age, Jackson Laboratories, Bar Harbor, Maine) by immunization with R at a single dose of 100 µg (on a dry weight basis) in a suspension of 5 mg of aluminium hydroxide (Amphojel, Wyeth Ltd., Toronto, Ontario, Canada), which was administered intraperitoneally (143). One hundred days later, at a time when the IgE antibody titer was at or near the maximum level, the mice were bled from the tail. The sera were pooled and stored in the frozen state. The levels of the murine anti-R IgE antibodies were measured by PCA analysis (to be described later, see pp. 58-59) in hooded rats.

2. Sheep precipitating antisera:

To prepare antisera containing precipitating antibodies to R, sheep (North American Laboratory Supplies, Gunton, Manitoba, Canada) were repeatedly immunized according to the schedule given in Table I. The antiserum employed in the studies to be described in this section (referred to S11) was obtained after the fifth course of immunization with R.

The immunoelectrophoretic pattern of R using anti-R (S11) serum revealed that most antigenic components had migrated in the anodic direction (Figure 3). However, one antigen had migrated in the extreme anodal direction and the major portion of the immuno-

TABLE I

Protocol for preparation of sheep anti-R (S11) serum

Day 1	First immunization* with R
12	Second immunization with R
27	Third immunization with R
56	Fourth immunization with R
131	Fifth immunization with R
138	Animal bled for antiserum

*A solution of R (0.5 mg) was emulsified with Freund's complete adjuvant and injected intradermally for the first immunization, and intradermally and intramuscularly for all subsequent immunizations.

Figure 3: Immunoelectrophoretic pattern of the retentate (R, central well) prepared from the aqueous extract of KBG pollen.
Sheep anti-R (S11) serum was used as the developing antiserum and was placed into the longitudinal trough. One of the immuno-precipitin bands has been labelled 'A' for ease of reference.

Figure 3a: Photograph of stained slide.

Figure 3b: Diagramatic illustration.

Figure 3a:

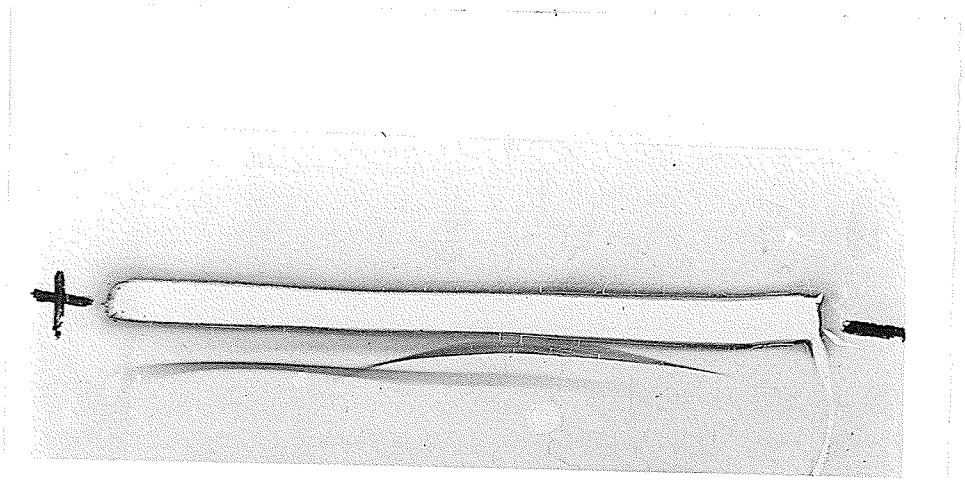
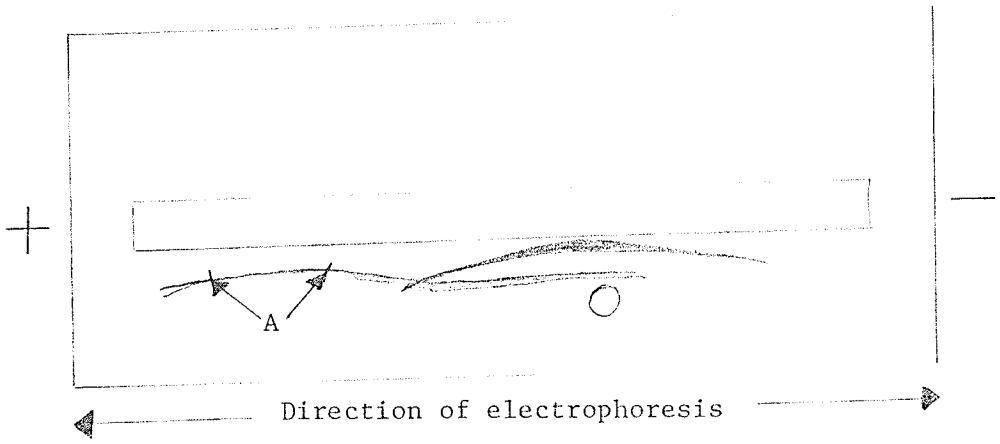


Figure 3b:



precipitin band (labelled 'A', Figure 3b) was well separated from other precipitin bands. Since each immunoprecipitin band represents the presence of a unique antigen, complexed with homologous antibody, the possibility was visualized that the precipitin band could be used for the immunization of animals to produce an antiserum specific to a unique antigen. For this purpose, the immunoelectrophoresis of R was done on a 'preparative' scale in order to obtain a sufficient number of the precipitin bands which, together with the agar, could be excised from the developed immunoelectrophoretic plate.

For this purpose, glass plates (20 cm X 20 cm) were precoated with an aqueous solution of agar (1%) and allowed to dry. Next, a solution of agar (1%) was prepared in veronal buffer* (1 part of stock solution : 3 parts of distilled water) by heating in a boiling water bath until the agar dissolved. A volume of 90 ml of the agar solution was poured onto the glass plate and allowed to solidify. A total of 16 wells for the antigen were punched into the agar and a volume of 30 µl of a solution of R (25 mg/ml) was added to each well. Electrical connection between the plate and buffer vessels was accomplished with paper wicks soaked in veronal buffer (full strength, stock solution). Electrophoresis was carried out for 3 hours at a potential of 5 volts/cm D.C. After this time, a total of 14 troughs alongside the antigen wells and parallel to the direction of electrophoresis were cut out of the agar and the γ-globulin fraction (refer to p.47) of the sheep antiserum to R (S11) was added. After the precipitin bands had fully developed, the plate was

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*Veronal buffer (stock solution): 20.618 g sodium barbital + 13.608 g sodium acetate.$3H_2O$ + 3.684 g barbituric acid + 1.0 g sodium azide, bring up to a final volume of 2 liters with distilled water (pH 8.6)

washed thoroughly to remove proteins with either unrelated or no antibody activity. The precipitin bands labelled 'A' (Figure 3) were carefully excised. A total of 28 precipitin bands were excised from the developed immunoelectrophoretic plate. No attempt was made to separate the antigen-antibody complexes from the agar; rather the excised agar was first frozen and thawed several times, and the resulting suspension was emulsified with Freund's complete adjuvant and used for immunizing a sheep according to the protocol described in Table II.

After obtaining the sheep anti-R (S11) serum which was used for the preparation of immunoprecipitin bands for use as an immunizing antigen, the sheep received 4 additional intramuscular injections of R, each at a dose of 5 mg emulsified in Freund's complete adjuvant, over a period of approximately 11 months. The antiserum obtained after this time was referred to as S11R and was employed in all subsequent immunodiffusion analyses (Ouchterlony, immunoelectrophoresis, crossed-immunoelectrophoresis and crossed-radioimmunoelectrophoresis) of R or its fractions.

Immunodiffusion

Immunodiffusion was carried out in agar gel on plastic petri dishes (5.50 cm in diameter). A solution of 1% agar in saline, containing 0.001% sodium azide was prepared by heating in boiling water until all the agar dissolved. A volume of 6 ml of the agar solution at a temperature of 80^{o}C was poured onto the petri dish placed on a levelled surface. After a time of 15 minutes, by which time the gel had solidified, wells were punched into the agar with a gel puncher (LKB-Produkter AB, Sweden). Due to the fact that antibodies possess a higher molecular

TABLE II

Protocol for preparation of sheep anti-component 'A' (S597) serum

Day 1	First immunization* with immunoelectrophoretic precipitin band 'A'
8	Second immunization with immunoelectrophoretic precipitin band 'A'
43	Third immunization with immunoelectrophoretic precipitin band 'A'
55	Animal bled; antiserum used to develop the precipitin bands following immunoelectrophoresis of R. Precipitin band 'A' cut out
64	Immunization with immunoprecipitin band 'A'
85	Immunization with immunoprecipitin band 'A'
92	Animal bled for antiserum

*Intradermal injections of immune precipitate 'A' (c.f. Figure 3) emulsified in Freund's complete adjuvant. Subsequent injections given both intradermally and intramuscularly.

weight and thus diffuse more slowly than the antigens in KBG aqueous extract (m.w. of R ranging from 10,000 to 60,000 as determined by SDS-PAGE, refer to Results, p.90), immunoprecipitin bands would be expected to form at the antibody wells and the patterns would be difficult to interpret. Therefore, the antiserum was placed into wells and allowed to diffuse for a period of 3 hours prior to the addition of antigen into the wells. The gel was allowed to incubate at room temperature in a humid chamber for 24-48 hours. In this manner, the immunoprecipitates formed in the region of agar between the antibody and antigen wells. The immunoprecipitin patterns were observed and recorded.

Immunoelectrophoresis

The analytical immunoelectrophoretic technique described by Williams and Grabar (144) was used. For this purpose, clean glass plates (3¼" X 4", Erie Scientific Corporation, Buffalo, New York) were coated with 1% agar (Special Agar-Nobel, Difco Laboratories, Detroit, Michigan) solution in water, at 80°C, by spreading a few drops of the solution evenly across the plates and allowing it to dry. A volume of 20 ml of a solution of agar (1% agar dissolved by heating Tris-barbital-lactate buffer* at pH 8.6) was applied onto each levelled agar-precoated glass plate and allowed to gel for about 15 minutes. A well 5 mm in diameter was cut into the middle of the agar bed. The gel plug was

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* Tris-barbital-lactate buffer at pH 8.6 was prepared by dissolving amounts of 8.843 g Tris, 4.42 g barbital, 1.848 g calcium lactate and 0.0195 g sodium azide in distilled water to a final volume of 1 liter.

removed by suction. A volume of 40 µl of the antigen solution containing protein at a concentration of the order of 1.5% was added to the well with a Micropet disposable pipette.

After application of the sample, the glass plate was placed on the electrode chamber (Gelman deluxe electrophoresis chamber, Gelman, Ann Arbor, Michigan) filled with the Tris-barbital-lactate buffer. The buffer-gel electrical connections were accomplished with paper wicks, wetted with the buffer. Electrophoresis was carried out for a period of 2 hours at a potential of 10 volts D.C. per cm and a current of 35 mA using the Canlab Model LR 23909-C power supply.

After the antigen solution had been electrophoresed, a lateral trough (7 mm X 2 mm) was cut into the agar gel alongside the antigen well in the direction of electrophoresis. The gel was removed from the trough with a knife and the appropriate antiserum was pipetted into the trough. Immunodiffusion was allowed to proceed for 2 days at room temperature in a humid chamber. The unreacted proteins were removed by washing overnight with several changes of saline containing sodium azide and finally rinsed in distilled water. The gel was covered with a wet filter paper (Whatman No.1) and then dried by pressing between several layers of dry tissue paper.

Prior to staining, the filter paper was removed and the gel was immersed for 5 minutes in a solution composed of methanol, acetic acid and distilled water in a ratio of 4.5 : 1 : 4.5, containing 0.5% Coomassie Brilliant blue R-250 (Bio-Rad Laboratories, Richmond, California). Excess stain was removed by washing the plates with the solution of methanol, acetic acid and distilled water until a clear background was obtained. The plate was finally rinsed with distilled

water, dried and stored for a permanent record.

Rocket Immunoelectrophoresis

The electrophoresis of an antigen in a gel containing the corresponding antibody results in the development of a long rocket-like immunoprecipitate. The length of the rocket is linearly correlated to the amount of antigen and inversely proportional to the amount of antibody.

Rocket immunoelectrophoresis was used to determine the optimal concentrations of the reactants to be used for crossed-immunoelectrophoresis,(refer to the next section). Combinations of different concentrations of antibody incorrporated in agarose gels and a range of antigen concentrations were employed. Rocket immunoelectrophoresis (145) was carried out on agar-precoated glass plates (3¼" X 4"), each overlaid with a volume of 20 ml of supporting matrix consisting of agarose gel (Indubiose A37, L'Industrie Biologique Francaise S.A.) at a concentration of 1% dissolved in a Tris-barbital-lactate buffer at pH 8.6, containing the appropriate sheep antiserum at different concentrations. After congelation of the agarose (5-10 minutes), evenly spaced wells with a diameter of 6 mm were punched out along a line at the center of the gel and constant volumes (40 µl) of solution of R in concentrations ranging from 0.5% to 2.5% was added. The glass plate with the gel was placed on the Gelman electrophoresis apparatus (described previously), and connected to the Tris-barbital-lactate buffer vessels by means of paper wicks. Electrophoresis was carried out at a potential of 2.5 volts D.C. per cm for a period of 20 hours employing water cooling (15^{o}C). After the completion of the electro-

phoresis, the wells were filled with water and the gel was covered with wet filter paper, avoiding the entrapment of any air bubbles. A 2 cm thick layer of cellulose paper was then placed on top and a pressure of about 10 g/cm^2 was applied and maintained for 30 minutes. The gel was squeezed in a very effective manner by this technique and the liquid phase of the gel containing non-immunoprecipitated proteins was absorbed into the paper. The gel was then washed in 0.1 M NaCl for 10 minutes, twice for 10 minutes each time in distilled water and finally was pressed (as described earlier), dried and stained. The procedures for staining and destaining were the same as that described for the immunoelectrophoretic gels.

The combination of antigen and antiserum concentrations which resulted in the longest precipitin arcs or rockets was regarded as the optimal conditions for use in the two dimensional or crossed-immuno-electrophoresis technique.

Crossed-Immunoelectrophoresis (or Two-dimensional electrophoresis)

Crossed-immunoelectrophoresis (146) was performed on 3¼" X 4" glass plates which had been precoated with aqueous agar solution (1%) and then overlaid with a volume of 20 ml of the 1% agarose solution in Tris-barbital-lactate buffer at pH 8.6 (as described on p.39). After congelation of the agarose, a well 5 mm in diameter was punched into the center of the agarose bed and a volume of 40 µl of the antigen solution (the optimal concentration of the antigen was determined by rocket immunoelectrophoresis as described in the previous section) was applied into the well by means of a Micropet disposable pipette and the gel was connected with the Tris-barbital-lactate buffer by means

of paper wicks. Water cooling was used (15°C). The electrophoresis of antigen in the first dimension was carried out at a potential of 10 volts D.C. per cm for a period of 2 hours.

After that time, a thin strip of agarose (1 X 8 cm) encompassing the electrophoresed antigens, in the direction of electrophoresis, was left on the plate. The rest of the agarose gel was discarded and the exposed plate was wiped clean. A volume of 20 ml of 1% agarose solution in Tris-barbital-lactate buffer was dissolved by boiling in a water bath. The agarose solution was cooled to 55°C, an appropriate amount of antiserum (optimal concentration was determined by rocket immunoelectrophoresis) was added and mixed thoroughly. A total volume of 18 ml of antibody-agarose gel solution was poured onto the glass plate to surround the antigen-containing strip. After a period of 5-10 minutes to allow the gel to solidify, the plate was placed on the electrophoresis apparatus and the gel was connected with the buffer by means of paper wicks, The electrophoresis in the second dimension (perpendicular to the first) was performed at a potential of 2.5 volts D.C. per cm (refer to Figure 4) at 10-20°C for a period of 24 hours.

At the end of the electrophoresis, the gel was pressed, washed, pressed, dried, stained and destained as described previously.

