The University of Manitoba

# IMMUNOCHEMICAL STUDIES ON KENTUCKY BLUE GRASS POLLEN ALLERGENS

by

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A Thesis

Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

Department of Immunology

Winnipeg, Manitoba

February, 1980

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

TO MY WIFE LILA

HER SUPPORT AND UNDERSTANDING HAVE MADE THIS WORK POSSIBLE

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. F.T. Kisil for his guidance and encouragement during the course of this study and especially for his help and constructive criticism in the preparation of this thesis.

I also wish to express my gratitude to Dr. A.K.M. Ekramouddoullah for his many valuable suggestions and stimulating discussions.

I am also grateful to Dr. K. Kelly for his technical advice in preparative isoelectrofocusing; and Dr. Valerie Strevens for her critical reading of this thesis.

I would also like to express my gratitude to Dr. A.H. Sehon for giving me the opportunity to enrich my learning experience at the Protein Laboratory, University of Copenhagen, Denmark.

The technical assistance of Mr. Tom Cook is also gratefully acknowledged.

## SCOPE OF THE INVESTIGATION

The principal objectives of this investigation were to (i) isolate haptenic components from Kentucky Blue Grass (KBG) pollen which are able to block the allergic reactions triggered by multi-valent allergens of KBG pollen; (ii) isolate an allergenic component in pure form from KBG pollen and to characterize this component; (iii) examine the allergenic and antigenic relationships of these components employing murine IgE antibodies, human IgE antibodies and rabbit precipitating antibodies.

This thesis is divided into four chapters. The first chapter is introductory in nature and contains a literature survey and general information.

The second chapter deals with methods of isolation of haptenic components from KBG pollen and the biological properties of these components.

The third chapter summarizes results of studies on the isolation and characterization of a purified allergen from KBG pollen.

The last chapter examines the allergenic and antigenic relationships of two purified allergens isolated from KBG pollen and the antigenic relationship of the haptenic components to these two purified allergens.

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## ABSTRACT

Components with haptenic properties were isolated from the non-dialyzable fraction, i.e. the retentate (R) and the dialyzable fractions of the aqueous extract of Kentucky Blue Grass pollen (KBG aq. ext.) by preparative isoelectrofocusing (Prep-ISO-EF) on Sephadex G-100 gel. The haptenic components could not elicit PCA reactions in rats passively sensitized with murine reaginic antisera to R but they could inhibit completely and specifically the PCA reactions normally elicitable with R. Some haptenic fractions which contained only a few components, detectable by analytical isoelectrofocusing (Anal-ISO-EF), could inhibit specifically and completely the PCA reactions normally elicitable with R which contained over 30 components. This clearly indicated that such haptenic fractions possessed all the allergenic specificities present in R. It was concluded that the specificity of murine IgE antibodies was directed to a determinant(s) which was common to either

allergenic or haptenic fractions. On the other hand, by employing a pool of human sera from individuals allergic to KBG pollen in the RAST procedure, it was apparent that most of the haptenic fractions lacked some of the specificities present on allergenic components of R that are recognized by the human IgE antibodies.

An allergenic glycoprotein (M.W. 11,000) designated as Allergen C was isolated from R by a combination of Prep-ISO-EF and gel filtration on Bio-Gel P-60. It possessed all the allergenic specificities of R recognized by the murine reaginic anti-R sera. On the other hand, Allergen C lacked some of the specificities recognized by the pool of human allergic sera. Allergen C contained all the naturally occurring amino acids with the exception of cysteine. The allergenic activity was found to be stable on exposure to extremes of pH and guanidine HCl treatment; since protease treatment completely destroyed its allergenic activity, it is suggested that allergenicity is associated with the protein moiety of this molecule. Enzymatic digestion of Allergen C revealed that the allergenic determinants recognized by the murine IgE antibodies were different from the antigenic determinants recognized by a rabbit precipitating antiserum to Allergen C.

Allergen C and another allergen, KBG-1 also isolated from KBG aq. ext. were found to be allergenically identical

in terms of their specificities evaluated with the murine reaginic sera; whereas they were only partially identical when evaluated with the human allergic sera. However, Allergens C and KBG-1 were antigenically distinct with respect to rabbit precipitating antisera produced separately to each allergen. Allergen C did not share any common antigenic specificities with the haptenic components; however, Allergen KBG-1 were found to share some common antigenic specificities with the haptenic components.

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## ABBREVIATIONS

| | |
|---|---|
| Anal-ISO-EF | Analytical isoelectrofocusing |
| BSA | Bovine serum albumin |
| CIE | Crossed immunoelectrophoresis |
| cpm | Counts per minute |
| CRIE | Crossed radio-immunoelectrophoresis |
| $D_{24}$ | 24 hour dialysate of KBG aq. ext. |
| $D_{48}$ | 48 hour dialysate of KBG aq. ext. |
| DNP | Dinitrophenol |
| hrs. | Hours |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| KBG aq. ext. | Aqueous extract of Kentucky Blue Grass pollen |
| MW | Molecular weight |
| mins. | Minutes |
| OA | Ovalbumin |
| PCA | Passive cutaneous anaphylaxis |
| P-K transfer test | Prausnitz-Küstner transfer test |
| Prep-ISO-EF | Preparative isoelectrofocusing |
| R | Retentate or non-dialyzable constituents of KBG aq. ext. |
| RAST | Radioallergosorbent Test |
| RIA | Radioimmunoassay |
| RPM | Revolutions per minute |
| secs. | Seconds |
| TCA | Trichloroacetic acid |
| V | Volts |

# CHAPTER 1

## INTRODUCTION

### IgE MEDIATED HYPERSENSITIVITY

Hypersensitivity can be defined as an altered state of immunity induced by an antigen in which anaphylactic reactions and symptoms of atopic sensitivity can be subsequently elicited by that antigen, or by structurally similar substances. At the end of the 19th century following the discovery of antitoxins and antimicrobial antibodies, the nature of the immune response was considered to be purely protective. However, at the beginning of this century it was realized that immune responses also possessed harmful potentialities. Portier and Richet (1902) proposed that certain immunological mechanisms similar to those involved in protection from microbial infection could also result in harmful reactions and effects which could be fatal. At the beginning of the 20th century, von Pirquet (1906) coined the term "allergy" (Greek for "altered action") to denote any harmful altered response to a substance induced by previous exposure to it. Through usage, "allergy" and "hypersensitivity" have become synonymous: both referred to the harmful altered response to a substance induced by previous exposure.

Hypersensitivity states can be classified according to the onset of the reaction following exposure of sensitized individuals to the offending antigen. Sensitization refers to the altered state of immunity induced by exposure to the offending antigen. The two main classes of hypersensitivity are: (i) of the immediate and (ii) of the delayed type. Immediate type hypersensitivity can be passively transferred with serum from a sensitized individual to a non-sensitive recipient and it is due to the presence of humoral antibodies in the serum. Anaphylaxis and atopic sensitivity are two examples of immediate type hypersensitivity. By contrast, humoral antibodies are not involved in delayed type hypersensitivity and its passive transfer is usually accomplished with white cells from a sensitized individual or with soluble extracts of these cells. Examples of delayed hypersensitivity are: tuberculin sensitivity, contact allergy, and phenomena related to transplantation immunity. The manifestation of different forms of immediate type hypersensitivities are described below.

Anaphylaxis

The term "anaphylaxis" was coined by Portier and Richet (1902) (Greek, ana = against; phylaxis = protection) to denote an increase in susceptibility to a toxic substance rather than the expected increase in resistance. They observed that dogs given a second injection of sea

anemone extract, several weeks after the first, became acutely ill, went into shock and died within a few minutes. Other investigators (Koch, 1890; Flexner; 1894) in the late 19th century had also observed similar phenomena upon reinjection of antigens into previously sensitized animals. When an animal is sensitized with an antigen, an interval of time is required before anaphylactic shock can be elicited on reinjection of the antigen into the animal. During this interval antibodies are formed which become fixed to tissues (target organ) rendering that animal prone to anaphylaxis. When the antigen is injected into the sensitized animal, the combination of antigen with tissue-fixed antibodies leads to 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, and prostaglandins] from target cells. These mediators diffuse through the extracellular fluid surrounding the target cell until contact is made with certain effector structures - smooth muscles and blood vessel walls. Contraction of the former and enhanced permeability of the latter give rise in turn to clinical symptoms characteristic of immediate hypersensitivity (wheal and flare skin reactions, itching, sneezing etc) or anaphylactic reactions.

## Atopic Sensitivity

The term atopy (meaning strangeness) was introduced

by Coca and Cooke (1923) to describe a type of hypersensitivity occurring mostly in man. Asthma, hayfever, urticaria, angiodema are some of the chief atopic conditions. The allergens responsible for this type of hypersensitivity are found in pollens, feathers, animal danders, house dusts, and in food such as milk and eggs. Simple chemicals and drugs such as penicillin which can react with the host's protein can also cause atopic sensitivity.

Prausnitz and Küstner (1921) first recognized that the condition of immediate hypersensitivity was brought about by the production of a serum factor 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. These skin-sensitizing antibodies were designated by Coca and Grove (1921) as reagin. Skin-sensitizing antibodies were found not to be able to cross the placenta and were retained by the choroid plexus (Bell and

Erikson, 1931; Sherman et al., 1940; London, 1940). They also migrated with the mobility of slow moving β-globulins (Loveless and Cann, 1953; Sehon et al., 1956).

Earlier studies (Sehon et al., 1958; 1959; Gordon, 1958) indicated that reaginic activity was associated with 19S immunoglobulins. Other studies indicated that reaginic antibodies might be associated with the IgA class (Heremans et al., 1962; Fireman et al., 1963). However, in 1966 the Ishizakas (Ishizaka and Ishizaka, 1966a; 1966b; Ishizaka et al., 1966c; 1966d; 1966e) demonstrated that reagin did not belong to the IgA class or to any of the known classes of immunoglobulins. Antiserum prepared by immunizing rabbits or guinea pigs with a reagin-rich fraction of atopic patients' sera did not react with any of the known classes of immunoglobulin, but reacted with a reagin-rich fraction from the sera of ragweed-sensitive patients. On the basis of these differences in antigenic properties, it was proposed that reagin belonged to a new class of immunoglobulin, designated as IgE. The discovery of IgE myeloma in human patient (Johansson and Bennich, 1967) had facilitated the elucidation of the structural features of the IgE molecule and the role it played in atopic allergy.

Properties of IgE and Receptor for IgE

IgE has a molecular weight of approximately 190,000 daltons and a sedimentation coefficient of 8S (Bennich

and Johansson, 1968). The immunoglobulin heavy chain is of the ε type and has four constant domains. 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. The concentration of IgE in serum is of the order of 0.1 - 0.4 μg/ml (Johansson, 1967). A statistically higher level of IgE has been found in the sera of atopic patients and those with parasitic infestations (Johansson, 1967; Johansson et al., 1968). IgE has also been detected in many animal species such as monkey, rabbit, guinea pig, rat, mouse, and cattle (Ishizaka, 1972).

The skin fixing or cytotropic activity of IgE antibodies is directed to specific receptor molecules present on the membrane of basophils and mast cells (Ishizaka et al., 1970; Sullivan et al., 1971). The cytotropic activity is likely to be associated with unique structures in the Fc portion of IgE molecules. The skin-sensitizing activity of IgE is lost at 56°C; digestion with papain or reduction with 0.1M mercaptoethanol also leads to loss of biological activity (Ishizaka et al., 1967;

Stanworth et al., 1970). It is generally accepted that histamine release from the target cells is triggered by bridging of cell-bound IgE molecules by multivalent ligands. The number of receptor sites for IgE on human basophilic granulocytes is in the order of 40,000 to 100,000 per cell (Ishizaka et al., 1973) and the number of receptors on rat mast cells and leukemia cells is in the order of 300,000 to 1,000,000 per cell (Kulczycki et al., 1974a; 1974b; Conrad et al., 1975). The binding of IgE molecules with receptors is a reversible reaction (Ishizaka et al., 1973; Ishizaka and Ishizaka, 1974). Membrane bound IgE on human basophils can be made to cap, indicating that the receptors for IgE are free to move on the surface of these cells (Becker et al., 1973). IgE antibodies combine with the receptors on target cells with high affinity; the equilibrium constant for the association reaction is in the order of $10^9$ to $10^{10}$ $M^{-1}$ in both human and rat system (Ishizaka et al., 1978).

At present the exact nature of these receptors for IgE is not known. Studies of these receptors on rat mast cells and rat basophilic leukemia cells have established that this receptor has a molecular weight of about 60,000 daltons and is, at least in part, a protein (Froese, 1977). Recent studies by Conrad et al. (1979) suggest that two receptor components, with molecular size of the order of 45,000 and 55,000 daltons, are present on the surface of both rat mast cells and

rat basophilic leukemia cells. When IgE antibodies are bound to the receptors, the 45,000 dalton component cannot be iodinated and therefore, cannot be detected by the cell surface iodination technique. The receptor for IgE has been shown to be glycoprotein in nature and bind to lentil lectin (Kulczycki et al., 1976; Helm et al., 1979).

## ANALYSIS OF ALLERGENIC POTENCY OF ALLERGENIC EXTRACT EMPLOYING IgE ANTIBODIES

Examples of immediate hypersensitivity states in man include hay fever, asthma and hives,and the inducing agents are called allergens. There are many environmental sources of allergens, such as pollens (of grasses, trees, weeds), animal danders, fungi, insects, foods, certain drugs and chemicals. It is generally accepted that the antibodies involved in atopic allergy belong primarily, if not solely, to the IgE class of immunoglobulins. Throughout this thesis, the term allergen will be used to denote substances that can cause atopic allergy and can combine with IgE antibodies in vivo or in vitro. The term antigen will be used to denote substances that can combine with antibody classes other than IgE. Since the molecular species which are responsible for inducing the formation of IgE or other classes of antibody have not been elucidated, the usage in this thesis of the term allergen and antigen makes no reference to their immunogenic properties. These definitions do not imply that antigenic and aller-

genic determinants are necessarily distinct and different from one another. They serve to distinguish the combination of IgE antibodies with their ligands from those combinations with ligands involving classes of antibodies other than IgE.

At present there is no universal standard used for evaluating the allergenic activity or potency of commercially available allergenic extracts which are marketed for diagnostic and therapeutic purposes. Extracts are sold on the basis of their protein nitrogen content or by dry weight to volume ratio, neither of which may necessarily have any direct, or more importantly, consistent relationship to the biological potency of the extracts (Baer et al., 1970). The lack of standardization of allergenic potency of allergenic extracts is due to the unavailability of purified, well defined allergens and the high variation among patients' allergic responses (Kisil et al., 1971; Marsh, 1974; Løwenstein, 1976; 1978a). This variation is probably due to the individual's unique genetic capability to mount an IgE response to the allergens in a complex allergenic extract (Marsh, 1975). In view of this variation, allergenic activity is best measured employing a large number of sera from allergic individuals with IgE specificities covering all the allergens of the allergenic extract in the analytical methods described below.

P-K Transfer Test and Direct Skin Test

The P-K transfer test (Prausnitz and Küstner, 1921) and direct skin test performed on allergic patients (Becker, 1948; Becker and Rappaport, 1948a, 1948b); were for many years the only method available for assaying allergenic activity. In the P-K transfer test serum from allergic patients is injected intradermally into a number of sites in the back of a normal human volunteer. 24 hours later these sites are challenged with an injection of allergenic extracts. The size of the reactions following challenge are then measured and graded as an indication of allergenic activity. In direct skin testing the allergens are introduced into the skin (prick, scratch or intradermal injection methods) of an allergic individual causing an immunologically specific reaction with mast cell-bound IgE whereby vasoactive substances such as histamine are secreted from the cell and elicit a wheal and flare reaction. The size of the wheal, as measured by its diameter, is taken as a measure of the allergenic activity.