Crossed-Radioimmunoelectrophoresis (147,148)

This method was used to determine which antigen-antibody precipitate(s) formed in crossed-immunoelectrophoresis possessed also allergenic activity i.e. were able to bind human serum reaginic IgE. For this purpose, crossed-immunoelectrophoresis of the antigen(s) was carried out. After immunodiffusion reactions had fully developed, the

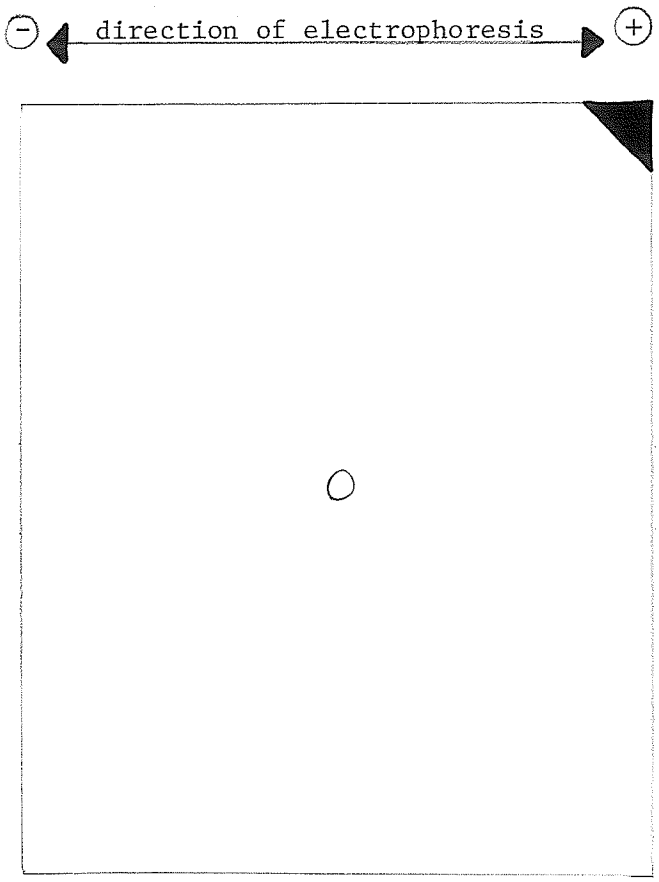
Figure 4: Schematic diagram of crossed-immunoelectrophoresis

+ = Anode; - = Cathode;

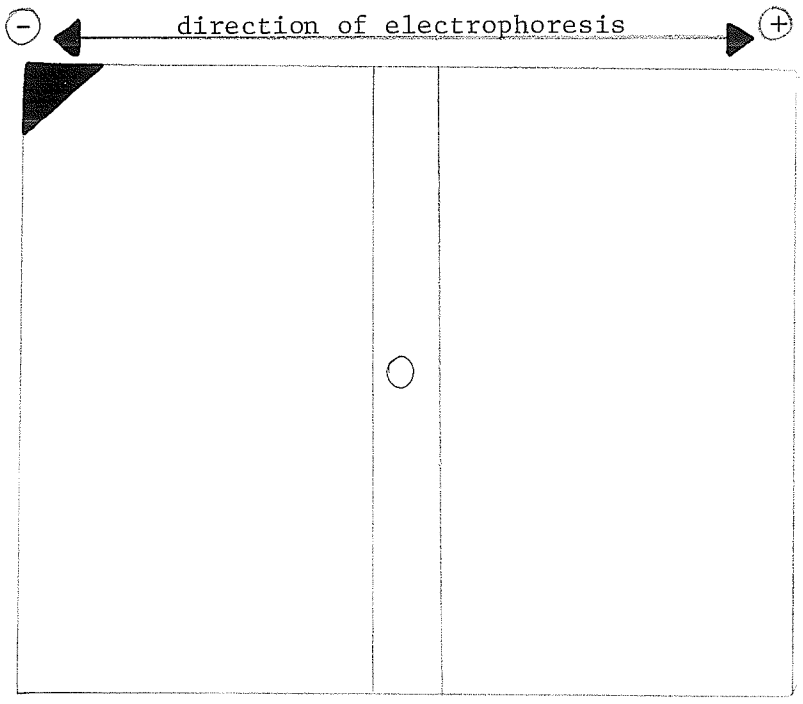
The solid triangle at the corner of the slide is used for orienting the directions of electrophoresis.

Drawn to scale.

First dimension:



Second dimension:



gel was pressed as already described, and washed thoroughly (at least 3 times for 30 minutes each time in fresh saline) to remove the unreacted components. The gel was then rinsed with distilled water, partially dried down and pressed to a thin transparent film on the plate without distorting the pattern of antigen-antibody precipitates.

To test for the presence of allergenic activity in the immunoprecipitins, a volume of 2 ml of a pool of sera obtained from 4 individuals allergic to KBG pollen was overlaid directly onto the gel by means of a Pasteur pipette and then incubated overnight at room temperature (18-24^{o}C) inside a humid chamber to permit the IgE antibodies (amongst other antibody classes) to react with their respective allergens. The serum proteins which had not been involved in the second immunoprecipitin reactions were removed by washing the gel with 100 ml portions of saline containing 1% Tween 20 (Fisher, Winnipeg, Manitoba), at least four times for periods of 10 minutes each. Finally 5 ml of incubation buffer* together with 0.35 µCi (corresponding to approximately 200,000 cpm) of ^{125}I-labelled rabbit antibodies specific for human IgE immunoglobulin (a gift from Pharmacia, Uppsala, Sweden) was overlaid directly onto the gel and allowed to react in an enclosed moist chamber at room temperature for at least one day.

To remove the unbound ^{125}I-anti-IgE, the gel was washed in 50 ml portions in incubation buffer, followed by saline and then with distilled water for periods of 30 minutes each. The gel was finally pressed and dried.

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* Incubation buffer: phosphate buffer 0.05 M, pH 7.5 containing 0.3% bovine serum albumin, 0.9% sodium chloride, 0.1% sodium azide and 0.1% EDTA.

Autoradiography was performed by placing the dried gel onto Kodak medical X-ray N-S film (Kodak, Rochester, New York) in a light-proof wooden box. The film was exposed for a period of the order of 10 days and then developed using Diafine 2 stage developer (Acufine, Inc., Chicago, Illinois) and fixed in Amfix (Acufine, Inc.).

After the radioautographs had been obtained, the gel was stained with Coomassie Brilliant blue. The immunoprecipitates made visible by staining were compared with the radioautographic patterns. In this manner, it was possible to identify those precipitates formed in crossed-immunoelectrophoresis which were able to bind human serum IgE antibodies to allergenic pollen components.

Determination of protein concentration

The protein concentration was determined with the Folin-Ciocalteu reagent (149) following the method of Lowry (150).

The following reagents were used for the Lowry protein concentration determination:-

(i) 2.0% sodium potassium tartrate

(ii) 1% $CuSO_4.5H_2O$

(iii) 2% sodium carbonate in 0.1 N sodium hydroxide

(iv) Reagent C was prepared by mixing 0.5 ml of (i), 0.5 ml of (ii) together with 50 ml of (iii)

(v) Reagent D was prepared by two-fold diluted Folin-Ciocalteu reagent (Fisher, Winnipeg, Manitoba) with distilled water

(vi) Bovine serum albumin (1 mg/ml) was used as a standard and distilled water served as blank

For determining protein concentration, a volume of 0.6 ml of

the protein solution (protein concentration in the range of 0.01-1 mg/ml) was mixed well with 3.0 ml of Reagent C.

After standing at room temperature for ten minutes, a volume of 0.3 ml of Reagent D was added and mixed well immediately. After incubation at room temperature for a period of 30 minutes, which was sufficient for the full development of the color, the optical density was measured by a spectrophotometer (Perkin-Elmer Model No.139, Coleman Instruments Division, Maywood, Illinois) at 690 mμ.

Salt fractionation of the gamma globulin fraction of the sheep antisera

Salt fractionation of the gamma globulin fraction of the sheep antisera was carried out according to Weir (151). A saturated solution of ammonium sulfate (SAS) was prepared by heating an amount of 100 g of $(NH_4)_2SO_4$ with stirring in approximately 100 ml of distilled water at 50°C until the salt was dissolved. It was then allowed to stand overnight at 4°C. The next day, crystals were formed and the pH was adjusted to 7.2 by dropwise addition of a 1 N solution of NaOH.

The globulin fraction of the sheep antiserum was prepared by adding a volume of 50 ml SAS dropwise to an equal volume of sheep antiserum with stirring at 4°C. After admixture the precipitation was allowed to proceed for 24 hours in the cold room at 4°C in order to achieve a coarsely dispersed sediment. The precipitate was then isolated by centrifugation at a speed of 1,800 rpm (equivalent to 1,000 g) for 30 minutes using head #259 in a PR-J refrigerated centrifuge (International Equipment Company, Needham, Massachusetts). The precipitate was washed with a solution of cold 50% SAS, centrifuged and dissolved in a final volume of 50 ml of distilled water. The salt was

removed by dialysis against phosphate buffered saline (PBS, 0.01 M, pH 7.2) for 24 hours in the cold with several changes of PBS.

Preparation of Reversed Immunosorbent

Since it was the intention to isolate one component from R, advantage was taken of the fact that the antiserum to A (sheep S597) recognized only one antigenic component in R (see Results, pp. 61,63; and Figures 7,8, pp. 64,66). This finding was exploited for the isolation of component A by immunospecific means employing a reversed immunosorbent prepared with sheep anti-A antiserum (S597) as described below.

I. Preparation of Reversed Immunosorbent using Sepharose 4B-CL* (152,153)

A volume of 100 ml of washed Sepharose 4B-CL (Pharmacia, Uppsala, Sweden) was activated with an amount of 20 g of cyanogen bromide at pH 11. The activated gel was quickly washed in a Buchner funnel with copious volumes of cold aqueous solution of 0.1% sodium carbonate followed by 2 liters of cold PBS. The gel was then incubated with 1.5 g of the gamma globulin fraction of the sheep anti-A (S597) antiserum for 24 hours in the cold ($4^{o}C$). The immunosorbent was washed with PBS until the filtrate was free of protein and was then reacted

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*Serious problems of desorption of the sheep γ-globulins occured with the reversed immunosorbent prepared with a Sepharose 4B-CL matrix activated by means of cyanogen bromide (see Results 'Reversed immunosorbent prepared with Sepharose 4B-CL' p.71). As a result, the attempts of using this reversed immunosorbent for the specific isolation of component A had to be abandoned in favour of employing a different matrix. The method described in this section serves to illustrate some of the difficulties and pitfalls of preparing useful reversed immunosorbents.

with 500 ml of 0.05 M ethanolamine at room temperature for 30 minutes. The immunosorbent was washed thoroughly with PBS, glycine-HCl buffer (0.2 M, pH 2.2) until the optical density of the eluates was zero at 280 mμ and subsequently with PBS until the pH of the eluate was 7.2.

II. Preparation of Reversed Immunosorbent using Affi-Gel 10 (154)

The globulin fraction of the sheep antiserum (S597) was coupled to the supporting matrix of Affi-Gel 10 (Bio-Rad, Richmond, California). For this purpose, a volume of 25 ml of Affi-Gel 10 suspension was placed into a Buchner funnel and was washed with 100 ml of cold ($4^{o}C$) distilled water under suction. The gel was then immediately transferred into a beaker and a volume of 2 ml of γ-globulin (S597) solution (equivalent to 100 mg protein) in 25 ml of 0.1 M phosphate buffer, pH 7.0, was added to the gel. Since Affi-Gel 10 is supplied as an agarose gel with 10 Å long aliphatic side chains terminated by reactive carboxy N-hydroxy-succinimide esters (155), no further activation step is necessary. The coupling reaction was allowed to proceed at $4^{o}C$ for a period of 24 hours with gentle mixing at a speed of 175 rpm on a horizontal shaker (Gyrotory shaker-Model G2, New Brunswick, New Jersey). The gel suspension was then washed thoroughly to remove uncoupled protein with 1 liter of PBS (0.05 M phosphate buffer containing 1 M NaCl, pH 7.0), under suction, until the absorbance of the filtrate at 280 mμ was zero. To block any remaining active ester groups, the immunosorbent was allowed to react with 25 ml of buffered 1 M ethanolamine-HCl, pH 8.0, at room temperature for 1 hour, with gentle mixing.

After this time, the immunosorbent was packed into a column (2.5 X 13 cm) and was washed extensively with 8 liters of PBS overnight

in the cold (4°C). Prior to use, the column was washed with 100 ml of glycine-HCl buffer (0.2 M, pH 2.2) followed by 100 ml of phosphate buffer (0.05 M, pH 7.0). This washing cycle was repeated 4 more times. The immunosorbent was then washed with 100 ml of buffered 2M potassium iodide (KI) and finally washed with 500 ml of phosphate buffer (0.05 M, pH 7.0).

Immunosorption

The reversed immunosorbent prepared with the γ-globulin fractions of the sheep antiserum (S597) was used immediately following the washing cycles. A solution of R (20 mg) in phosphate buffer (0.05 M, pH 7.0) was applied and permitted to percolate into the column. The column flow was adjusted to 1 drop per 30 seconds and allowed to incubate for 1 hour at room temperature. After this time, components which had not become adsorbed were removed by washing the column with 500 ml of 0.05 M PO_4 buffer, pH 7.0, until the optical density at 280 mμ of the eluates was zero.

Elution of adsorbed antigen

Adsorbed antigen was eluted with a volume of 50 ml of 2M KI in 0.05 M phosphate buffer, pH 7.0. The KI eluate containing the protein was collected and dialyzed immediately against large volumes of distilled water in the cold room at 4°C for 24 hours with frequent changes of water and finally lyophilized. The protein concentration was determined according to the method of Lowry.

The immunosorbent column was further washed with a large volume of PBS and was stored in phosphate buffer (0.05 M, pH 7.0)

containing 0.001% sodium azide at a temperature of 4°C for later use.

Before reuse, the column was regenerated by washing extensively with 4 liters of PBS followed by washing with the eluting agents according to the cycles previously described. The regenerated immunosorbent was then used immediately after equilibration with phosphate buffer.

Estimation of molecular weight by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight of allergen A eluted from the reversed immunoadsorbent was evaluated by SDS-PAGE using the method described by Weber and Osborn (156).

(i) Reagents:

(a) Incubation Buffer A

Incubation buffer A consisted of sodium phosphate buffer (0.01 M, pH 7.0) containing SDS (0.1%) (BDH Chemicals, Poole, England) and β-mercaptoethanol (0.1%) (Bio-Rad).

(b) Incubation Buffer B

Incubation buffer B consisted of sodium phosphate buffer (0.01 M, pH 7.0) containing SDS (1%) and β-mercaptoethanol (1%).

(ii) Preparation of Gel:

The gel buffer contained 7.8 g of $NaH_2PO_4.H_2O$ (Fisher), 38.6 g of $Na_2HPO_4.7H_2O$ (Fisher) and 2.0 g of sodium dodecyl sulfate (95%) dissolved in distilled water up to a final volume of 1 liter.

The acrylamide solution contained 22.2 g of acrylamide (Bio-Rad)

and 0.6 g of methylenebisacrylamide (Bio-Rad) dissolved in water to give 100 ml of solution. Insoluble material was removed by filtration through Whatman No.1 filter paper. The filtrate was kept at $4^{o}C$ in a dark bottle.

Glass tubes, 10 cm long with an inner diameter of 6 mm, were used to form the gels. For a typical preparation of 8 gels, a volume of 10 ml of gel buffer was mixed together with 9.1 ml of the acrylamide solution and were deaerated for 15 minutes. To this solution was added a volume of 1.0 ml of freshly made ammonium persulfate (Fisher) solution (15 mg per ml) and 0.03 ml of N,N,N',N'-tetramethylethylenediamine (Bio-Rad). After mixing, each tube was filled with 2 ml of the solution and immediately overlayered with one drop of water. After 10 to 20 minutes a stable interface between the acrylamide gel and the water layer could be seen indicating that the gel had polymerized. Just before use the water layer was sucked off and the tubes with the gel were placed in the electrophoresis apparatus (Canalco, Rockville, MD).