The sensitivity of the P-K transfer test and direct skin testing is high; ng quantities of allergen can be detected. In view of the variation in the IgE antibody response among allergic individuals (Marsh, 1975), allergenic activity is best measured employing the P-K transfer test on normal volunteers employing a large number of

allergic sera with IgE specificities covering all the allergens of the allergenic extract. These tests presuppose that the skin reaction measured is an effect exclusively caused by type I reaction which may be doubtful since allergen extracts possibly contain low molecular weight compounds which directly or indirectly cause formation of urticarial reactions (Aas, 1975). Other type of non-immunological reaction might be caused by lectins which stimulate mast cells by binding to the Fc fragment of IgE thus eliciting histamine release.

Histamine Release Assay from Human Leucocytes

Measuring histamine release from allergic human leucocytes (Lichtenstein et al., 1964; 1966; 1969; Osler et al., 1968) has many properties in common with skin test assays. Leukocytes from normal donors may also be used for the histamine release assay after they have been passively sensitized with allergic serum. On exposure of the sensitized cells to the homologous allergens, an immunologically specific reaction between the allergens and IgE bound to basophilic granulocytes occur, thereby releasing active substances including histamine, the amount of which is measured chemically. The relative allergenicity of different allergen preparations may be determined from the ratio of the respective concentrations which correspond to 50% of maximum histamine release. Due to genetic differences between different allergic

subjects (Marsh, 1975), different individuals exhibit unique response patterns to different allergens. Therefore, in order to investigate thoroughly the relative activities of different allergenic fractions, during fractionation procedures, it is necessary to test a number of subjects - preferably at least 10 when investigating major allergens and 100 or more for minor allergens (Marsh et al., 1973). This method presupposes that the histamine release is caused exclusively by the immunologically specific type I reaction, but, as mentioned for the skin reaction above, it may also be triggered unspecifically by low molecular agents or lectins.

Passive Cutaneous Anaphylaxis (PCA) and Inhibition of PCA

The PCA test developed by Ovary (1964) who utilized the local anaphylactic reaction in the skin as a highly sensitive test for the presence of homocytotropic antibody. Mice have been found to be a very suitable animal model for IgE antibody production (Levine et al., 1970; Vaz and Levine, 1970; Ekramoddoullah et al., 1977a; 1977b). For the PCA procedures employing murine reaginic sera, the sera are serially diluted and injected intradermally into skin sites on the back of a rat. 24 hours after sensitization the rat is challenged intravenously with a solution of allergenic extract and Evan's blue dye. The titer of the reaginic sera can be expressed as the highest dilution that will elicit a positive skin reaction.

This technique is sensitive and can detect as little as 1 µg of allergen. The anaphylactic reactions elicited in the rat skin detect solely the presence of IgE antibodies (Wolfgang et al., 1973; Ovary et al., 1975). Allergenic potency of different allergenic extracts can thus be evaluated in terms of the PCA titers elicited in rats employing a standard murine reaginic sera.

Neutralization of PCA reactions is a variation of the PCA technique which can be used to compare the allergenic activity of different extracts, or different fractions isolated from an allergenic extract. The allergenic activity or potency of different extracts or fractions is evaluated in terms of their ability to combine with the reaginic antibodies produced against the original whole extract and thus neutralize the PCA reactions. Extracts or fractions are incubated with the murine reaginic sera prior to intradermal sensitization of the rat. The degree to which the PCA reactions normally elicitable with the whole extract are inhibited due to neutralization of reaginic antibodies by the allergens being tested, provides an indication of the level of allergenic activity. The potency of certain allergens as measured by PCA inhibition, employing murine reaginic sera, has been found to be fairly well correlated to that as measured by the inhibition of RAST (Akiyama et al., 1979). Using a murine system to standardize allergens will eliminate the difficulties experienced in using

human subjects (in skin testing or P-K transfer test) such as discomfort to patients and the possibility of transmission of viral hepatitis. However, it is important to resolve whether or not murine and human IgE antibodies share common allergenic specificities. Standardization of allergens employing reaginic sera from mice or other experimental animals is feasible only if the animals' sera share the same allergenic specificities with human sera.

Radioallergosorbent Test (RAST)

RAST, developed by Wide et al. (1967), has proved to be well suited for the analysis of allergenic activity. Allergens are covalently coupled to cyanogen bromide activated polysaccharide particles (cellulose, agarose) which are used as allergosorbent in the test. The solid-phase allergens are used to absorb reaginic sera; the amount of allergen-specific IgE absorbed is then quantitated by the addition of radioactive-labelled rabbit anti-human IgE.

This method assumes that all potential allergens should contain equal statistical probabilities for reaction through their combining α- and ε-amino groups to the cyanogen bromide-activated solid support. This is never the case. First, carbohydrate allergens which lack protein moiety (Gelfand, 1943; Kabat et al., 1957) have been reported which have to be coupled through chemical

reactions other than the cyanogen bromide reaction. Second, many proteins especially from cereals, have no or extremely low content of lysine which can cause biased results as demonstrated in the case of wheat flour allergy (Blands et al., 1976) where some major allergens are underrepresented on the solid support. In spite of these limitations RAST procedures have been very useful for the analysis of allergenic activity. However, as was the case with skin tests and histamine release assays, the serum pool used for the RAST tests has to be selected carefully to cover all the individual patient's unique IgE responses. A useful modification of the RAST procedure is referred to as inhibition of RAST; that is, allergens in solution compete with solid-phase allergens for the binding of specific IgE (Foucard et al., 1972; Yman et al., 1973; Gleich and Yunginger, 1974). Standardization of extracts can be accomplished by the RAST inhibition technique. In this procedure, varying quantities of soluble allergen are incubated with the allergic sera before adding to the allergosorbents and the degree of inhibition is then calculated from the uptake of radioactive-labelled anti-IgE. Inhibition curves can be established for various extracts, and these curves are linear between 30 and 70 per cent inhibition when plotted in a log-linear fashion (Yunginger et al., 1976). A good estimation of relative allergenic

activity is the concentration of extracts which gives 50% inhibition (Aronsson et al., 1974). This 50% RAST inhibition method is especially recommended by the International Committee of Allergen Standardization as a good method for measurement of the potency of allergenic extracts.

The inhibition of RAST method is also particularly useful for allergen purification studies as only one solid-phase allergosorbent has to be prepared which is used to test all fractions. For an allergenic extract containing many different allergenic components, the maximal inhibition of RAST by each allergen will be less than that attained by the mixture of allergens. In addition to its use for the diagnosis of allergies (i.e. detection and determination of the levels of specific IgE antibodies in serum), RAST may also bé used to monitor the results of efforts to alter the production of IgE antibodies by immunotherapy (i.e. monitoring the levels of specific IgE antibodies).

Varga and Ceska (1972) and Topping et al. (1978a; 1978b) have used a combination of isoelectrofocusing on polyacrylamide gel and RAST procedures to analyze allergenic activity present in complex allergenic extracts. The components present in an allergenic extract are first separated by isoelectrofocusing on poly-acrylamide gels according to their pI values. The gels are then sliced into 1 mm thick sections and each gel

section is incubated in contact with cyanogen bromide activated paper discs. In this manner the separated components are covalently attached to the discs to form allergosorbents. RAST is then carried out as described. This method is very useful in establishing and comparing the allergenic profiles of different allergenic extracts according to pI values of the allergenic components.

## ANALYSIS OF ANTIGENIC COMPLEXITY OF ALLERGENIC EXTRACT EMPLOYING IgG ANTIBODIES

The most important factor in the analysis of complex allergenic extracts by using precipitating IgG antibodies is the success in raising antibodies against all potential allergens. At present there is no evidence to indicate that allergens are a special class of antigens. Although allergens generally fall within certain molecular size range, no allergens which have been isolated and characterized so far, have been shown to possess chemical features that are distinct from antigens. The lower molecular weight of allergens is limited to those substances which have a sufficient structural complexity to retain their immunogenic properties (Crumpton, 1974); the upper limit is determined by their capability to penetrate mucosal membranes and is generally of the order

of 40 - 60,000 daltons. It is the genetic capability of an individual's IgE producing system which appear to be the important factor in recognizing an antigen to be allergen (Marsh, 1975). In the analysis of allergens employing IgG antibodies by the various methods described below, the allergens are precipitated by the antibodies to what are referred to as antigenic determinants in this thesis.