(iii) Preparation of sample solutions

One drop of glycerol (Fisher), 1 drop of 1% mercaptoethanol, 1 drop of tracking dye (0.05% Bromophenol blue (Bio-Rad) in water), 50 µl of incubation buffer A together with a volume of 50 µl of the protein solution containing an amount of 50 µg of protein were mixed together and pipetted onto the top of the gel. Gel buffer, diluted 1:1 with water, was carefully layered on top of the sample to fill the tube. The two electrode compartments of the electrophoresis apparatus were filled with gel buffer diluted with an equal volume of distilled water.

Electrophoresis was performed at a constant current of 8 mA per tube with the positive electrode in the lower chamber. The electrophoresis was run for approximately 4 hours until the marker dye had moved three-quarters of the length of the gel in the direction towards the anode. The following proteins with molecular weights indicated within the brackets were used as standards to calibrate the SDS-PAGE system: cytochrome c (11,700), ovalbumin (43,000), aldolase (40,000), pepsin (35,000) and ribonuclease (13,700).

(iv) Staining and destaining

Following extraction of the gels from the tubes, the gel lengths and the distance of the migration of the dye were measured. The gels were stained by immersing in a staining solution containing 1.25 g of Coomassie brilliant blue, 454 ml of 50% methanol and 46 ml of glacial acetic acid for a period of 3 hours at room temperature. The gels were rinsed with distilled water and placed in destaining solution containing 75 ml of acetic acid, 50 ml of methanol and 875 ml of water for 5 hours. Further destaining was accomplished electrophoretically using the Bio-Rad Model 172A Gel Electrophoresis Diffusion Destainer (Bio-Rad) in 7% acetic acid solution until the background was completely clear. After destaining, the length of the gels and the positions of the blue protein zones were recorded. The gels were stored in 7.0% acetic acid solution.

Since the gels swelled approximately 5% in the acidic solution used for staining and destaining, the electrophoretic mobility of the protein was calculated according to the following formula:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale.

Radioallergosorbent Test (RAST)

The ability of R to combine with human IgE antibodies to KBG pollen allergens was evaluated by RAST as described by Ceska et al (157).

(i) Activation of paper discs

10 g of cellulose paper discs (0.6 cm in diameter, Whatman No.1 filter paper) were allowed to swell in 200 ml of distilled water for 30 minutes. A volume of 200 ml of 5% cyanogen bromide solution in distilled water was added dropwise to the paper discs with gentle stirring. To maintain the pH in the range of 10.0 to 10.5, a 1 N solution of NaOH was added dropwise, as required. The discs were then immediately and thoroughly washed with a volume of 2 liters of a cold ($4^{o}C$) aqueous solution of sodium bicarbonate (0.005 M). This washing procedure was repeated for 4 more times and followed by washing 4 times with a volume of 500 ml of cold acetone (reagent grade) each time. The paper discs were drained on a filter paper in the cold room at $4^{o}C$ for 3 hours and finally dried over calcium chloride ($CaCl_2$) in a desiccator overnight at $4^{o}C$. The dry paper discs were stored at $-20^{o}C$ for later use.

(ii) Coupling of allergens to the CNBr-activated discs (Preparation of Allergosorbent)

R was insolubilized by its covalent attachment to the activated cellulose discs. This was accomplished by incubation of a volume of 0.2 ml of the allergen solution (100 µg) in PBS (0.02 M, pH 7.2) with a CNBr-activated paper disc on a horizontal shaker (Gyrotory Shaker-Model G2, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at a speed of 100 rpm in the cold room at $4^{o}C$ for 20 hours. (For simplicity, the amounts of the reagents given here are those for one disc; in actual practice and to ensure uniformity, a larger number of allergosorbent discs sufficient for the experiment at hand, was prepared at one time.) After this time, the allergosorbent disc was washed with a volume of 2.5 ml of a solution of $NaHCO_3$ (0.1 M). To block any remaining activated sites on the cellulose disc to which constituents of allergen had not become attached, the disc was reacted with a volume of 1 ml of a solution of β-ethanolamine (0.05 M in 0.1 M $NaHCO_3$) on a horizontal shaker at room temperature for 3 hours. The paper disc was washed successively with volumes of 2.5 ml of 0.1 ml $NaHCO_3$, three times with acetate buffer (0.1 M, pH 4.0) and finally twice with incubation buffer*.

To establish the maximum quantity of IgE antibodies present in a volume of 50 µl of a pool of human allergic sera from individuals allergic to KBG pollen, which would bind to the insolubilized R, allergosorbent discs were prepared with different amounts of R. As can be seen

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*Incubation buffer containing 0.9% NaCl, 0.05 M phosphate buffer pH 7.4, 0.3% human serum albumin and 0.05% sodium azide.

in Figure 5, 100 µg is the minimum amount of R per disc which resulted in the maximum binding of radiolabelled anti-human IgE antibodies in the RAST procedure.

(iii) Adsorption of human allergic sera

A volume of 50 µl of a pool of the KBG allergic sera was diluted in a volume of 75 µl of the incubation buffer, added to each allergosorbent disc and incubated with agitation on a horizontal shaker at a speed of 100 rpm overnight at room temperature. Each paper disc was then washed three times with a volume of 2.5 ml of incubation buffer containing 1% Tween 20.

(iv) Incubation with ^{125}I-labelled anti-human IgE

(Detection of human IgE antibodies bound to the allergosorbent)

The extent to which the IgE antibodies bound to the insolubilized allergens was evaluated by the addition of a volume of 200 µl of ^{125}I-labelled anti-human IgE immunoglobulin which had been diluted in incubation buffer to contain approximately 40,000 cpm. After incubation with labelled anti-human IgE on a horizontal shaker at a speed of 100 rpm overnight at room temperature, each paper disc was washed three times with volumes of 2.5 ml of a solution of 1% Tween 20 in 0.9% NaCl. The radioactivity bound to the allergosorbent-antibody complexes was measured with a Beckman Gamma 300 system counter (Beckman Instruments, Inc., Fullerton, California).

RAST Inhibition: The fraction isolated from the reversed immunosorbent (i.e. allergen A) was tested for allergenicity in terms of its ability

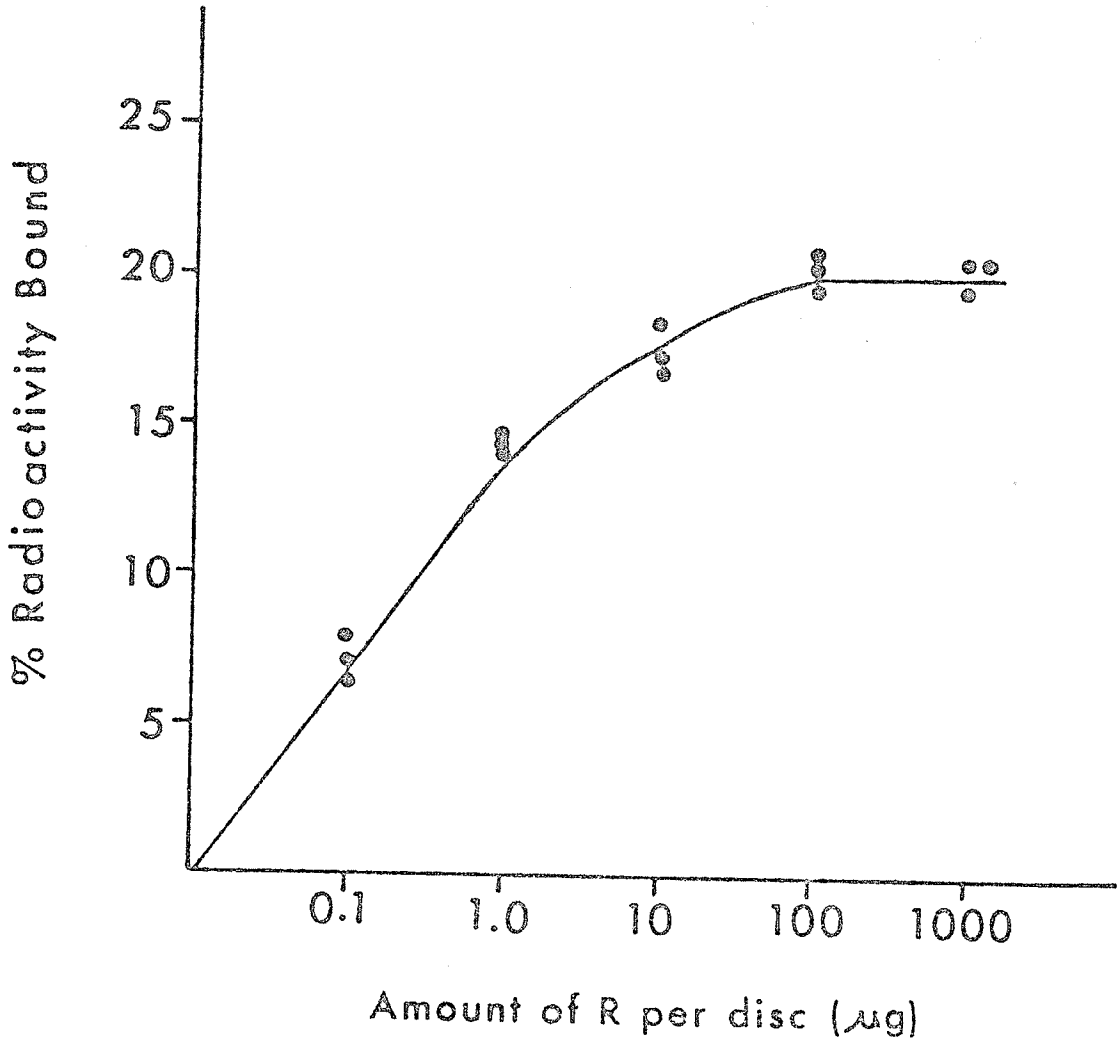


Figure 5: Relationship between the amount of R coupled to activated paper discs and the amount of ^{125}I-labelled rabbit anti-human IgE antibodies bound to the allergosorbent discs (for conditions refer to text pp.55-56).

to bind to IgE antibodies. The fraction was first separately incubated with a volume of 50 µl of a pool of the KBG human allergic sera together with a volume of 50 µl of incubation buffer at room temperature on a horizontal shaker at a speed of 100 rpm for 2 to 3 hours and then in the cold room at $4^{o}C$ overnight before incubating with the allergosorbent disc. The percentage of RAST inhibition is directly proportional to the allergenic activity of the fraction.

Each sample was analysed by inhibition of RAST in quadruplicate and the average value for the radioactivity bound to the allergosorbent-antibody complexes was used.

Passive Cutaneous Anaphylaxis (PCA)

The allergenicity of the isolated fraction was also evaluated by means of PCA and Neutralization of PCA.

The titers of the murine reaginic antisera to R (refer to the section under 'preparation of antisera to R' on p.33) were established by PCA analysis (158,159) in random bred hooded rats (obtained from North American Laboratory Supply Co.,Gunton, Manitoba, Canada).The reasons for performing the PCA in rats rather than using the homologous PCA test in mice are two-fold: (i) the rat skin fixed only the IgE class of mouse antibody, and (ii) the PCA titers obtained in rats with the murine reaginic serum are higher than the corresponding PCA titers obtained in mice.

The rats were passively sensitized by intradermal injections (i.e. local passive sensitization) of volumes of 50 µl of the serially diluted murine reaginic anti-R antisera. At least two animals were sensitized with the antiserum diluted identically. A period of 24 hours

was allowed to elapse before the PCA reactions, if any, were elicited by challenging these sensitized animals with an intravenous injection of a volume of 1 ml of a solution containing an amount of 100 µg of R or the isolated fraction together with Evan's blue dye (0.25%) (Matheson, Coleman and Bell, Norwood, Ohio). Antigen-antibody complexes are formed when the injected antigen comes into contact with the IgE antibody which was fixed to the mast cells in the skin. Local skin reactions result when the antigen-antibody combination triggers the mast cells to release vaso-active substances such as histamine and serotonin. Due to increased capillary permeability, the Evan's blue dye bound to serum proteins is extravasated from the capillaries into the surrounding tissues, blue spots are visualized on the skin at the site of the local antigen-antibody reaction. The end-point of the titrations were expressed as the reciprocal of the highest dilution of the reaginic serum capable of sensitizing a skin site so as to give a PCA reaction of 5 mm in diameter or greater.

Neutralization of Passive Cutaneous Anaphylaxis

Different amounts of the isolated fraction (ranging from a concentration of 10 µg/ml to 8 mg/ml) were mixed with constant volumes (200 µl) of the murine anti-R reaginic antiserum (PCA titer of 640, refer to Results, p.78) at a dilution of 1:10. The mixture of antiserum and fraction was serially diluted two-fold up to the maximum final serum dilution of 1:640 and was incubated at room temperature for a period of 3 hours. Hooded rats were locally sensitized by intradermal injections of a volume of 50 µl of the mixture. Twenty-four hours later, PCA reactions, if any, were elicited in these rats by intravenous challenge

with a volume of 1 ml of a solution containing an amount of 1 mg of R and Evan's blue dye (0.25%).

Production of rabbit antisera to allergen A isolated by the reversed immunosorbent prepared to the sheep anti-A serum (S597)

In order to examine for the purity of allergen A eluted from the reversed immunosorbent, two New Zealand white rabbits (Female Albino Rabbits, 5-6 lbs, Canadian Breeding Laboratories, Montreal, Quebec) were hyperimmunized with this fraction by subcutaneous injections, into multiple sites. An emulsion was prepared with 1 ml of a solution of allergen A (containing an amount of 1 mg) and 1 ml of Freund's complete adjuvant. Each animal received intramuscular injections of a volume of 1 ml of the emulsion (containing 0.5 mg of allergen A) at intervals of three weeks. The animals were bled two weeks after each immunization. Four or more immunizations were required before antibodies to allergen A could be detected by immunoelectrophoresis using R as the antigenic preparation. The animals were reimmunized, at appropriate intervals, 3 more times after antibody had been detected in their sera (i.e. to produce hyperimmune antisera).

RESULTS

I. Evaluation of sheep antisera to R by immunoelectrophoretic analysis

a. Antiserum S11

The immunoelectrophoretic analysis of sheep antiserum S11 using R as the antigenic preparation revealed the presence of four major immunoprecipitin bands (Figure 3, p.35). One of the precipitin bands (at the extreme anodal region) which for the most part appeared to be distinct and separate from the other precipitin bands, was for convenience, referred to as precipitin A. It should be noted that this precipitin band A was observed to split into two at its extreme anodal position. From this immunoelectrophoretic profile alone, it was not possible to determine whether the split was due to two different antigens precipitating at the same position or to denaturation of a single antigen which resulted in the unmasking of additional antigenic determinants.

b. Antiserum S11R

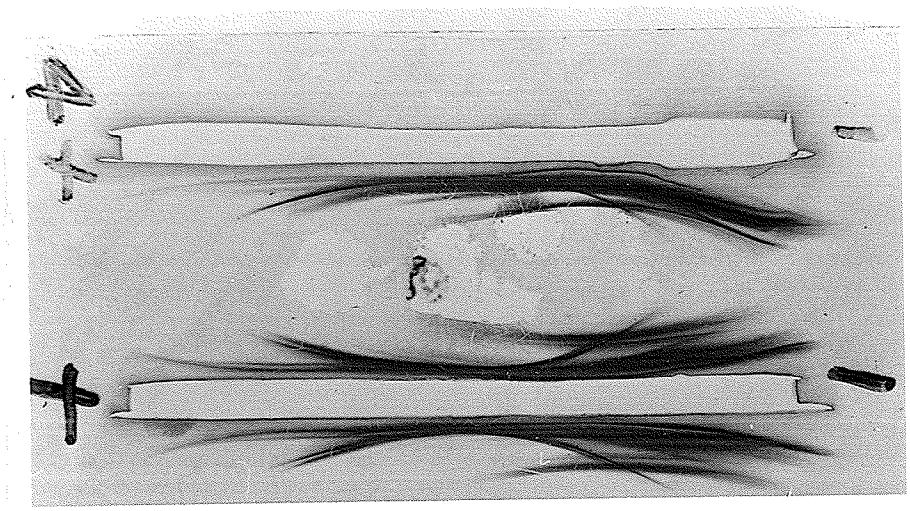
The immunoelectrophoretic pattern elicited with sheep antiserum S11R using R as the antigenic preparation revealed the presence of at least 10 immunoprecipitin bands predominantly in the region cathodic to the sample well (Figure 6).