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 (1948; 1949). 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 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 (1969) 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 (1974) 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.

Immunoelectrophoresis

Immunoelectrophoresis (Grabar and Williams, 1953) was developed from the technique of double diffusion according to Ouchterlony. The relatively low resolving power of the double diffusion technique is increased by separating the antigens by electrophoresis prior to the immunodiffusion as performed in 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. Immunoelectrophoresis has been used in the characterization of allergenic extracts (Augustin, 1959a; 1959b; Hussain et al., 1972; King et al., 1964). Kisil et al. (1971) 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.

Crossed Immunoelectrophoresis and Crossed Radio-Immunoelectrophoresis.

The immunoelectrophoresis technique described above is a qualitative method only. In 1960, Ressler 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. A few years later, a high resolution quantitative immuno-electrophoresis, crossed immunoelectrophoresis (CIE) was developed (Laurell, 1965; Clarke and Freeman, 1967).

In the CIE technique, antigens are first separated by electrophoresis in one dimension followed by electrophoresis into antibody containing gel in a second dimension perpendicular to the first. Immunoprecipitates are formed following the second dimension electrophoresis. The area given in each immunoprecipitate can be correlated with the concentration of the individual antigens. The high resolving power and the possibility of quantification of individual antigens without previous separation have made this method widely used (Axelsen et al., 1973). Precipitin reactions of identity, non-identity and partial identity can be identified by CIE in various modifications (Løwenstein, 1978b). For example, Løwenstein (1978c) has made use of the CIE technique to study the immunological relationship of timothy pollen allergens to that of allergens from false oat, orchard and rye grass.

Crossed radio-immunoelectrophoresis (CRIE) introduced by Weeke and Løwenstein (1973) allows the identification of individual allergens in a complex antigenic mixture. The mixture is first separated by crossed immunoelectrophoresis in duplicates. One gel is stained with dye to reveal the immunoprecipitate peaks elicited

with the different antigens. The duplicate unstained gel is next incubated with sera from allergic patients and this is followed with application of radiolabelled anti-human IgE antibodies. By autoradiography, those immunoprecipitates which are allergens by their binding to the radio-label (and therefore the IgE antibodies) can be identified as containing allergenic components. This method can also be used to survey serum samples from different individuals to establish the extent of variation in the individual unique response to different allergens. The CRIE technique also allows the identification of major and minor allergens in a complex allergenic extract without their prior separation (Løwenstein, 1978b). Major allergens are those allergens to which at least 90% of a population of allergic individuals are allergic to, and minor allergens represent those allergens to which only a limited number of allergic individuals are allergic to (Marsh, 1975). An example of a major allergen of ragweed is Antigen E (King et al., 1964) and minor allergens of ragweed are Ra 3, Ra 4 and Ra 5 (Lichtenstein et al., 1973; Santilli et al., 1975; Marsh et al., 1973). The identification of major and minor allergens in a complex allergenic extract is accomplished by means of a grading system according to the intensity of the radiostaining of allergens on the CRIE plates after various days of exposure. Major allergens that bind a high level of IgE have a high intensity of radiostaining and

minor allergens that bind less IgE have a lesser intensity of radiostaining.

According to Løwenstein (1978b) there is no chemical basis which distinguishes allergens as being different from antigens. However, the possibility cannot be excluded that in different species of animals under different immunization conditions, the allergenic determinants that induce IgE formation in one species may be different from that of the antigenic determinants that induce IgG formation in another species. Evidences to support this hypothesis will be presented in chapter IV of this thesis. It is conceivable that if the antigenic and allergenic determinants are of similar or identical in nature, the precipitating antibodies may block the subsequent binding of reaginic antibodies with their homologous allergenic determinants. If the allergenic determinants to which the IgE antibody specificity is directed is different from the antigenic determinants to which the IgG antibody is directed, there should be no competition for combination for the respective determinants by the reaginic and precipitating antibodies. However, steric hindrance resulting from the close proximity of different determinants may 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 autoradiograph.

Mancini Technique and Rocket Immunoelectrophoresis

Such techniques employing monospecific precipitating antibodies have been used in the identification and quantification of allergens (Baer et al., 1970; Berlin et al., 1971; Center et al., 1974). Monospecific antibodies against one antigen in an allergenic extract may be produced by immunizing animals with partially purified components followed by immunoabsorption of the resulting antisera using appropriate fractions of allergenic extract which are devoid of the antigen of interest. Alternatively, monospecific antibodies may be obtained by immunizing animals with immunoprecipitates following immunodiffusion or immunoelectrophoresis (Shivers and James, 1967; Kum, 1979).

In single radial immunodiffusion - Mancini technique (Mancini, 1965) - the area of the diffusion ring formed by the precipitated antigen in antibody-containing gels is correlated with the amount of antigen used. Similarly, in rocket immunoelectrophoresis (Laurell, 1966; Weeke, 1973) the height of the precipitate formed by electrophoresing antigen into the antibody-containing gel is proportional to the antigen concentration. Antigens in the concentration range of 1 - 10 µg per ml can be detected by these techniques employing a high titered

antiserum obtained after careful immunization (Mancini, 1965; Weeke, 1973). The advantages of these techniques are their simplicity and convenience for fast detection of the antigen of interest without its isolation from a complex allergenic extract.

## CHARACTERIZATION OF ALLERGENS BY CHEMICAL AND PHYSICAL METHODS

Allergens isolated from allergenic extracts can be characterized by physical and chemical methods. Most of these methods are also widely used in the field of protein chemistry. The molecular size of allergens can be determined by sedimentation equilibrium ultracentrifugal analysis and sedimentation diffusion analysis (van Holde et al., 1958; Schachman, 1959) using the ultracentrifuge. Molecular size can also be estimated using more easily performed techniques such as gel filtration (Porath and Flodin, 1959; Andrews, 1964) and sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (Shapiro et al., 1967). Isoelectric point of an allergen can be established by isoelectrofocusing in polyacrylamide gel or granulated gel (Vesterberg, 1972; 1973; Radola, 1973). Electrophoretic homogeneity of an allergen can be determined by acrylamide disc electrophoresis (Ornstein, 1964; Davis, 1964), starch gel electrophoresis (Smithies, 1959),

and agarose gel electrophoresis (Johansson, 1972).

Chemical analysis of allergens may include amino acid composition and sequence analysis of amino acids in the allergenic molecule (Hirs and Timasheff, 1972). Carbohydrate moiety if present may also be qualitatively or quantitatively determined (Dische, 1955; Gottschalk, 1966).

## CHEMICAL AND BIOLOGICAL PROPERTIES OF SOME PURIFIED POLLEN ALLERGENS

The chemical nature of pollen allergens remained unknown prior to 1960 despite painstaking effort to elucidate the nature of these allergens (Richter and Sehon, 1960). With the development of more refined techniques, such as gel filtration, ion-exchange chromatography and electrophoresis in the sixties, a renewed effort was launched on to isolate and characterize pollen allergens.

### Ragweed Pollen Allergens

Due to the wide spread distribution of ragweed pollens and the large number of individuals who develop allergic reactions to it, they have been the most extensively studied of all pollens. A purified allergenic fraction was isolated by paper block electrophoresis from a dialyzed water soluble ragweed pollen extract by Lea and Sehon

(1962). This fraction, referred to as delta fraction, had a sedimentation coefficient of 3.2S and contained 80% protein and small amount of carbohydrate consisting of arabinose and galactose. When examined by passive transfer cross-neutralization tests with sera of untreated ragweed allergic patients, the delta fraction appeared to contain all the allergenic components of ragweed pollen (Lea et al, 1962).

There are at least 14 antigens in short ragweed pollen extract (Gussoni, 1966). Twenty or more protein bands have been detected by isoelectrofocusing (King, 1972). An important protein allergen, antigen E (AgE) was isolated by King and Norman (1962). King et al. reported that removal of AgE from whole ragweed pollen extract by precipitation with specific rabbit anti-AgE serum removed at least 90% of the allergenic activity of the whole pollen extract. This study suggested that AgE was the major allergen of ragweed pollen. AgE was shown to possess several isoallergenic forms. In 1967 King and associates (1967a; 1967b) reported the isolation from ragweed pollen of another purified allergen, Antigen K, which was partially related antigenically and allergenically to AgE. Like AgE, AgK also possessed several isoallergenic forms. Based on quantitative comparison of antigens E and K by leukocyte and intradermal skin test assays, King and associates concluded that AgK was the second most potent allergen in ragweed pollen.