II. Evaluation of sheep antiserum to 'A'

Antiserum S597 was raised in a sheep by immunization with the immunoprecipitin band labelled 'A' (Figure 3, p.35). In view of the

Figure 6: Immunoelectrophoretic pattern of R (central well) using sheep anti-R (S11R) as the developing antiserum (longitudinal trough).

At least 10 immunoprecipitin bands could be visualized. Most of the precipitin bands formed in the region cathodic to the sample well.



split in precipitin band A (see above), only that segment of precipitin band A which avoided the split was excised (as indicated in Figure 3, p.35) from the developed immunoelectrophoretic gel, and used as an immunogen for the production of an antiserum to 'A'. The immunoelectrophoretic analysis of anti-A antiserum (S597), using R as the antigenic preparation, revealed the presence of one single immunoprecipitin band in the anodic region (Figure 7).

III. Determination of the optimal concentrations of the reactants to be used in crossed-immunoelectrophoresis (CIE)

The combination of the amounts of the antiserum and antigen which resulted in the largest rockets were considered as the optimal conditions. In this manner, the concentration of an aqueous solution of R was found to be 15 mg/ml (the amount of R actually electrophoresed was 0.6 mg) and the amount of the sheep antiserum incorporated into a volume of 20 ml of 1% agarose solution was 1.2 ml for anti-R antiserum (S11R) and 5.0 ml for anti-A antiserum (S597) (Table III).

IV. Crossed-immunoelectrophoretic analyses of R using anti-A (S597) and anti-R (S11R) sera

CIE analysis of R using sheep anti-A (S597) as the developing antiserum revealed the presence of one immunoprecipitin peak in the anodic region (Figure 8). By comparison, the CIE profile of R using sheep anti-R (S11R) as the antiserum revealed the presence of at least fifteen immunoprecipitin peaks, 7 in the anodic region and 8 in the cathodic region (Figure 9).

Figure 7: Immunoelectrophoretic pattern of R (central well) using sheep anti-A (S597) as the developing antiserum (longitudinal trough).
One immunoprecipitin band in the anodic region could be visualized.

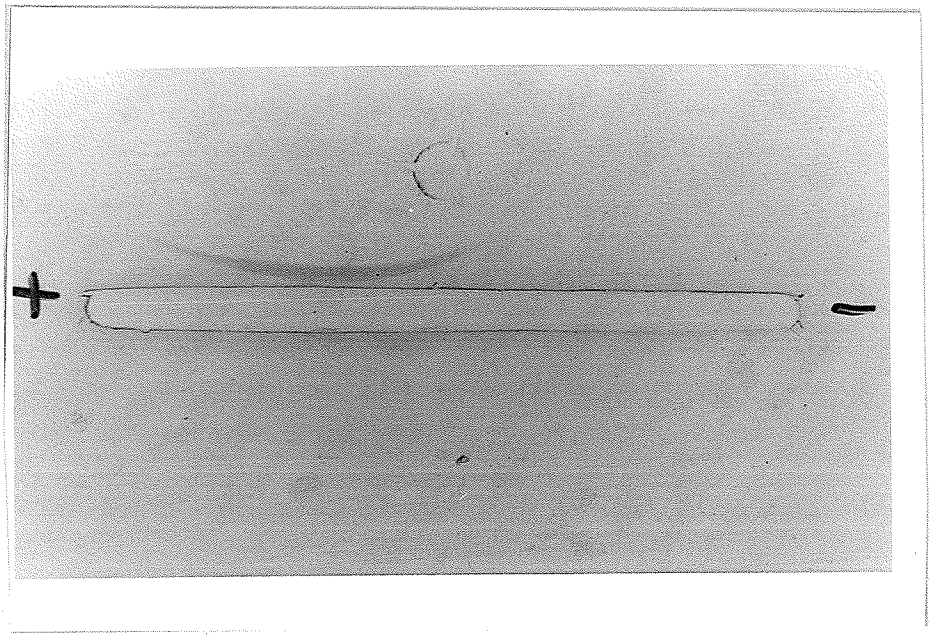


TABLE III

Relationship of rocket precipitin heights to concentration of R and amount of sheep antisera used in rocket immunoelectrophoresis

Amount of R[a] (mg)	Sheep antiserum (S11R) to R Volume (ml)			Sheep antiserum (S597) to A Volume (ml)		
	1.0[b]	1.2	1.4	2.5	5.0	7.5
0.4	1.80[c]	2.40	1.70	1.30	2.40	1.20
0.5	1.90	2.45	1.80	1.60	2.80	1.50
0.6	2.20	2.60	1.95	1.80	3.20	1.80
0.7	2.25	2.20	1.80	2.00	3.00	1.75
0.8	2.20	2.20	1.80	1.90	2.90	1.70

[a]Aqueous solution of R in a volume of 40 µl.

[b]Amount of antiserum (ml) incorporated into 20 ml of 1% agarose solution.

[c]Height (cm) of rocket measured from stained slide.

Figure 8: Crossed-immunoelectrophoretic pattern of R (central well) using sheep anti-A (S597) as the developing antiserum.

One immunoprecipitin peak in the anodic region could be visualized.

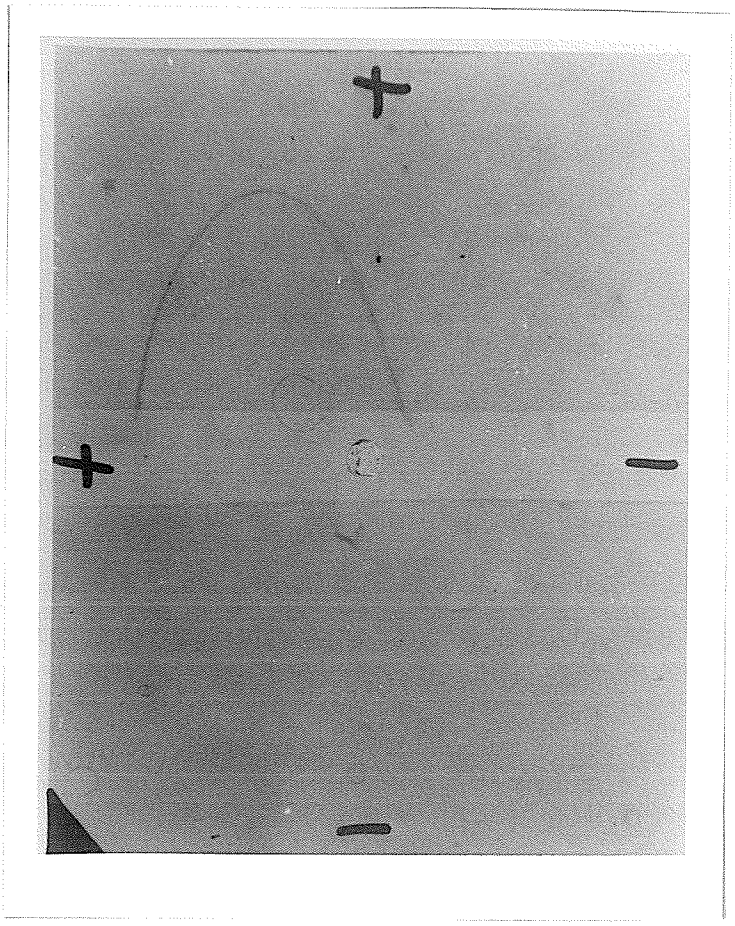


Figure 9: Crossed-immunoelectrophoretic pattern of R (central well) using sheep anti-R (S11R) as the developing antiserum.

Figure 9a: Photograph of stained slide.

Figure 9b: Diagramatic illustration: A total of at least 15 immunoelectrophoretic peaks (at the anodic and cathodic regions) were obtained. The immunoelectrophoretic peaks were numbered arbitrarily.

Figure 9a:

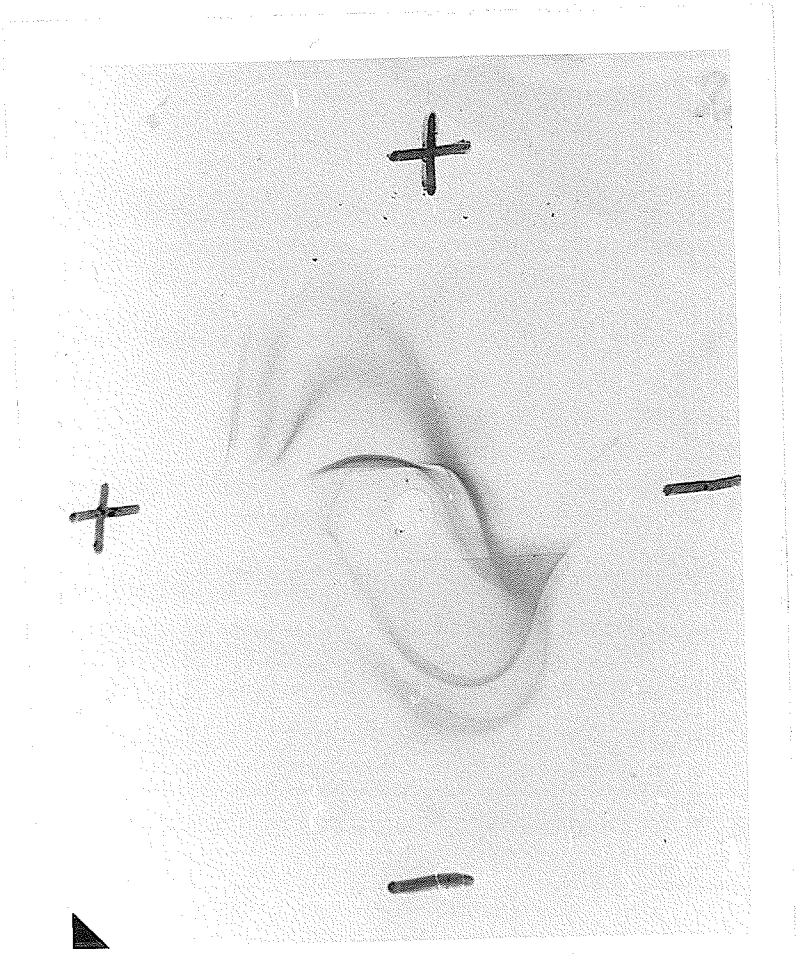
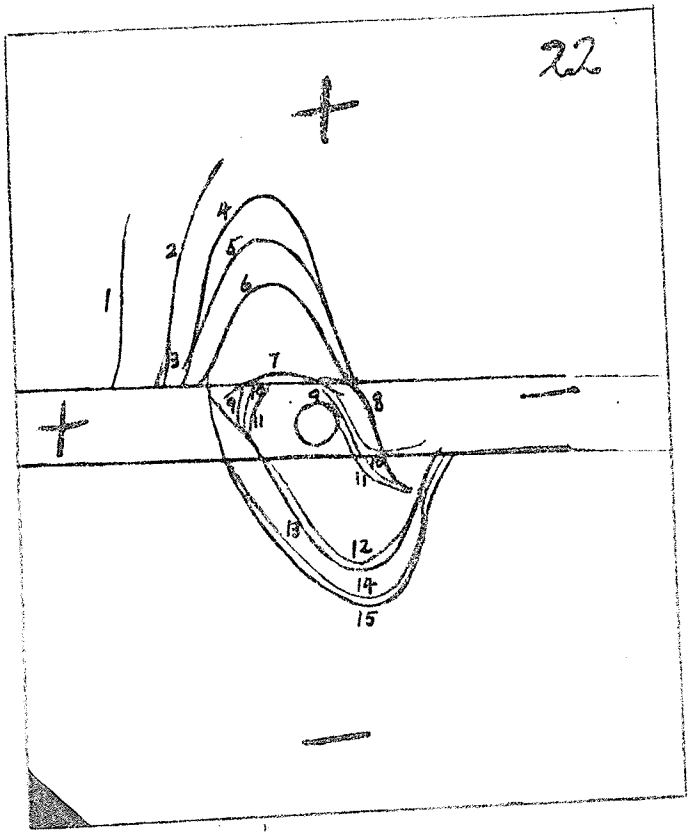


Figure 9b:



V. Crossed-radioimmunoelectrophoretic (CRIE) analyses of R using anti-A (S597) and anti-R (S11R) sera

To determine whether the antigenic components in R detected by CIE using the antiserum to A (S597) (c.f. Figure 8, p.66) had also the ability of combining with human IgE antibodies to KBG pollen, the technique of CRIE was used. Radioautograph (Figure 10) of the gel, obtained after an exposure time of 10 days, revealed that the radiolabel was incorporated into the immunoprecipitate visualized on the stained slide and, in fact, the radioautograph and stained immunoprecipitate were superimposable. From these observations it was concluded that the antigen which had been precipitated by sheep anti-A serum (S597) also possessed allergenic determinants (and therefore was designated as allergen A) which could combine with human IgE antibodies to KBG pollen. By comparison, the CRIE pattern of R using sheep anti-R serum (S11R) revealed the presence of at least ten radioimmunoprecipitin peaks (Figure 11), i.e. at least 10 of the total of 15 antigenic components recognized by the sheep anti-R serum (S11R) had also allergenic determinants recognized by the IgE antibodies present in the pool of human sera from individuals allergic to KBG pollen.

In order to determine whether any nonspecific binding of the radiolabelled anti-human IgE antibodies had possibly occurred, CRIE was performed using the same procedures and reagents as already described, except that the step of overlaying the human allergic sera onto the gel was omitted. Following this protocol, radioactive peaks were no longer observed and it was concluded, according to the interpretations of Weeke and Løwenstein (147), that nonspecific binding of the radiolabelled anti-human IgE antibodies to the antigen-antibody precipitates had not occurred.

Figure 10: Identification of allergens in R by crossed-radio-immunoelectrophoresis (CRIE).

Radioautograph of R (central well) obtained with sheep anti-A (S597) as the initial developing antiserum. The slide was then developed with a pool of human sera from individuals allergic to KBG pollen, followed by development with an ^{125}Iodine-labelled rabbit antiserum to human IgE. One single radio-immunoprecipitin peak could be visualized.