Antigens E and K are acidic proteins with molecular weights of the order of 38,000 daltons and their pI values are in the range of 4.8 to 6.0 (King et al., 1964; 1967a; King, 1972). These 2 antigens represent 6 and 3% of pollen proteins, respectively. Both antigens were found to contain less than 0.6% carbohydrate. In each case 99% of the weight of the sample can be accounted for as common amino acid residues. AgE is very unstable toward physical denaturation, including lyophilization, heating, 8M urea treatment, and exposure to buffers outside the range of pH 6 - 8.5 (King et al., 1964). This instability arises because the AgE molecule is comprised of 2 polypeptides, α (MW 21,800) and β (MW 15,700), held together by non-covalent forces (Griffiths, 1972; King et al., 1974; King, 1974). Under appropriate denaturing conditions the molecule first dissociates into its component chains and subsequently polymerizes, resulting in almost complete loss of biological activity. The AgE molecule is, however, unusually resistant to digestion by trypsin, chymotrypsin, or papain at neutral pH, although it is inactivated by pepsin at pH 2 and by the bacterial protease, Nagarse (King et al., 1967b).

Three other allergenically active proteins designated as Ra3 (Underdown and Goodfriend, 1969), Ra5 (Goodfriend and Lapkoff, 1974; Roebber et al., 1975), and BPA-R or Ra4 (Griffiths and Brunet, 1971; Griffiths, 1972;

Roebber, 1975) have been isolated and characterized from ragweed pollen. They are basic proteins with molecular weights of 11,000, 4,970, and 23,000 daltons, respectively. They represent, respectively, 0.4, 0.1 and 0.9% of proteins extractable from ragweed pollen. Their pI values range from 8.0 to 9.6. Antigens Ra3 and Ra4 may be glycoproteins, as these allergenic preparations contain small amount of carbohydrate. Ra5 does not appear to contain any carbohydrate. Antigen Ra4 was found to share common antigenic determinants with antigen E, but antigens Ra3 and Ra5 are antigenically distinct from each other and from antigen E.

Results on the studies of the allergenic activities of Ra3 (Lichtenstein et al., 1973), Ra4 (Santilli et al., 1975) and Ra5 (Marsh et al., 1973) showed that these antigens were highly allergenic in about 20 - 30% of ragweed sensitive individuals as contrasted to the high activity of antigen E in the majority of the individuals tested (95%). Since a small percentage of sensitive individuals reacted with these basic protein allergens, they are termed minor allergens as contrasted to antigen E which is the major allergen in ragweed pollen.

Allergen Ra5 is of particular importance in our understanding of the allergic response on the basis of genetic background because of its very simple molecular structure (MW 5000; 45 amino acids). The complete amino acid sequence of Ra5 has been elucidated (Mole et al.,

1975). Ra5 exists in two different isoallergenic forms. 30% of the sample of Ra5 sequenced has leucine substituted for valine at position 2. The molecule possesses an unusually large proportion of half-cystine residues (8) which form four disulfide bonds.

The purified ragweed allergens discussed above by no means represent all the allergens in ragweed pollen. There are probably many other allergens in ragweed pollen yet to be identified. It had been observed that during fractionation procedures, allergenic activities were distributed over all the fractions (King and Norman, 1962). It has also been reported (Marsh, 1975) that a brief, 16 minute aqueous extraction of ragweed pollen released less than 1% of the total extractable antigen E. Yet this extract, when expressed in terms of its antigen E content, was found to have a higher allergenic activity than antigen E alone. These results would suggest the presence of other ragweed allergen(s) having higher allergenic activity than antigen E and serve to emphasize the enormity of the problems which can be encountered in the pursuit of pure allergens.

## Grass Pollen Allergens

Out of a total of several thousand grass species, only about 20 produce small wind-distributed pollen in sufficient quantities as to be important in inducing allergy (Wodehouse, 1971). Like ragweed, grass pollens are

antigenically complex and most of them contain at least 14 distinct antigenic components all of which may be regarded as potential allergens (Augustin, 1959a, Feinberg, 1960). Most grass pollen allergens, unlike Antigen E of ragweed, are very stable towards physical denaturation, including lyophilization, heating, 8M urea treatment, and exposure to buffers outside the pH range of 6 - 8.5 (Marsh, 1975).

Timothy Grass Pollen Allergens

Purified allergens from timothy pollen have eluded isolation for many years. Timothy pollen extract was found to contain at least 27 antigens using the technique of crossed immunoelectrophoresis (Løwenstein et al., 1974). Malley and associates isolated two timothy pollen allergens designated as antigens A and B (Malley et al., 1962; Malley and Dobson, 1966). Immunodiffusion analysis revealed that antigens A and B were partially related; AgA was impure and AgB was homogeneous by this criterion. Antigen B consisted of three isoallergenic forms (Marsh, 1974). These two allergens are acidic proteins with molecular weights of about 30,000 and 16,000 daltons respectively (Malley and Harris, 1967; Marsh, 1974). Malley and Harris (1967) also reported the isolation of a low molecular fraction designated AgD which was structurally closely related to AgB and possessed hapten-like activity.

In 1975 Malley and associates (Malley et al., 1975;

1977) reported the isolation of a low molecular weight (MW 1000) antigen $D_3$ from timothy pollen. Antigen $D_3$ was allergenically closely related to AgB. Preliminary characterization suggested that $D_3$ consisted of a flavonoid pigment, quercetin, and a disaccharide of cellobiose linked to threonine. Quercetin was found to represent the major portion of the antigenic determinant of AgB.

Recently three highly purified allergens -allergen no.3,25 and 30 were isolated from timothy pollen by Løwenstein (1978a) by a combinations of ion-exchange and gel chromatography. These allergens were protein in nature and contained less than 2% (W/W) immunochemically detectable impurities. Molecular weights were found to be less than 10,000 for allergens 3 and 15,000 and 34,000 for allergens 25 and 30 respectively; their pI values were 3.9, 4.5 and 9.4 respectively. Allergen 25 was found to be the major component in Malley's AgB. By means of RAST inhibition and prick tests, allergen 25 was found to be the most important allergen of timothy pollen.

Rye Grass Pollen Allergens

Four groups of rye grass allergens designated as Groups I, II, III and IV have been isolated (Johnson and Marsh, 1965; 1966; Marsh et al., 1970; Marsh, 1975). With the exception of Group IV antigen, each one is isolated in multiple electrophoretic forms. Groups I and II are acidic proteins with molecular weights of about

27,000 and 11,000 daltons respectively. Groups III and IV are basic proteins with molecular weights of about 11,000 and 50,000 daltons respectively. These four rye grass allergens have been found to be antigenically and allergenically distinct from one another as shown by immunodiffusion studies with rabbit anti-rye grass sera (Marsh, 1974) and by leukocyte histamine release assays with rye grass sensitive patients (Lichtenstein et al., 1969). Group I antigen was found to be the major allergen of rye grass pollen. Over 95% of 250 rye grass sensitive individuals tested (skin testing and leukocyte histamine release assay) were found to be highly reactive to Group I. The other groups of antigens showed variable responses in individuals sensitive to Group I antigen.

Kentucky Blue Grass (KBG) Pollen Allergens

KBG pollen consists of a complex mixture of allergenic or antigenic components with pI values ranging from 3.0 - 10 as shown by isoelectrofocusing on polyacrylamide gel (Chakrabarty et al., 1978). Antigenically at least 10 different antigens can be identified by crossed immunoelectrophoresis employing sheep precipitating antiserum to KBG pollen (Kum,1979). Allergen A was isolated from the non-dialyzable fraction called the retentate (R) (Kum, 1979) by a reverse immunosorbent procedure employing a monospecific antiserum to one of the antigenic components of R. Allergen A was found to possess 94% of the aller-

genic activity of R as measured by inhibition of PCA assay employing a pool of murine reaginic anti-R sera. It possessed 67% of the allergenic activity of R when measured by the inhibition of RAST method employing a pool of human allergic sera. Allergen A was found to have a molecular weight of the order of 16,500 daltons.