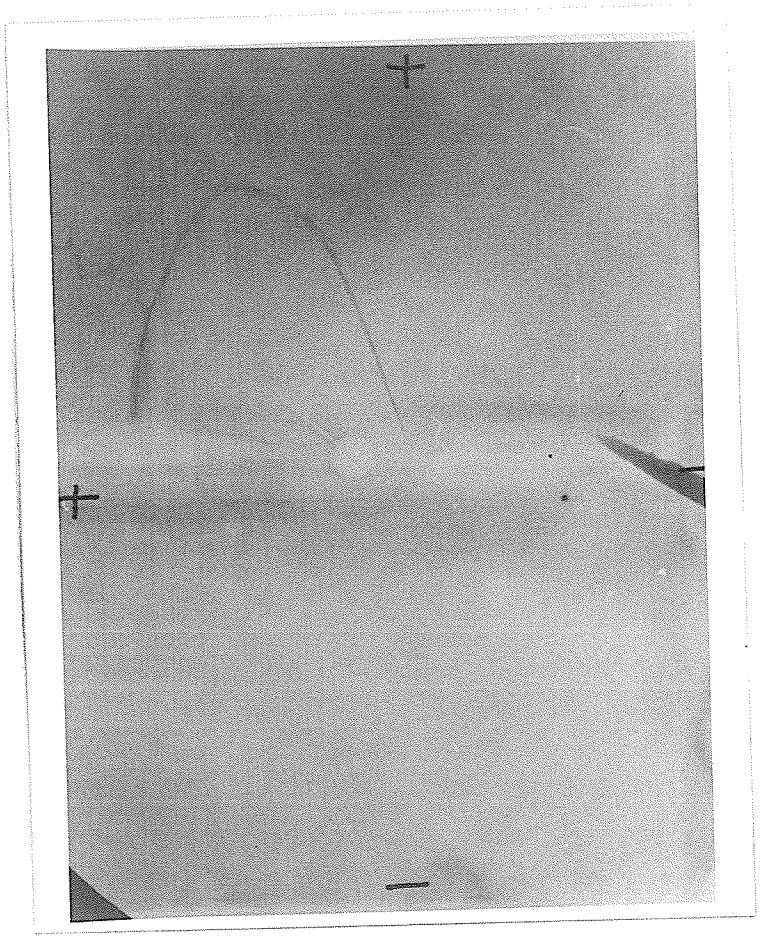
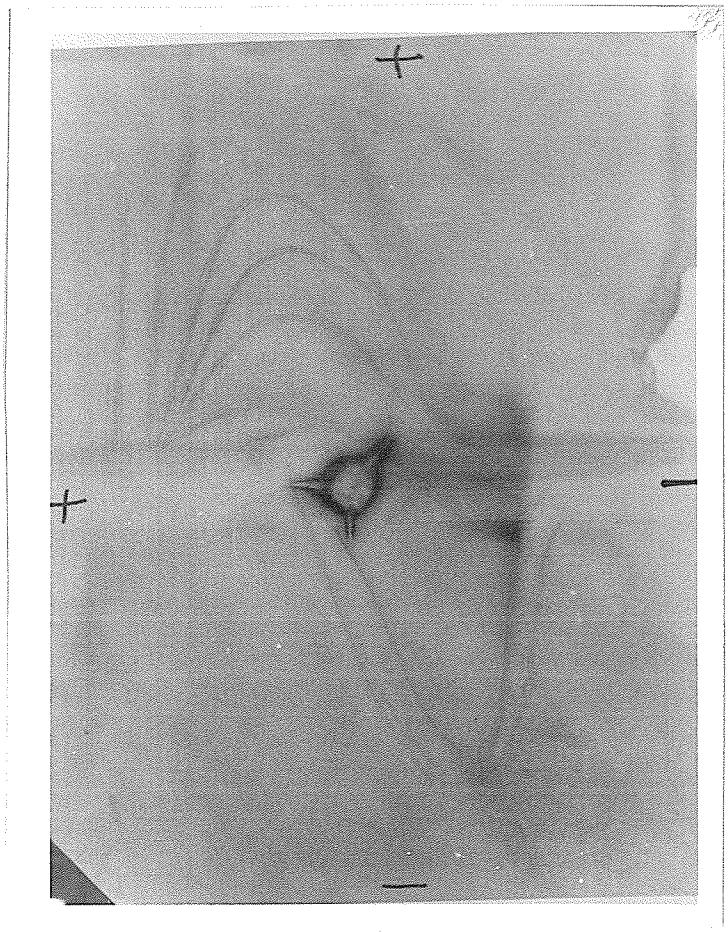


Figure 11: Radioautograph of crossed-radioimmunoelectrophoretic pattern of R using sheep anti-R (S11R) as the developing antiserum.



VI. Isolation of allergen A from R by means of a reversed immunosorbent

Since it was the intention to isolate one allergenic component from R, advantage was taken of the observation that the sheep antiserum to A (S597) recognized only one antigenic component in R. This finding was exploited for the isolation of allergen A from R by immunospecific means employing a reversed immunosorbent prepared with the γ-globulin fraction of sheep anti-A antiserum (S597).

(a) Reversed immunosorbent prepared with Sepharose 4B-Cl

A reversed immunosorbent was prepared with Sepharose 4B-Cl as the supporting matrix to adsorb a solution of R. After washing thoroughly to remove unadsorbed material, elution of the specifically adsorbed antigen was accomplished with an acidic solution of gly-HCl (pH 2.2). Ouchterlony analysis of the eluate revealed the presence of components which reacted with an antiserum to sheep gamma globulins indicating that sheep gamma globulins had become desorbed from the reversed immunosorbent (Figure 12). Although antigneic components of R were also recovered in the same eluate, the contamination with the sheep proteins suggested that this particular matrix was not suitable for preparing a reversed immunosorbent for the isolation of allergen A from R.

(b). Reversed immunosorbent prepared with Affi-Gel 10

Affi-Gel 10 was used as a supporting matrix to prepare a reversed immunosorbent with the γ-globulin fraction of sheep anti-A antiserum (S597) to specifically adsorb allergen A from R. For this purpose, an amount of 100 mg protein (S597) was added to 25 ml of Affi-Gel 10. From an analysis of the amount of protein which remained in the

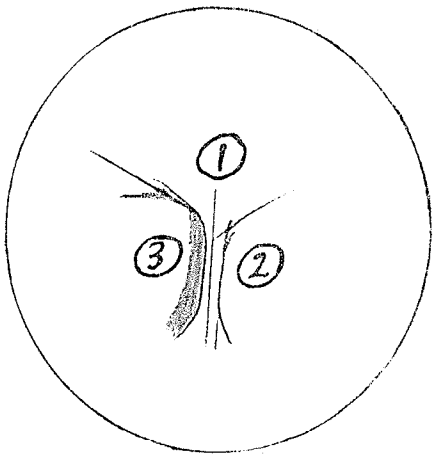


Figure 12: Diagramatic illustration on Ouchterlony Analysis of the eluate obtained from reversed immunosorbent prepared by attaching the γ-globulin fraction of sheep antiserum to A (S597) to Sepharose 4B-Cl as the supporting matrix.

Wells: 1. Allergen A recovered from the reversed immunosorbent.

2. Sheep antiserum to R (S11R).

3. Rabbit antiserum to sheep γ-globulins.

supernatant after incubation of the globulin preparation with the Affi-Gel 10, it was determined that about 70 mg of the globulin protein had been coupled to the insoluble matrix.

A solution of R (20 mg) was incubated with the reversed immunosorbent. After thorough washing, about 1 mg of protein was recovered by elution with a solution of 2M KI buffered at pH 7.0. In the lyophylized state, this material had a light yellow color. On Ouchterlony analysis of the eluate with rabbit antiserum to sheep globulins, precipitin formation was not detected (Figure 13). This indicated that the immunosorbent prepared with Affi-Gel 10 was not shedding the sheep globulins during the elution step.

VII. Antigenic analysis of allergen A eluted from the reversed immunosorbent

Using the multispecific sheep anti-R antiserum (S11R) for CIE analysis of allergen A eluted from the reversed immunosorbent, one immunoprecipitin peak (Figure 14) was obtained. It was therefore concluded that only one component possessing the antigenic determinant was associated with allergen A.

To establish that the immunoprecipitin peaks obtained on one hand with the immunosorbent-purified allergen A and the antiserum to R (S11R) and on the other hand with the unfractionated R and the antiserum specific to A (S597), were due to identical antigen-antibody specificities, the technique of CIE with intermediate gel was employed. In this procedure, the intermediate gel contained sheep antiserum S597 and the main body of the gel contained sheep antiserum to R (S11R). For this purpose, the location of the antigen well was moved to the position as shown in Figure 15. After electrophoresis of allergen A in

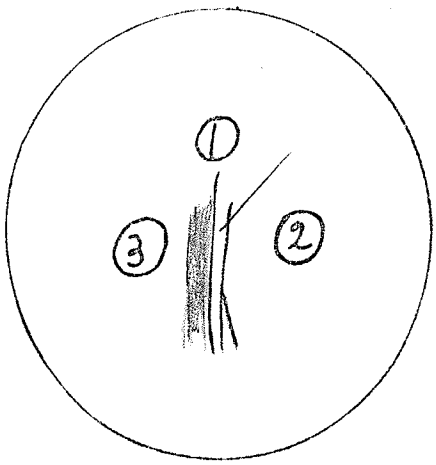


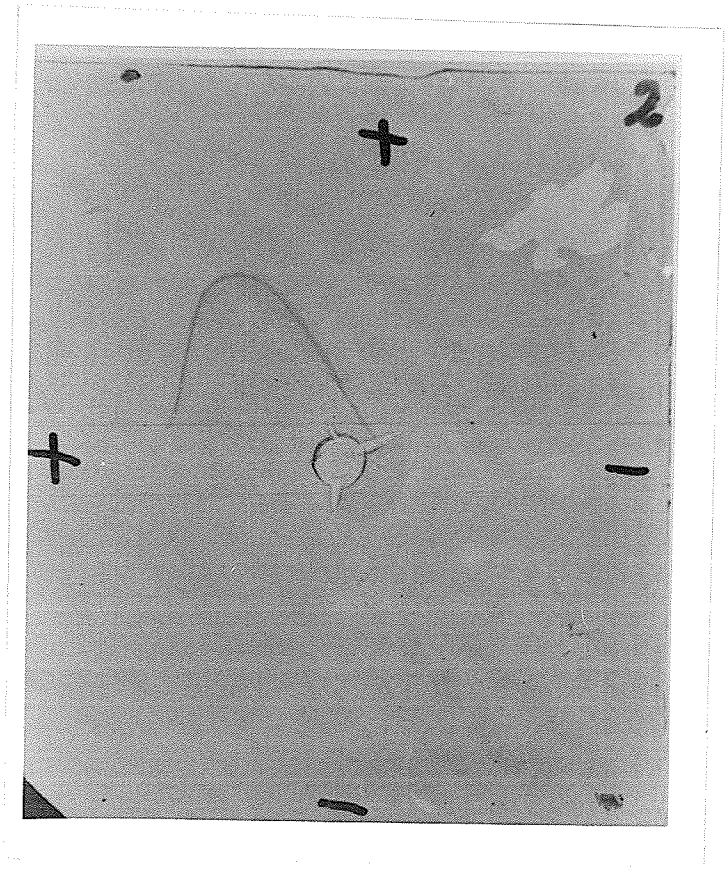
Figure 13: Diagramatic illustration on Ouchterlony Analysis of the eluate obtained from reversed immunosorbent prepared by attaching the γ-globulin fraction of sheep antiserum to A (S597) to Affi-Gel 10 as the supporting matrix.

Wells: 1. Allergen A recovered from the reversed immunosorbent.

2. Sheep antiserum to R (S11R).

3. Rabbit antiserum to sheep γ-globulins.

Figure 14: Crossed-immunoelectrophoretic pattern of Allergen A (central well) using sheep anti-R (S11R) as the developing antiserum.
One immunoprecipitin peak at the anodic region could be visualized.


2
+
+
-
-

the first direction, a thin strip of agarose (2 X 8 cm) encompassing the electrophoresed antigen, along the direction of electrophoresis, was left on the plate, Figure 15, section A. The rest of the agarose gel was removed and the exposed glass was wiped clean. A volume of 16 ml of a 1% agarose solution containing 1 ml of sheep anti-R antiserum (S11R) was layered onto the exposed surface. After the gel had solidified, a thin strip (Figure 15, section B) (1½ X 8 cm) of the antibody containing agarose gel was removed from the edge alongside gel A. The exposed glass was washed carefully with distilled water and wiped dry. A volume of 3 ml of the 1% agarose solution containing 0.75 ml of the sheep anti-A antiserum (S597) was pipetted onto the space labelled B, (Figure 15), and served as the intermediate gel. After congelation, electrophoresis was performed in the second dimension. As shown in Figure 15, a reaction of identity between the precipitate in the intermediate gel (S597) and the precipitate in the gel containing sheep anti-R (S11R) antiserum was obtained. According to the interpretation of CIE with intermediate gel patterns by Axelsen (160), this observation suggested that the precipitin peak as detected by CIE using R as the antigenic preparation and sheep anti-A (S597) (c.f. Figure 8, p.66) was the same as the one obtained on CIE of allergen A using sheep anti-R (S11R) (Figure 14).

VIII. Allergenic evaluation of allergen A

1. Passive Cutaneous Anaphylaxis (PCA)

PCA analysis employing murine reaginic antisera to R was performed in random bred hooded rats to evaluate the allergenic activity

Figure 15: Crossed-immunoelectrophoresis of Allergen A using the intermediate gel technique.

Figure 15a: Photograph of stained slide.

Figure 15b: Diagramatic illustration of arrangement of the 3 different sections of the gel.

(A) Agarose gel containing antigen well. Electrophoresis in the first direction was along the long axis of this gel:

(B) The intermediate gel contained sheep antiserum (S597) to A.

(C) Gel contained sheep antiserum (S11R) to R.

Figure 15a:

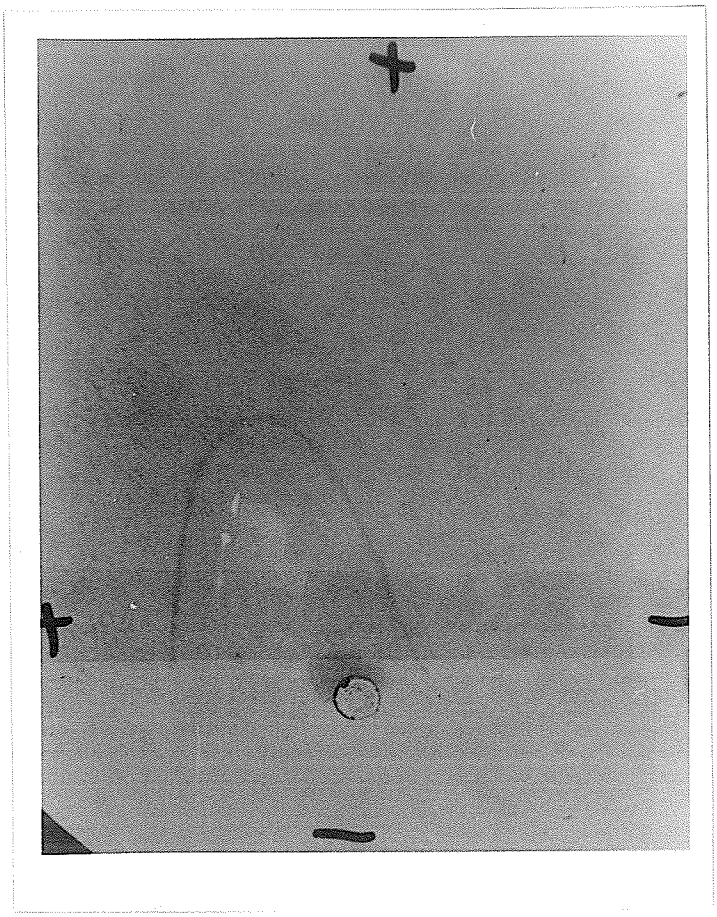
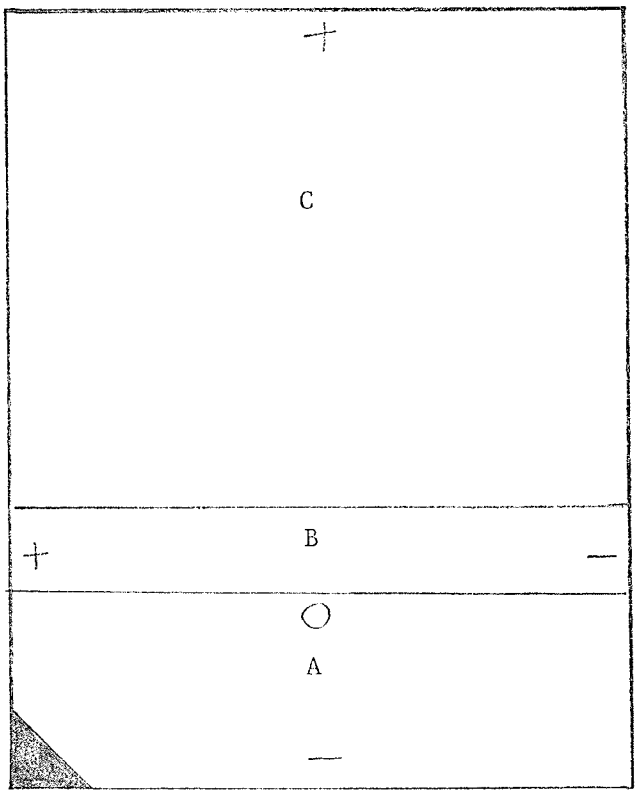


Figure 15b:



of allergen A. The PCA titer elicited with allergen A (100 µg) was found to be 320 (Table IV). By comparison, a PCA titer of 640 was elicited in rats sensitized with the murine anti-R reaginic serum and challenged with R at a dose of 100 µg.

2. Neutralization of PCA

The ability of allergen A to neutralize the anti-R IgE antibodies present in a murine reaginic serum (PCA titer of 640 elicited with R, c.f. Table IV) was assessed by the procedure of PCA neutralization. Different amounts of allergen A (ranging from a concentration of 10 µg/ml to 8 mg/ml) were mixed with constant volumes (200 µl) of the murine anti-R reaginic antiserum at a dilution of 1:10 (see Table V). Each mixture was serially diluted to the maximum final serum dilution of 1:640. As shown in Table V, incubation of allergen A (at a concentration of 4 mg/ml) with the murine anti-R antiserum reduced the PCA titer from 640 to 40, i.e. allergen A could neutralize the PCA reaction normally elicitable with R by 94%. By comparison, R employed at a concentration of 1 mg/ml was sufficed to neutralize the PCA to the extent of 94% and at a concentration of 4 mg/ml, R could neutralize the PCA reaction completely (Table VI).

3. Inhibition of RAST by allergen A

The allergenicity of allergen A was also evaluated in terms of its ability to combine with and to neutralize human IgE antibodies to KBG pollen. For this purpose, the inhibition of the RAST procedure was used. Various amounts of allergen A (ranging from 10 to 200 µg) were incubated separately with 50 µl of the allergic sera before addition

TABLE IV

Allergenic evaluation of Allergen A and R by PCA analysis

Reciprocal of serum dilution[a]	PCA reaction in diameter (mm) elicited on challenge with	
	Allergen A	R
10	13.0	16.5
20	12.0	15.0
40	10.0	12.0
80	9.0	10.0
160	7.0	9.0
320	6.0	8.0
640	-[b]	6.5
1280	-	-
2560	-	-
Control (saline)	-	-

[a] A pool of murine reaginic antiserum to R prepared by immunization of A/HeJ mice with 100 µg of R in 5 mg of aluminium hydroxide gel.

[b] Reactions resulting in intradermal extravasation of Evan's blue dye of a diameter of 5 mm or less were considered to be negative.

TABLE V

Neutralization of murine anti-R reaginic antiserum by Allergen A

Reciprocal of final dilution of murine anti-R antiserum admixed with Allergen A	Concentration of Allergen A[a]						
	10 µg/ml	100 µg/ml	500 µg/ml	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml
10	14.0[b]	13.5	12.5	10.0	7.5	4.5	3.5
20	13.5	12.5	11.5	8.5	6.5	4.0	3.5
40	12.5	11.0	10.5	7.5	4.5	3.5	2.5
80	10.0	10.0	9.5	5.5	4.0	-	-
160	9.5	9.0	6.5	3.5	3.0	-	-
320	5.5	5.5	4.5	3.0	-[c]	-	-
640	5.5	5.5	3.5	2.5	-	-	-

Controls: 1. A solution of saline was injected into the skin sites of rats. A PCA reaction was not observed following challenge with R.

2. Sensitization with murine reaginic anti-R antiserum (1:640 dilution) gave a PCA reaction of 6.5 mm in diameter on challenge with R.

[a] Concentration of Allergen A mixed with a volume of 200 µl of murine reaginic anti-R antiserum diluted 1:10. The mixtures were then serially diluted and injected intradermally into rats.

[b] Size (diameter, mm) of PCA reaction elicited on challenge with R (1 mg/ml).

[c] Absence of PCA reaction.

TABLE VI

Neutralization of murine anti-R reaginic antiserum by R

Reciprocal of final dilution of murine anti-R antiserum admixed with R	Concentration of R[a]						
	10 µg/ml	100 µg/ml	500 µg/ml	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml
10	10.0[b]	8.5	7.5	5.5	3.5	-	-
20	9.5	8.0	6.5	4.0	-	-	-
40	8.5	6.5	6.0	3.0	-	-	-
80	7.5	6.0	5.5	-[c]	-	-	-
160	6.5	4.5	4.0	-	-	-	-
320	6.0	4.0	3.5	-	-	-	-
640	5.5	4.0	2.5	-	-	-	-

Controls: 1. A solution of saline was injected into the skin sites of rats. A PCA reaction was not observed following challenge with R.

2. Sensitization with murine reaginic anti-R antiserum (1:640 dilution) gave a PCA reaction of 6.5 mm in diameter on challenge with R.

[a] Concentration of R mixed with a volume of 200 µl of murine reaginic anti-R antiserum diluted 1:10. The mixtures were then serially diluted and injected intradermally into rats.

[b] Size (diameter, mm) of PCA reaction elicited on challenge with R (1 mg/ml).

[c] Absence of PCA reaction.

to the allergosorbent disc prepared with R. As shown in Figure 16, a maximum inhibition of RAST to the extent of 67% was obtained when an amount of 100 µg of allergen A was used. By comparison, an amount of 100 µg of R could inhibit RAST by 100%.

IX. Production of antiserum to allergen A in rabbits

In order to further examine the purity of allergen A, this material was used as the immunogen to produce antisera in rabbits. For this purpose, 2 New Zealand White rabbits were repeatedly immunized by intramuscular injections of allergen A at a dose of 0.5 mg (per rabbit) emulsified in Freund's complete adjuvant, administered at intervals of three weeks. The animals required four such immunizations before antibodies to R were detected by immunoelectrophoresis. One immunoprecipitin band was obtained by immunoelectrophoresis using R as the antigenic preparation and rabbit anti-A as the antiserum (Figure 17). Hyperimmune antisera,obtained from these rabbits after they had undergone 3 additional immunizations with allergen A, were evaluated by CIE using R as the antigenic preparation. Identical results were obtained with both hyperimmune antisera (the CIE pattern for one of these antisera is illustrated). As can be seen in Figure 18, one single immunoprecipitin peak was observed. On the basis of the observation that hyperimmunization with allergen A resulted in the formation of rabbit antisera specific for allergen A, it was concluded that allergen A consisted of only one antigenic component.

X. Quantitation of allergen A in R by rocket immunoelectrophoresis

The technique of rocket immunoelectrophoresis was employed to

Figure 16: Inhibition of radioallergosorbent test (RAST) by Allergen A and R.

Different amounts of either Allergen A or R were incubated with a constant amount of a pool of sera from individuals allergic to KBG pollen, prior to addition to the allergosorbent discs prepared with R. The degrees to which the human IgE antibodies were bound to the allergosorbent discs were determined with an ^{125}Iodine-labelled preparation of antibodies specific for human IgE.

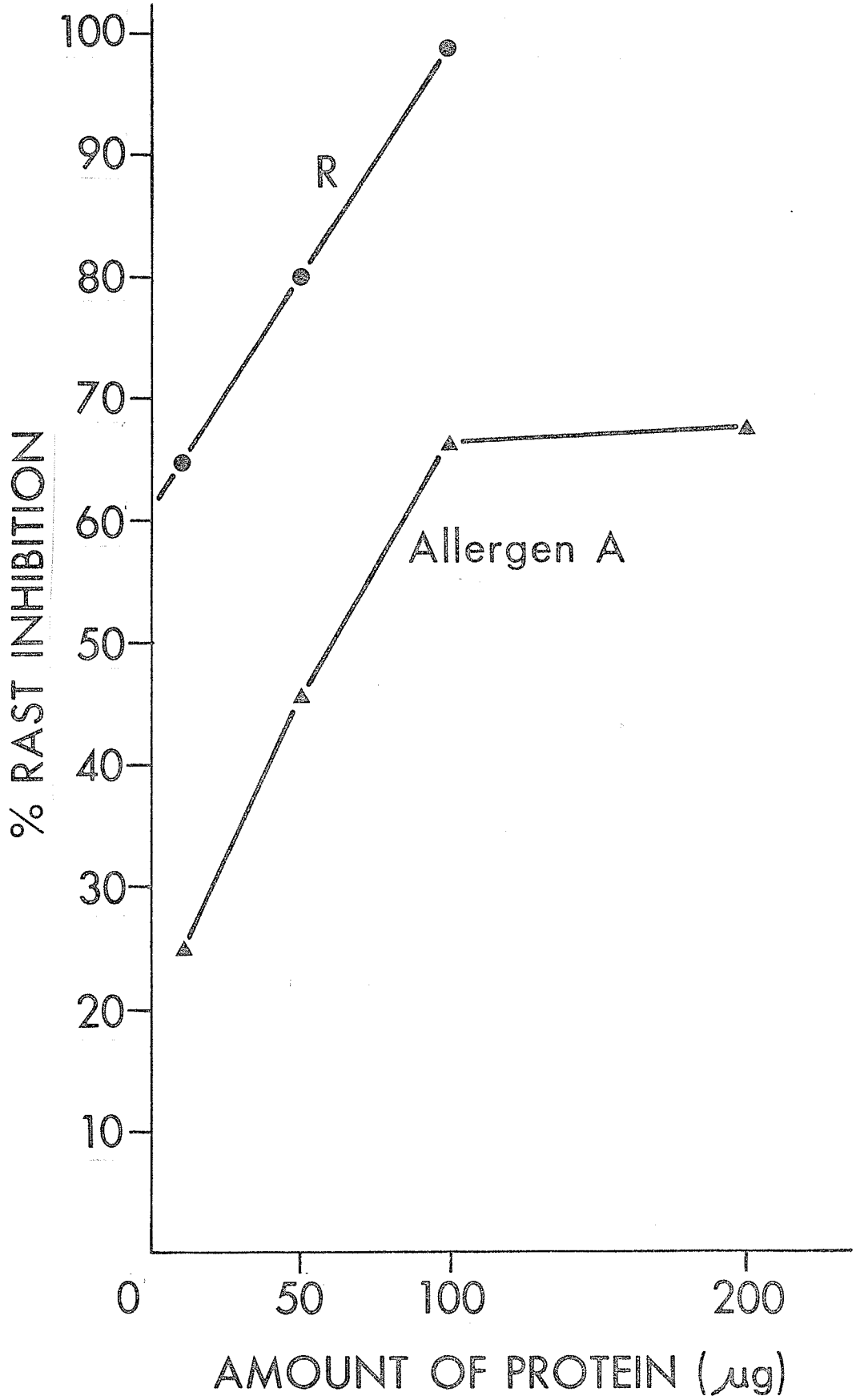

100
90
80
70
60
50
40
30
20
10
% RAST INHIBITION
R
Allergen A
0
50
100
200
AMOUNT OF PROTEIN (µg)

Figure 17: Immunoelectrophoretic pattern of R (central well) using rabbit anti-A as the developing antiserum (longitudinal troughs).

The distances of the sample wells from their corresponding antiserum troughs were different.

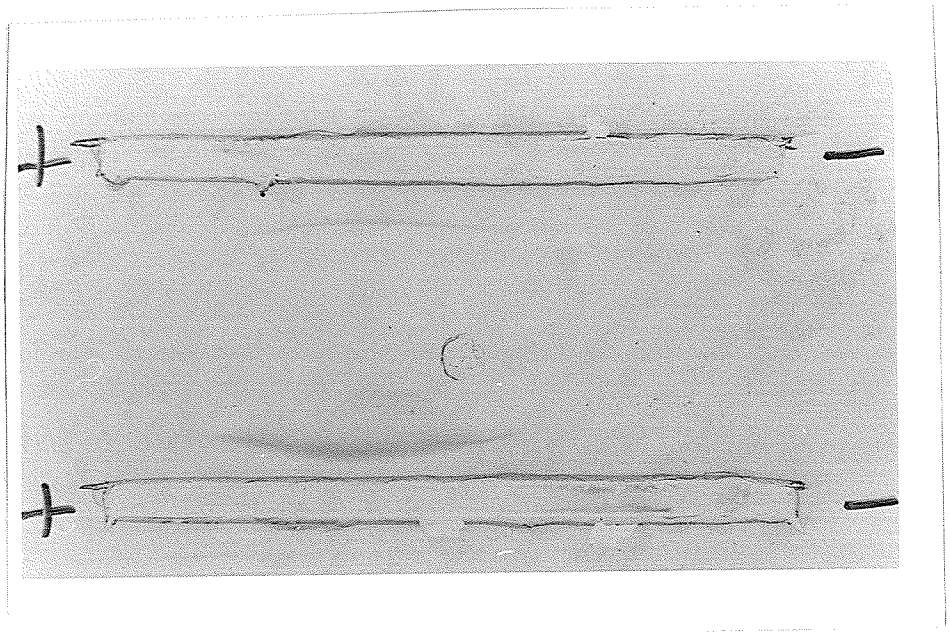
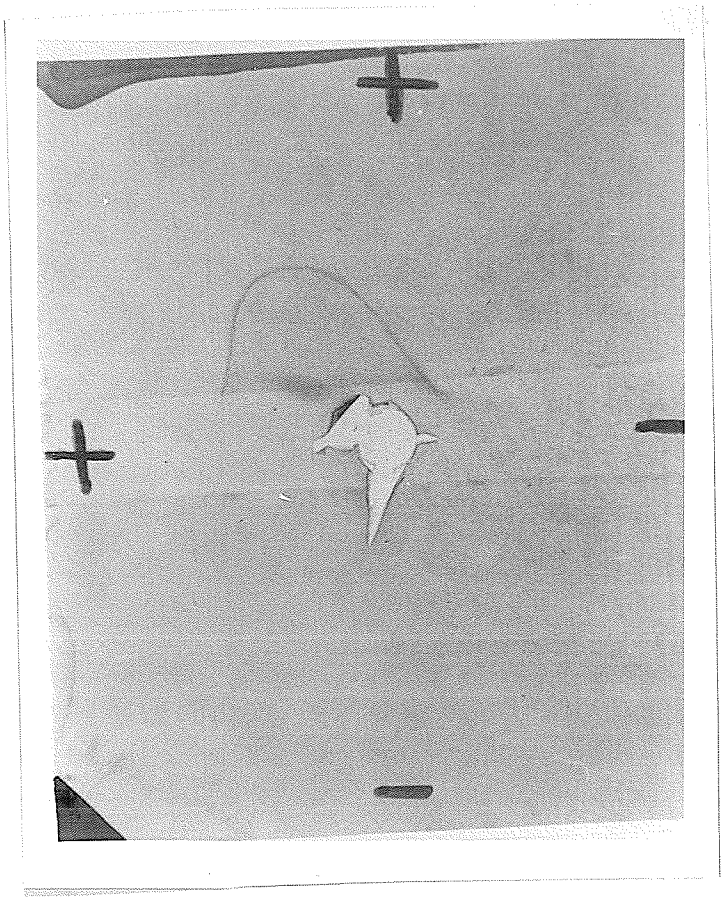


Figure 18: Crossed-immunoelectrophoretic pattern of R (central well) using rabbit anti-A as the developing antiserum (incorporated into the agarose gel). One immunoprecipitin peak was seen at the anodic region. (For values see Table VII).



quantitate the amount of allergen A present in R. Briefly, a volume of 20 ml of 1% agarose solution containing a volume of 5.0 ml of the sheep anti-A antiserum (S597) was overlaid onto the glass plate (3¼" X 4"). After congelation, five antigenic wells each at 1.0 cm apart were punched out along a line at the center of the gel. Constant volumes (40 µl) of a solution of allergen A in concentrations of 7.5 mg/ml, 3.75 mg/ml, and 1.875 mg/ml (protein basis) were added to three of the holes and served as reference concentrations for calibration of the rocket technique. Volumes (40 µl) of solutions of different batches of R (R_1 and R_2) at a protein concentration of 15 mg/ml were placed in the remaining holes. Rocket immunoelectrophoresis was carried out according to the procedures described in the Methods (refer to pp.41-42).