Another allergen referred to as KBG-1 (Ekramouddoullah et al.,1979) was isolated from the dialysate of KBG pollen. It is an acidic protein with a pI value of 4.7 and a molecular weight of the order of 10,000 daltons. It appears to possess all the allergenic activity of KBG pollen as measured by the inhibition of PCA procedures employing murine IgE antibodies, and possess 50% of the allergenic activity as measured by RAST inhibition employing a pool of human allergic sera. The differences in the allergenic specificities that were detected by the PCA and RAST procedures will be discussed in another section (chapters II and III) of this thesis.

## ALLERGENIC VALENCE AND HAPTENS

The bridging of the cell-bound IgE antibodies by means of allergen provides the initial stimulus that triggers a series of biochemical events which culminates in the release of pharmacologically active mediators. An observation by Ovary and Taranta (1963) showed that antigens need to be at least divalent in order to elicit anaphylactic reactions

in guinea pigs. Bridging of the cell-bound IgE antibodies by means of the allergen appears to be pre-requisite in triggering the release of pharmacologically active mediators from mast cells and basophills. This concept has been strongly supported by experimental evidences. Levine (1966) and de Weck (1974) showed that at least two penicilloyl groups were required to elicit the production of anaphylactic reactions in guinea pig and man following previous sensitization with penicillin. Thus, multivalent allergens, divalent haptens and the F(ab')2 dimer of antibodies specific to IgE immunoglobulin which can result in the cross-linking or bridging of the membrane bound IgE molecules can trigger mediator release (Ishizaka et al., 1969; Becker et al., 1973).

On the other hand, whilst univalent haptens can combine with the antibody combining sites on IgE antibodies they are unable to effect the cross-linking or bridging of IgE molecules on the cell membrane. Similarly, while the univalent Fab fragment of an antibody to the Fc portion of IgE can bind to the homologous antigenic determinants, it cannot bridge two adjacent membrane-bound IgE molecules. Consequently, the univalent agents lack the ability to provide the triggering stimulus for mediator release. Furthermore, univalent agents can inhibit the reactions normally elicitable by their multivalent forms. de Weck has applied this principle to prevent anaphylaxis

in penicillin-allergic patients who required prophylactic administration of penicillin (de Weck and Schneider, 1972; de Weck and Girard, 1972). The prior administration of high doses of a monovalent derivative of penicillin, i.e. benzylpenicilloylformyl lysine (BPO-FLYS), precluded bridging of the IgE antibodies specific for penicillin. Consequently, subsequent administration of penicillin did not elicit allergic reactions mediated by IgE antibodies.

Grass pollens have been found to contain components with haptenic activity. Feigen et al.(1967) found that in vitro anaphylactic reactions in sensitized tissues, which were mediated by IgG antibodies, could be inhibited by hapten-like components obtained from ragweed extract. Attallah and Sehon (1969) reported that hapten-like components isolated from short ragweed pollen were not able to elicit skin reactions in allergic patients who reacted strongly to the aqueous extract of ragweed pollen. The ragweed haptens were also able to inhibit the Prausnitz-Küstner (P-K) transfer test in normal volunteers sensitized with sera from ragweed sensitive individuals. Using an animal model system, Malley and Harris (1967) demonstrated that hapten-like components isolated from the dialyzable fraction of timothy grass pollen were capable of blocking PCA reactions and systemic anaphylaxis normally inducible in appropriately sensitized guinea pigs on challenge with the non-dialyzable, i.e. multivalent timothy grass pollen allergens. These haptenic materials could also inhibit

the in vitro release of histamine from monkey lung tissue passively sensitized with anti-timothy antibodies following challenge with the crude timothy pollen extract. Malley and his associates (1971) also showed that suppression of human IgE formation was achieved with the concomitant clinical improvement by treatment of patients allergic to timothy grass pollen over a period of two years with a low molecular weight hapten-like fraction of the pollen extract. Moreover, they demonstrated that the peripheral leucocytes of patients so treated, by contrast with the cells of patients who had received hyposensitization therapy with the conventional whole water soluble extract of the pollen, lost their ability to undergo blast transformation and to take up tritiated thymidine when cultured in vitro with the regular whole water soluble extract of timothy grass pollen. From these results, it was concluded that interaction of the monovalent fraction with the receptors of IgE forming cells had prevented the reaction of these cells with the polyvalent constituents of timothy grass pollen leading to the eventual suppression of reagin formation. Maintenance of the anergic state of the patients, who had undergone a complete season of immunotherapy with the hapten-like fraction, was achieved with as few as four injections per year.

On the basis of the observations that haptens are unable to elicit allergic or anaphylactic reactions and they are capable of inhibiting these reactions, which are

normally elicitable with the multivalent allergens, it would appear that haptenic components could provide protection from allergic reactions.

## RATIONALE FOR PURIFYING ALLERGENS

Purified allergens are essential for the standardization of allergenic extract used in immunotherapy. At the present day, information on commercial allergenic extracts is inadequate. This information such as weight: volume ratio or the protein nitrogen content (PNU), or the total nitrogen content do not correlate with biological activity (Baer et al., 1970). Commercial extracts which have the same protein nitrogen content or weight: volume ratio could differ hundreds or even thousands folds in their biological activity. If pure allergens are isolated, we can use these allergens to standardize allergenic extracts - for example AgE has been identified as the major allergen in ragweed; the measure of AgE has been proposed as a method to standardize ragweed pollen extract (King et al., 1964; Baer et al., 1970).

Pure allergens, e.g. Ra5 isolated from ragweed, will facilitate studies on the immunogenetic basis for the immune recognition of allergenic determinant on the allergen molecule (Marsh, 1975). They will allow us to understand the physical and chemical nature of these allergenic determinants and may enable us to modify the

allergenic determinants in such a way as to reduce the incidence of anaphylactic shock when used in immunotherapy (Marsh, 1975). Pure allergens will also facilitate our understanding of the molecular basis of interaction between allergen and IgE antibodies on the membrane of basophilic leukocytes and mast cells; this will allow us to understand the way in which the cell receives the signal to release pharmacologically active substances such as histamine (Marsh, 1975).

At the present day almost all commercial allergenic extracts for clinical use are crude, heterogeneous mixtures with all the extractable components of the original material. The crude extract might contain materials which are not allergenic or might even be toxic. Hyposensitization treatment with the crude allergenic extract may result in the sensitization of the patients to some component(s) of the extract to which they may not have been sensitive before treatment. If allergens in an extract are characterized and purified, the ideal situation would be to treat the patients with only the components that they are allergic to.

Recently, abrogation of the ongoing reaginic responses to haptens, such as the benzylpenicilloyl and 2,4-dinitrophenyl groups in experimental animals (mice, rats and dogs), have also been achieved by the injection of conjugates of the appropriate hapten with nonimmunogenic carriers, such as isologous γ-globulins (Lee and Sehon,

1975), polyvinyl alcohol (Lee and Sehon, 1979) or the synthetic copolymer of D-glutamic acid and D-lysine (Katz et al., 1973; Tse et al., 1978). Clearly, this therapeutic modality could be extended to a wide spectrum of allergies on condition that the determinants of the appropriate allergens were available in the form of compounds which could be incorporated into tolerogenic conjugates. In essence, this approach would involve the establishment of the chemical structure of the relevant allergenic determinants and the subsequent synthesis of the appropriate compounds, as has been achieved, for example, for the determinants of sperm whale myoglobin (Atassi, 1977).

# CHAPTER II

## FRACTIONATION OF AQUEOUS EXTRACT OF KENTUCKY BLUE GRASS POLLEN: ISOLATION OF HAPTEN-LIKE COMPONENTS

### INTRODUCTION

Preliminary investigation revealed that the aqueous extract of Kentucky Blue Grass pollen (KBG aq. ext.) contained components which were separable by analytical isoelectrofocusing (Anal-IS0-EF) on polyacrylamide gel on a pH gradient established with ampholine in the pH range of 3.5 - 10. 30 - 40 components were detected over this pH range. Attempts to fractionate KBG aq. ext.by gel filtration or ion-exchange chromatography were not successful i.e. poor resolution of the eluates were obtained. Eluted fractions were found to contain many components when analyzed by Anal-IS0-EF or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Since the components of KBG aq. ext were found to be very well separated by Anal-IS0-EF i.e. sharply focused discrete bands were obtained on the analytical gel, it was decided to fractionate KBG aq. ext. employing preparative isoelectrofocusing.

Fractions with allergenic and hapten-like properties were isolated from KBG aq. ext. This chapter describes

the fractionation of KBG aq. ext. and the properties of the isolated hapten-like fractions.

## METHODS AND MATERIALS

### Preparation of KBG aq. ext.

An aqueous extract of KBG pollen (Hollister-Stier Laboratory, Mississauga, Ontario, Canada) was prepared according to procedures previously established in this laboratory (Ekramouddoullah et al., 1977a; 1977b). 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 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.

### Preparation of Dialyzable and Non-dialyzable Fractions of KBG aq. ext.

The non-dialyzable and dialyzable constituents were obtained according to procedures previously established in

this laboratory (Ekramouddoullah et al., 1977a; 1977b). KBG aq. ext. was dialyzed through Visking tubing, No. 20 (Union Carbide Canada Ltd., Lindsay, Ontario, Canada) against distilled water at 4°C. The dialysate obtained after dialysis of KBG aq. ext. for a period of 24 hrs. is referred to as $D_{24}$; the fraction obtained following further dialysis of KBG aq. ext. for an additional 24 hrs. is referred to as $D_{48}$ and the non-dialyzable components are referred to as the retentate (R). All fractions were stored in the lyophilized state.

Determination of Protein Concentration

The procedure of Lowry et al. (1951) was employed. Unless stated otherwise, amounts of concentrations are expressed on the basis of protein content. Samples containing 10 - 40 µg protein were diluted to a volume of 0.6 ml with water. Three ml of Reagent C* was added. The solution was mixed and allowed to stand at room temperature for 10 minutes. For color development 0.3 ml of 1N Folin-Ciocalteau Phenol reagent was added with immediate and rapid mixing. After standing for 30 minutes at room temperature the optical densities (O.D.) of the samples at 690 nm were measured in a Coleman UV-VIS spectrophotometer. A standard curve for protein concentration versus O.D. was obtained

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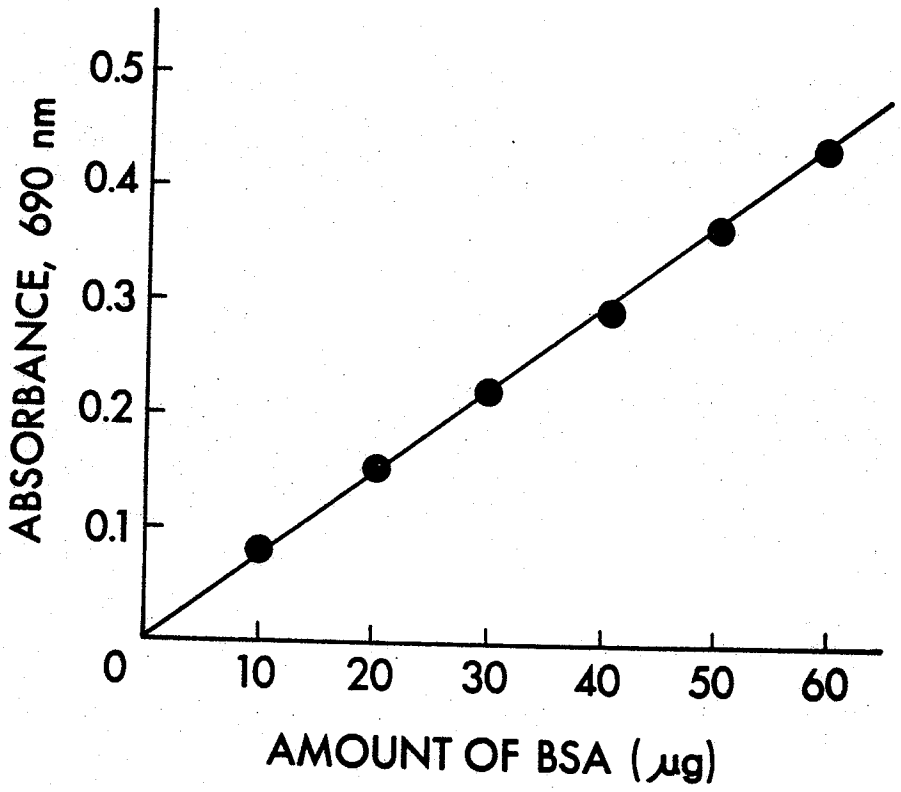
* Reagent C.....0.5 ml 0.5% $CuSO_4 \cdot 5H_2O$ in water plus 0.5 ml 1.0% $Na_2$ or K tartrate in water plus 50 ml 2.0% $Na_2CO_3$ in 0.1N NaOH

using Bovine serum albumin (Figure 1).

Analytical Isoelectrofocusing (Anal-ISO-EF) (Vesterberg, 1972; 1973.)

This analytical technique was performed on polyacrylamide gel rods as follows: a concentrated gel mixture was prepared by mixing 30 ml of 28% acrylamide (Bio-Rad Laboratories, Richmond, California) containing 0.735% bisacrylamide (Bio-Rad), with a volume of 8 ml of a solution of TEMED; 1%, (N:N:N'-N'-Tetramethylenediamine, Bio-Rad), and 3 ml of ampholine, (40%, pH range 3.5 - 10, LKB-Producter AB, Stockholm-Brommal, Sweden). A volume of 0.75 ml of the concentrated gel mixture was then mixed with a volume of 1.35 ml of an aqueous solution of ammonium persulfate, 0.1% (Fisher Scientific Co., Fairhaven, N.J.) containing sucrose at a concentration of 19%, and a volume of 0.1 ml containing the sample (100 - 500 µg protein). This mixture was immediately pipetted into glass tubes (0.6 x 12.8 cm) and allowed to gel at room temperature for 30 mins. The Bio-Rad electrophoresis cell (model 300A) was employed, with a solution of 0.1 N $H_2SO_4$ at the anode and a solution of 0.1 N NaOH was used at the cathode. In order to achieve a consistent high resolution and reproducible separation a pulsed constant power supply (ORTEC 4100, ORTEC Incorp., Oak Ridge, Tennessee) and a flow of cold tap water (9$^{o}$C) surrounding the gel tubes were employed to reduce heat created during focusing. The

Figure 1. Reference curve for the determination of protein content by the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used for the standardization.

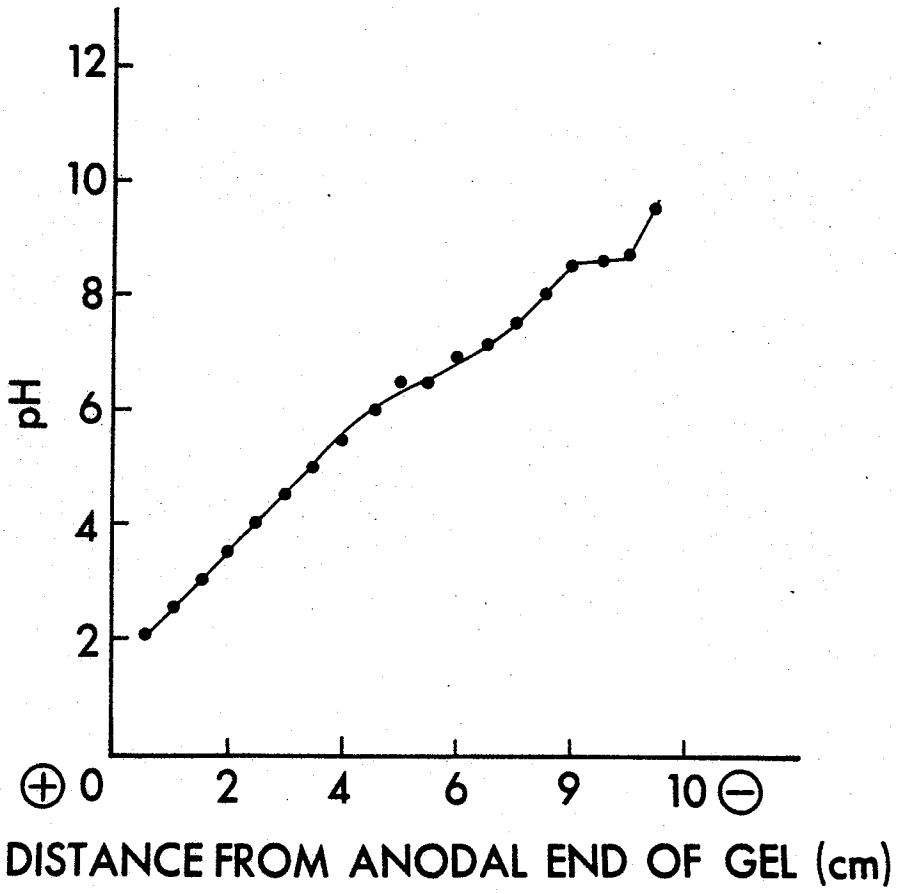

ABSORBANCE, 690 nm
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
AMOUNT OF BSA (μg)