The results of the rocket immunoelectrophoresis are given in Figure 19 and Table VII. A standard curve showing the relationship of the area enclosed by the precipitates to the concentration of allergen A is illustrated in Figure 20. It was estimated that R_1 and R_2 at a concentration of 15 mg/ml contained allergen A at concentrations of 2.9 mg/ml and 3.0 mg/ml respectively, i.e. 19.2% of R_1 was composed of allergen A and 20.3% of R_2 was composed of allergen A. These differences were regarded as not significant.

XI. Determination of protein content of allergen A and R

By Lowry's method, it was found that 56% of the dry weight of allergen A consisted of protein. By comparison, 70% of R consisted of protein.

Figure 19: Quantitation of Allergen A in R by rocket immunoelectrophoresis.

Sample wells 1,2,3 : Different amounts of Allergen A were used as reference concentrations (for values see Table VII).

Sample wells 4,5 : Constant (known) amount of R (R_1 and R_2 refer to different batches of retentate).

Sheep antiserum to A (S597) was used as the developing antiserum.

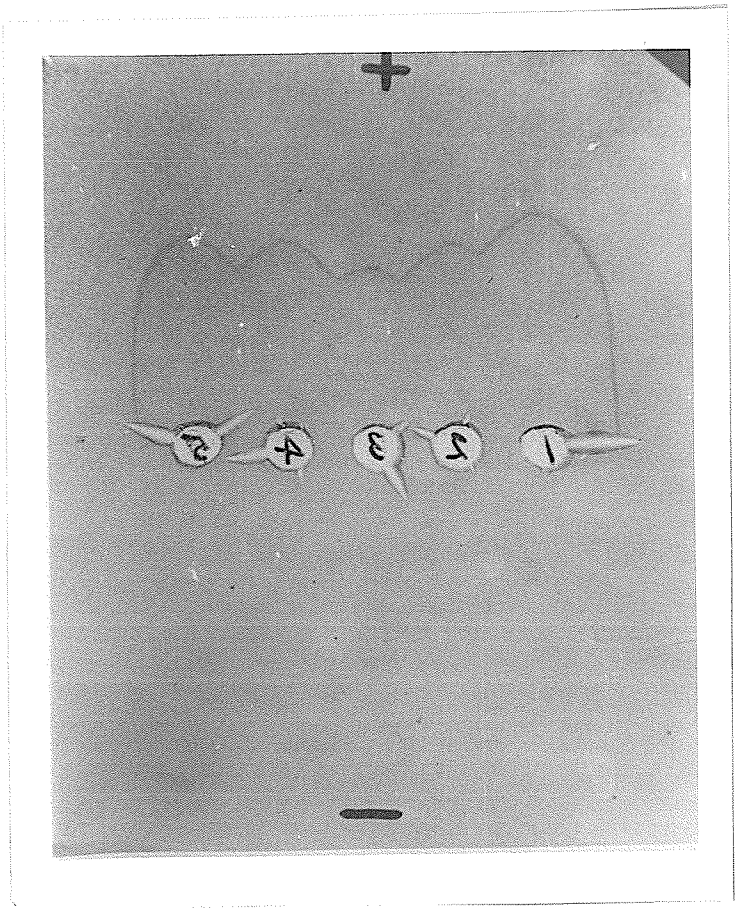

+
1
2
3
4
5
—

TABLE VII

Quantitation of Allergen A in R by rocket immunoelectrophoresis

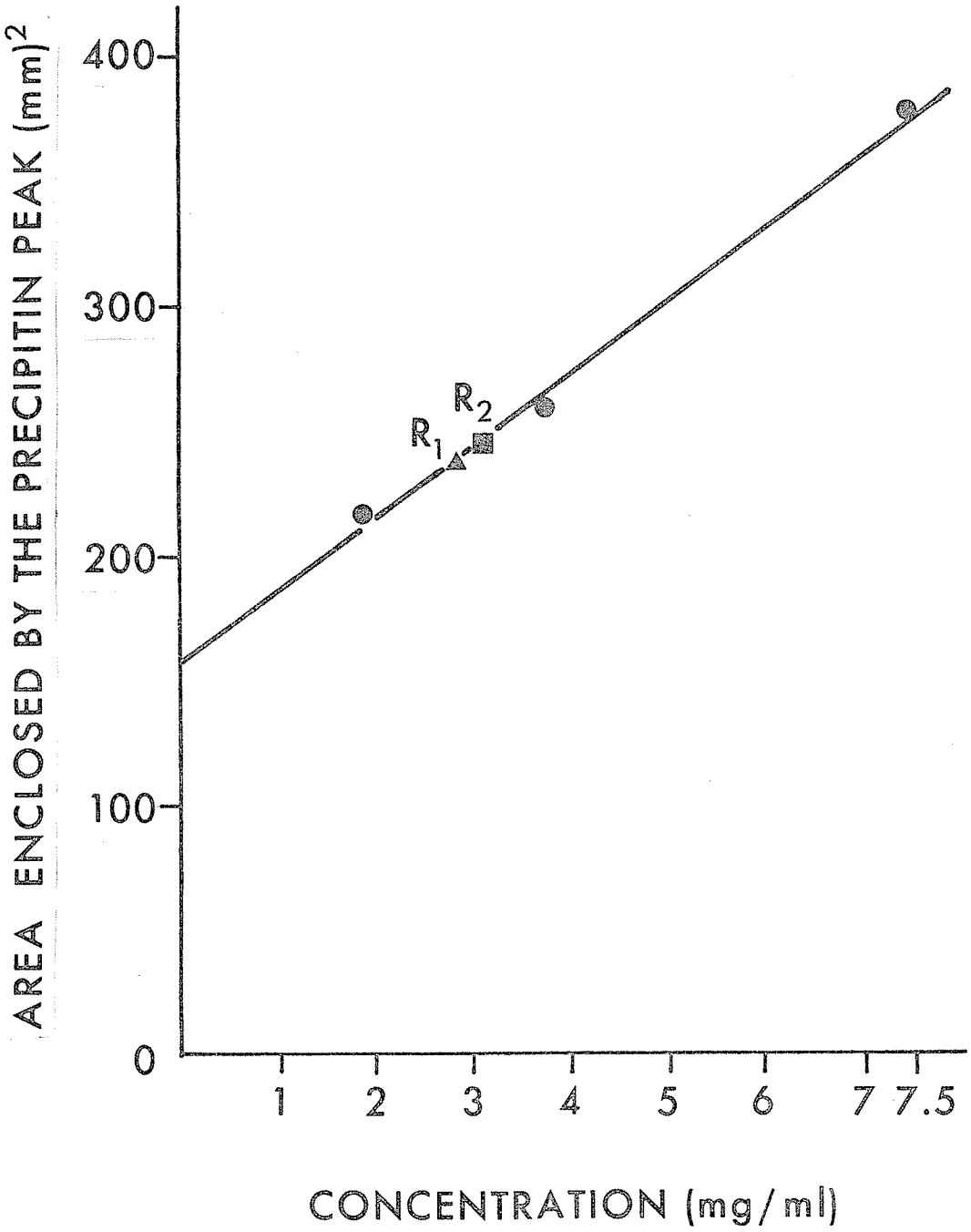
Concentration (mg/ml)	Allergen A	Sample R_1	R_2
15.0	-[a]	240	246
7.5	379[b]	-	-
3.75	259	-	-
1.875	216	-	-

[a] Not done.

[b] Area (mm^2) enclosed by the immunoprecipitin peak.

Figure 20: Quantitation of Allergen A in R by rocket immunoelectrophoresis. Relationship between area enclosed by the immunoprecipitin peak (rocket) and amount of Allergen A ● (see also Table VII).

▲ = R_1 and ■ = R_2 refer to different batches of retentate.


AREA ENCLOSED BY THE PRECIPITIN PEAK (mm)2
400
300
200
100
0
R2
R1
1
2
3
4
5
6
7
7.5
CONCENTRATION (mg/ml)

XII. Estimation of molecular weight of allergen A by SDS-PAGE

On SDS-PAGE of allergen A, one major protein band (Figure 21a) was visualized on the stained gel and it corresponded to a molecular weight of 16,500 daltons (Figure 22). Several minor bands were also observed. SDS-PAGE of R revealed the presence of at least twenty bands (Figure 21b) and the molecular weights of these components ranged from 10,000 to 64,000 daltons.

Figure 21: Estimation of molecular weight of Allergen A and R by SDS-PAGE.

(a) Allergen A: One major band and several minor bands were obtained.

(b) Retentate (R): At least 20 bands were obtained.

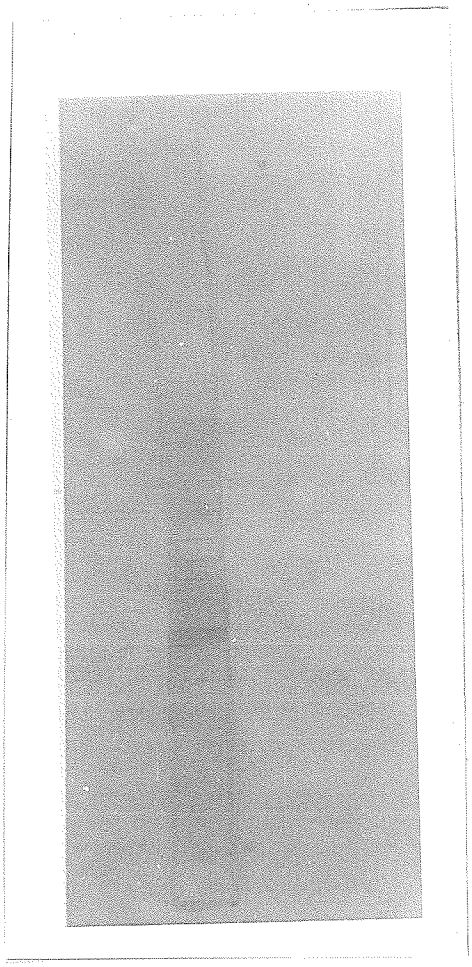


Figure 21a

CATHODE

Direction of migration

ANODE

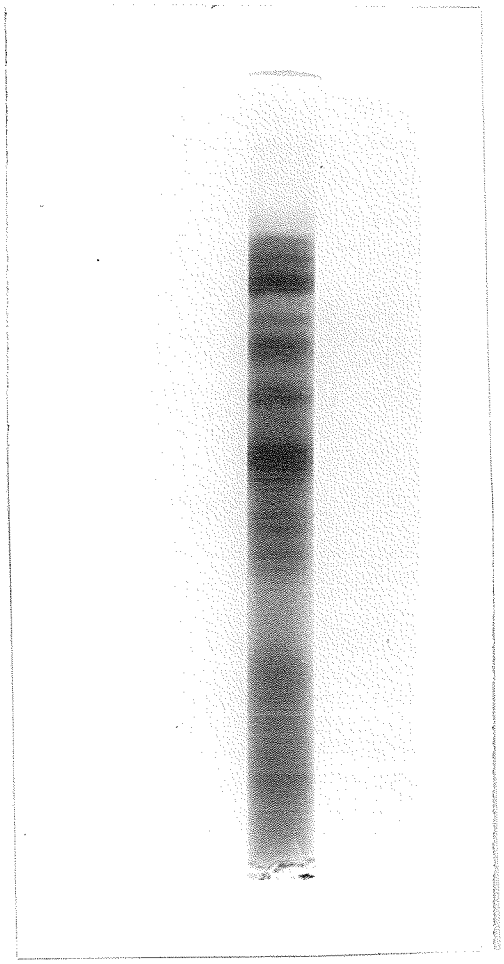
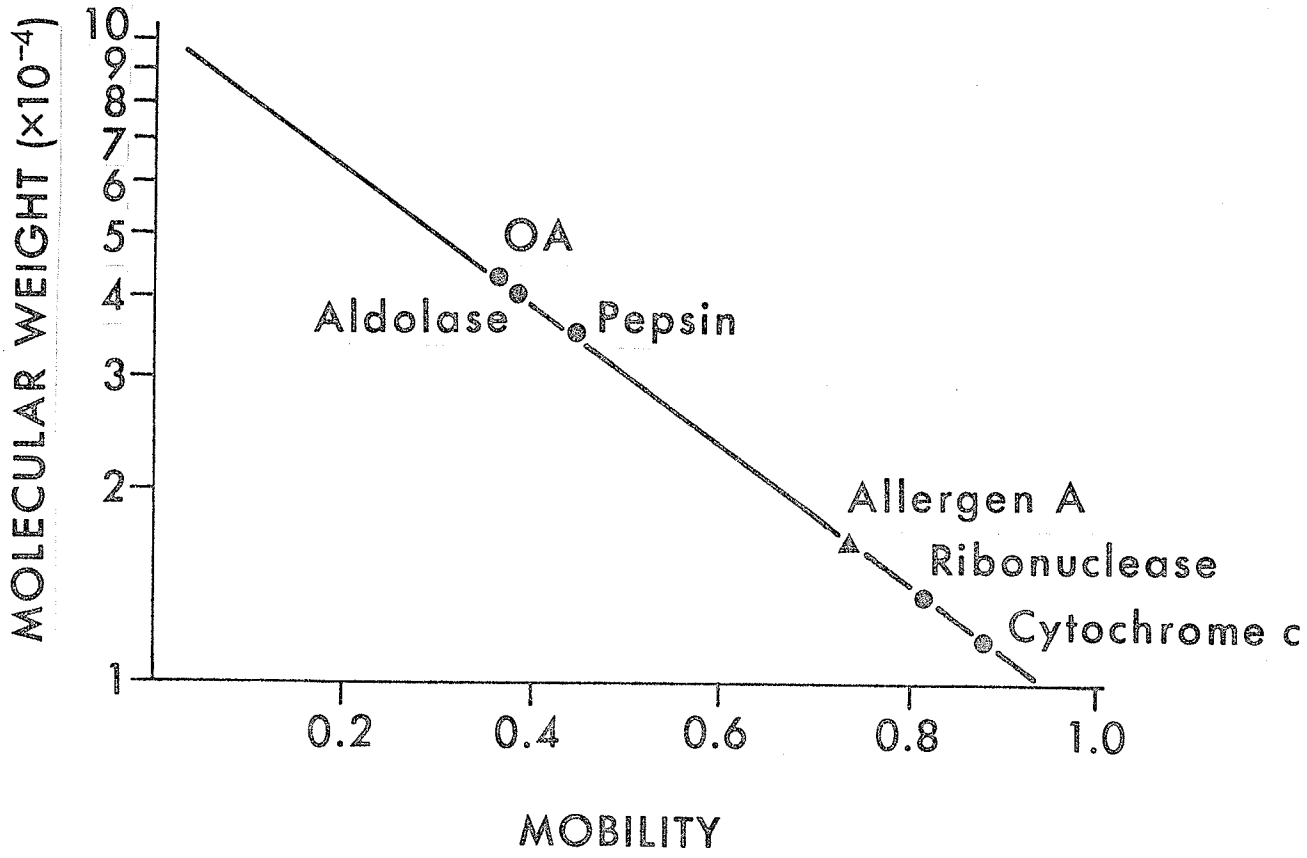


Figure 21b

Figure 22: Estimation of molecular weight of Allergen A in relation to that of standard proteins.

Mobility was determined by the formula given in Methods (p.54).


MOLECULAR WEIGHT (×10⁻⁴)
10
9
8
7
6
5
4
3
2
1
OA
Aldolase
Pepsin
Allergen A
Ribonuclease
Cytochrome c
0.2
0.4
0.6
0.8
1.0
MOBILITY

DISCUSSION

In addition to the intrinsic value of characterizing the nature of pollen allergens which would broaden our understanding of allergens in general, purified allergens are required for the standardization of extracts intended for the evaluation and as a treatment for pollen allergies in man. The observations that hyposensitization of hay fever patients with crude pollen extracts could result in the formation of skin sensitizing antibodies to allergenic components to which they were not previously sensitive (139,140), serve to emphasize the need for using well-characterized allergens for the therapy. It is clear that treatment with purified allergenic preparation(s) which are devoid of components to which the allergic individual is not sensitive, would eliminate the possibility of inducing IgE antibody formation with new specificities. In order to undertake characterization of the different allergenic components present in a crude pollen extract, it is necessary to obtain the different allergens in a pure form.

The analysis of the components present in the retentate, R, obtained by dialysis of the aqueous extract from Kentucky Blue grass pollen by analytical isoelectrofocusing revealed the presence of more than 30 components ranging from a pI value of 3 to 10 (161). SDS-PAGE analysis of R detected the presence of at least 20 components with molecular weights ranging from 10,000 to 64,000 daltons. These observations indicated that the components present in R are physicochemically heterogeneous.

In addition to the physicochemical heterogeneity, R was found to contain at least 10 antigenic components distinguished by immunoelectro-

phoresis. The technique of crossed-immunoelectrophoresis was found to be even more useful than the classical single dimension immunoelectrophoretic method for resolving the heterogeneous mixture of components in R and identifying the individual antigenic components. At least 15 antigenic components were revealed in R by crossed-immunoelectrophoresis, using a sheep antiserum to R (S11R). This suggested that at least some of the precipitin bands obtained in the single-dimensional immunoelectrophoresis were composed of several overlapping and thus unresolved immunoprecipitates. In evaluating the number of different antigenic components, consideration must be made of the fact that although the anti-R antiserum (S11R) employed in this study was prepared by the hyperimmunization of a sheep with R, the antiserum might not have necessarily contained antibodies against all the different antigenic components present in R. Consequently the fifteen antigenic components detected in R by crossed-immunoelectrophoresis provided an indication of the minimum number of antigens.

Physicochemical techniques commonly employed for the isolation of allergens from a complex mixture present in a crude pollen extract have included salting-out, gel filtration and ion-exchange chromatography. However, in view of the heterogeneous nature of the pollen extracts, these techniques have not proven adequate to separate from each other components (allergens) with similar physicochemical properties. Therefore, an immunologically specific method which could discriminate one component from another on the basis of unique antigenic identity, was employed to selectively isolate a distinct antigenic component from a heterogeneous mixture of antigens.

Advantage was taken of the fact that some antigens in R could

be resolved from one another on the basis of differences of their electrophoretic mobility and their distinct localization following immunoprecipitation with a polyspecific antiserum (S11R). An antiserum to one of these components was prepared by the immunization of a sheep with the complexes of a discrete antigen-antibody system obtained on immunoelectrophoresis of R and developed with the sheep anti-R polyspecific antiserum (S11R). Such an antiserum (S597, anti-A), on immunoelectrophoresis with R, elicited a single immunoprecipitin band. On crossed-immunoelectrophoresis with R, using antiserum S597, one immunoprecipitin peak was also obtained. It was demonstrated by crossed-radioimmunoelectrophoresis of R that the antigenic component precipitable with the sheep antiserum (S597) was also capable of combining with human IgE antibodies to Kentucky Blue grass pollen. This procedure established the fact that the antigenic component referred to as 'A' also possessed allergenic activity which was relevant in terms of the human allergic response to KBG pollen.

Of the 15 immunoprecipitin peaks which developed on crossed-immunoelectrophoresis of R with antiserum (S11R), only 10 immunoprecipitin peaks could bind human IgE antibodies to KBG pollen. The fact that not all antigenic components recognized by antiserum (S11R) could bind the human IgE antibodies may be explained by one or more of the following possibilities:- (i) some of the antigenic components present in the retentate are not allergenic; (ii) the pool of human allergic sera used to evaluate the property of allergenicity may not have contained all the respective IgE antibody specificities to all of the different allergenic components present in R; (iii) following the formation of immune complexes by reacting the antigen (allergen) with the precipitating sheep antibodies,

antigenic (allergenic) (refer to p.16) sites must still be accessible in the immune complex to permit any subsequent binding of the IgE antibodies present in the human allergic sera. However, if the human IgE antibodies have the same specificity as the sheep precipitating antibodies, i.e. directed to the identical determinant(s), the combination of the IgE antibodies to the allergen would be blocked by the prior exposure of the allergen to the sheep antibodies. Obviously, this blocking of further combination would be dependent on the presence of sufficient sheep antibodies to completely saturate all antigenic (allergenic) determinants. Furthermore, one must also consider that precipitating human IgG antibodies as well as antibodies of the other immunoglobulin classes, which are present in the human allergic sera, may, by competition, for the same or adjacent determinants, also adversely affect the binding of IgE antibodies to the immune complex. In principle, these antibodies could compete with the IgE antibodies for the binding to the sheep anti-R-antigen complexes and thus reduce the degree of specific binding by the IgE antibodies. From a consideration of these possibilities, it is clear that the radioimmunoprecipitin peaks seen on the radioautograph are an indication of the minimum number of allergenic components present.

Aukrust et al (124) recently demonstrated that co-precipitation of an allergen with other nonallergenic antigens could occur in CRIE and result in radio-labelling of a number of immunoprecipitin peaks which was greater than the number of allergens actually present. To resolve this ambiguity, the technique of crossed-immunoelectrophoresis with intermediate gel (CIEWIG) in which an antiserum specific to one of the antigens was incorporated into the intermediate gel, demonstrated that the immobilization of the homologous antigen (allergenically active)

by its immunoprecipitation in the intermediate gel, precluded its co-precipitation with other nonallergenic antigens in the main gel. This suggested that the different precipitates giving rise to radio-staining in CRIE do not necessarily represent distinct allergens.

Although it is conceivable that the radioactively labelled antihuman IgE antibody preparation could have bound nonspecifically to the immune complexes within the agarose gel, this possibility appeared to be remote since omission of incubation of the immune complexes with the human allergic sera pool, prior to the incubation with the labelled anti-IgE antiserum, did not result in the incorporation of the radioactive label into the sheep anti-R-antigen complexes.

The analysis of sheep anti-A (S597) antiserum by immunoelectrophoresis, CIE, and CRIE using R as the antigenic preparation, all indicated that antiserum (S597) was indeed monospecific for one antigenic component, i.e. 'A'. The salient feature of the aspect of this study is that a monospecific antiserum was produced to one antigenic component without first purifying or isolating the homologous antigen from R. To selectively isolate this antigenically unique component from KBG pollen, a reversed immunosorbent was prepared by coupling the globulin fraction of sheep antiserum (S597) to an insoluble matrix. Cyanogen bromide activated Sepharose 4B-Cl (Pharmacia) was first used as the supporting matrix but the problem of the leakage of ligand from such an immunosorbent as has been reported in the literature (93-97), was also experienced in the present study, and necessitated the use of an alternate source of the matrix and/or coupling procedure. The activated form of Affi-Gel 10 (Bio-Rad) was chosen for use as an inert supporting structure in the preparation of immunosorbents. One salient feature of

this gel is the 10 Å long spacer arms anchored to the agarose matrix by extremely stable ether linkage which are intended to help prevent steric hinderance between the matrix and the ligand.

Prior to its use, the reversed immunosorbent was washed extensively with a buffer at low pH (Glycine-HCl, pH 2.2) followed by a phosphate buffer, pH 7.0. This abrupt change in pH was intended to displace from the matrix the non-specific and/or weakly coupled protein. The immunosorbent was then washed extensively with those agents which would subsequently be used for the dissociation of the antigen-antibody complexes. It was finally washed with phosphate buffer, pH 7.0, and used immediately. In this manner it was expected that the risk of desorption of proteins coupled to the matrix would be minimized, if not eliminated, during the use of the immunosorbent.

The elution of the specifically adsorbed components of R from the reversed immunosorbent in this study was achieved with a solution of potassium iodide (2M) at neutral pH. This eluting agent was chosen rather than the glycine-HCl buffer since a solution containing chaotropic ions had previously (162) been found to be a mild and effective agent and it was considered that any denaturation of antigenic and/or allergenic activity would be minimal.

No attempt was made to determine the actual capacity of the reversed immunosorbent for its homologous antigen. It was simply considered that an amount of 20 mg of R would contain allergen A in an amount far in excess of that required to completely saturate the insolubilized antibodies to A. (The amount of allergen A in 20 mg of R as determined by rocket immunoelectrophoresis was of the order of 4 mg). In practice, it was found that an amount of the order of 1 mg of

allergen A (on protein basis) could be recovered from the reversed immunosorbent.

To obtain additional amounts of allergen A, the reversed immunosorbent was simply reused. It is well known that reversed immunosorbents, by nature, have low capacity for their homologous antigen. This is due in part to the fact that (i) only a small fraction of the γ-globulin proteins being attached to the insoluble matrix have antibody activity, and (ii) since there is no control over the spacial orientation of the antibodies during the process of attachment to the matrix, the antibody combining sites may have been sterically hindered from combining with its homologous antigen. It can be suggested that the capacity of the reversed immunosorbent may be increased by using only immunoglobulins with antibody activity to allergen A. These would be specifically isolated from the antiserum to A, using an immunosorbent prepared by attaching allergen A to an appropriate insoluble matrix.

Allergen A is considered to possess allergenic properties since (i) it binds human IgE antibodies as demonstrated by CRIE, and (ii) it is able to elicit PCA reactions. The fact that allergen A could not elicit a PCA titer in sensitized hooded rats as high as that when using R as the challenging allergen may be due to one or more of the following possibilities:- (i) allergen A is not as potent as R; (ii) some of the allergenic activity in allergen A may have been denatured during the elution process using the 2M KI buffer.

The level of allergenic activity present in allergen A was evaluated by PCA neutralization and inhibition of RAST. The observations that R could neutralize completely the PCA reaction at a concentration of 4 mg/ml while allergen A at the same concentration could neutralize the

PCA reaction by a maximum of 94% was interpreted to mean that R was more effective than allergen A in reducing the PCA titer of the antiserum to R. Since the maximum neutralization of PCA obtained with allergen A remained at 94% even at a concentration as high as 8 mg/ml, this finding suggested that allergen A lacked some allergenic determinants present in R, and therefore was unable to completely neutralize all the reaginic antibodies to R. In comparable experiments of the inhibition of RAST, no apparent increase in percentage of inhibition greater than the maximum amount of 67% was obtained even on further increase of the amount of allergen A beyond 200 µg. This was interpreted to mean that allergen A lacked some antigenic determinants found normally in R. The fact that allergen A could inhibit RAST by 67% while it neutralized PCA to an extent of at least 94% may be due to differences in either the avidity and/or specificities of the different IgE antibodies (human IgE for RAST, murine IgE for PCA) used in these two procedures.

In experiments designed to examine the antigenic purity of allergen A isolated by means of reversed immunosorbent, CIE employing an antiserum to R revealed only one immunoprecipitin peak. This result suggested that allergen A was antigenically pure and that contamination, if any at all, by other antigens, was below the limits of detectability by this technique. To rule out the possibility that trace contaminants possessing antigenic determinants different from those found in allergen A were present, a more stringent criteria for antigenic purity was used; i.e. allergen A was used to prepare a hyperimmune rabbit antiserum and the specificity of the antibody(ies) elicited was evaluated by CIE using the crude antigenic preparation, R.

The technique of eliciting antibody formation to the antigenic component(s) present in a purified antigenic preparation is far superior to the procedure of direct examination for detecting the presence of antigenic contaminants using an available antiserum. The shortcomings of the direct procedure lie in the fact that trace amount of antigenic contaminants may not give rise to visible precipitins even if the homologous antibody(ies) is present in sufficient amount. By immunization with the 'purified' antigen, antibodies to the antigenic contaminants, if any, may be formed in amounts which reflect their immunogenicity rather than their amount. Consequently, with an immunogenic contaminant, large amounts of antibody(ies) may be formed to relatively small amounts of contaminating antigen(s). This antibody(ies) will form visible precipitins with the crude antigenic preparation which contains the antigens in amounts larger than that present as contaminants in the purified antigenic fraction. Thus, by analysis of the specificity(ies) of the antibody(ies) elicited by the hyperimmunization with the 'pure' antigen, an indication of the antigenic purity can be obtained. In the present study, this technique verified that allergen A isolated from R was indeed antigenically pure, i.e. the rabbit antiserum to the purified allergen A was found by immunoelectrophoretic and crossed-immunoelectrophoretic analyses to contain one immunoprecipitin peak using R as the antigenic preparation. Therefore, in terms of the specificity of the antibody, the antiserum was considered to be monospecific and the antigen (allergen) A purified by means of the reversed immunosorbent consisted of a single antigenic component.

In a separate experiment, in which a sheep antiserum was prepared to allergen A by hyperimmunization with the appropriate immuno-

precipitin band, it was found by CIE of the crude antigenic preparation, R, that the sheep antiserum to A (S597) was able to detect only allergen A (as evidenced by a single immunoprecipitin peak). This indicated that sheep antiserum S597 was indeed specific for allergen A and served to illustrate that a monospecific antiserum could be produced by a relatively simple procedure which did not require the prior isolation of the antigen. A single precipitin peak was also obtained in the analysis of the purity of allergen A by CIE using the polyspecific antiserum to R (S11R). It was assumed that the immunoprecipitin peak obtained in either case was formed by the identical specificity of the antigen-antibody system (i.e. allergen A-anti-A). This assumption was proven to be correct by the technique of CIE with intermediate gel which conclusively demonstrated that the precipitin peak elicited with allergen A and the antiserum to A or to R was indeed immunologically identical.

In view of the heterogeneity of components present in R, the finding that the protein content of allergen A was different from the 'average' protein content present in R was not unexpected. On the basis of other investigations carried out in this laboratory (163) which revealed that several allergens are glycoproteins, it was assumed that the non-protein portion of allergen A consisted of carbohydrates and that allergen A is a glycoprotein. Further studies will be required to solve the long-standing question as to the significance or contribution of the carbohydrate moiety to the property of allergenicity.

The fact that only one antigenic component was detected by the crossed-immunoelectrophoretic analysis of allergen A while 4-5 bands were obtained on SDS-PAGE was not surprising since CIE does not discriminate on the basis of differences in molecular weight. In addition,

one or more of the following should be considered as possible explanations for the difference in the number of components detected by CIE in comparison with the technique of SDS-PAGE:- (i) the sheep anti-R antiserum (S11R) used for crossed-immunoelectrophoresis may not contain antibodies against all the antigenic components present in R, therefore the immunoprecipitin pattern obtained for crossed-immunoelectrophoretic analysis revealed only the minimum number of antigenic peaks; (ii) proteins with different molecular weight may have the same antigenic determinant reacting with the same antibody specificity. Therefore, several bands will be obtained on SDS-PAGE analysis, with each band indicating the presence of a component with a different molecular weight, while only one immunoprecipitin peak will be obtained on crossed-immunoelectrophoresis.

In view of the fact that pollens are highly complex materials and since a single isolation procedure (i.e. immunosorption chromatography) was employed, it is not surprising to have several components revealed by SDS-PAGE analysis while only one antigenic component was detected on crossed-immunoelectrophoretic analysis. It is obvious that in order to obtain an antigenic component which is unique in terms of either molecular size, or isoelectric value, or carbohydrate content, a combination of physical and chemical techniques in addition to immunological techniques will have to be employed.

The immunochemically pure antigen (allergen A) isolated by means of a reversed immunosorbent will facilitate standardization of the RAST system for measuring its allergenic potency. Moreover, allergen A can itself be employed for preparing allergosorbent discs for use in evaluating the amount of IgE antibodies to A present in the sera of allergic individuals. In this manner it will serve to establish its

allergenic importance in terms of the proportion of allergic individuals who respond to this allergen by producing IgE antibodies.

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