Anal-ISO-EF was carried out at a potential of 350 volts, D.C., with a discharge capacitance of 0.5 μ Farads at an initial frequency of 50 pulses/sec. for the first hour, then raised to 100 pulses/sec. for the second hour and continued at 200 pulses/sec. for 22 hrs.

At the completion of the electrofocusing, the gels were removed from the tubes and fixed in a solution of 10% trichloroacetic acid (TCA) for 1 hr. Following a brief washing with distilled water to remove the major portion of the TCA, staining was accomplished by immersing the fixed gels for 2-3 hrs., in a solution of Coomassie blue R250 (AMES Company, Division Miles Laboratories, Inc., Elkhart, Indiana) at a concentration of 0.2% (w/v) dissolved in a mixture of ethanol, water and glacial acetic acid in a ratio of 45:50:5 (v/v). The gels were destained in an aqueous solution of ethanol, water and glacial acetic acid in a ratio of 40:55:5 for 12 hrs. and then further destained in a diffusion destainer (Bio-Rad, Model 172) with 7% acetic acid in the presence of activated charcoal as adsorbant. The pH gradient established in the gel was determined by extracting a focused gel from the glass tube, cutting it into 0.5 cm lengths, adding a volume of 0.2 ml of distilled water and measuring the pH. The pH gradient of the analytical gel was illustrated in Figure 2.

Figure 2. pH gradient of the Anal-ISO-EF polyacrylamide gel established with ampholine (pH range 3.5 - 10) after electrofocusing for a period of 24 hours.

(+) = Anode
(-) = Cathode


12
10
8
pH 6
4
2
⊕ 0
2
4
6
9
10 ⊖
DISTANCE FROM ANODAL END OF GEL (cm)

Preparative Isoelectrofocusing (Prep-ISO-EF)

The method described by Radola (1973) was adapted for the Prep-ISO-EF fractionation of R, $D_{24}$ and $D_{48}$. An amount of 11.5 g of Sephadex G-100 superfine (Pharmacia, Uppsala, Sweden) was rehydrated in a volume of 200 ml of distilled water. A volume of 7.5 ml of a stock solution of ampholine (40%, pH 3.5 - 10; LKB, Stockholm, Sweden) was added to the gel and the suspension was deaerated under vacuum. This gel was poured onto a level glass trough (20 x 40 x 1 cm), spread uniformly to a depth of approximately 2 mm and dried to the extent of a weight loss of 25 - 30%. The gel was considered as suitable for use when it did not slide when the glass trough was tilted at an angle of 45 degrees. Adherence to this drying procedure was important for achieving uniform and reproducible separations.

An amount of 500 mg of R, $D_{24}$ or $D_{48}$ dissolved in a volume of 2 ml of distilled water was applied dropwise over the middle area of the gel along a direction transverse to that of electrofocusing; a margin of 0.5 cm wide along the sides of the gel was kept free of the sample. The gel with the applied sample was allowed to dry for an additional 5 mins. before the glass trough was placed on the precooled metal block of the electrophoresis chamber (Desaga, Heidelberg, West Germany). Cold tap water (9$^{o}$C) was circulated through the cooling block. Electrical connection

was made with the gel at both ends of the trough by means of cellulose acetate bridges moistened with a 0.2 M aqueous solution of $H_2SO_4$ for the anode and a 0.4 M aqueous solution of ethylenediamine for the cathode. Platinum electrodes were laid on top of the membrane bridges and the Prep-ISO-EF was carried out at a constant voltage setting (Brinkman, Voltage and Current Regulated Power Supply, Brinkman, Rexdale, Ontario) starting at 50 V/cm for the first day, 100 V/cm for the second day and 125 V/cm for the third day. The electrofocusing was considered to be completed after a period of 72 hrs., since by that time the current had reached a constant lowest level attained during the focusing.

The gel was divided transversely into 15 equal sections which were separately removed from the glass trough. A volume of 5 ml of distilled water was added to each separate section and the pH of the suspension was measured (pH meter E300B and single electrode EA147 Metrohm, Herisau, Switzerland). The gel sections were each extracted with a volume of 15 ml of distilled water and the Sephadex gel was removed by filtration through filter paper and the ampholine was removed by dialysis against water. Visking tubing was used for the dialysis of the focused components of R. Spectrapor membrane (Spectrum Medical Industries,

Inc., Los Angeles, molecular size cut-off 3,500), which was found to be permeable to the ampholine, was used for the dialysis of the focused components of $D_{24}$ and $D_{48}$. All fractions were lyophilized and stored in the dry state. For simplicity, the term "focused fraction" will be used in reference to the component(s) extracted from a 2.5 cm segment of the gel.

Preparation of Murine Reaginic Antisera

Male A/HeJ mice (Jackson Laboratories, Bar Harbor, Maine), 6-7 weeks of age, were used for the production of reaginic antibodies. Each mouse was immunized with R at a dose of 100 µg (dry weight) adsorbed onto 5 mg of $Al(OH)_3$ gel administered i.p. One hundred days later, at a time when the anti-R IgE antibody titer was at or near the maximum level (Ekramouddoullah et al., 1977a; 1977b), the mice were bled from the tail. The sera were pooled and stored in the frozen state.

Passive Cutaneous Anaphylaxis (PCA)

The levels of the murine anti-R IgE antibodies were measured by PCA analysis in rats. Random bred hooded rats (North American Laboratory Supply, Co., Gunton, Manitoba, Canada) were passively sensitized by intradermal injections of volumes of 50 µl of the serially diluted murine reaginic anti-R sera. At least two animals were sensitized with

the sera diluted identically. The PCA reactions were elicited 24 hrs. later by challenging these sensitized animals with an intravenous injection of a volume of 1 ml of a solution containing an amount of 1 mg of R (dry weight) and Evan's blue dye (0.25%)(Matheson, Coleman & Bell, Norwood, Ohio). The end point of the titration was expressed as the reciprocal of the highest dilution of the reaginic sera capable of sensitizing a skin site so as to give a PCA reaction of 5 mm in diameter or greater. In most cases the values for the PCA titers of a murine reaginic anti-R sera determined in duplicate were identical; when variations were seen, the titers did not differ from each other by more than one serial dilution. Similarly, using the same reaginic antisera the titers elicited in experiments on different occasions were highly reproducible.

The PCA procedure was employed to examine for the allergenic activity of the focused fractions. For this purpose, rats were passively sensitized and challenged as described, with an amount of 1 mg of a focused fraction. The assessment of allergenic potency was made on the basis of the PCA titer elicited.

Allergenic Composition of Focused Fractions Relative to Allergens Present in Retentate

To establish the allergenic composition of each focused fraction of R, $D_{24}$ and $D_{48}$, relative to the allergens

present in the unfractionated R, the procedure of neutralization of PCA was employed. The fractions, in different amounts, were mixed with the murine reaginic anti-R antisera* such that the final antisera dilution was 1:320. This dilution of antisera was chosen so as to minimize the amount of each focused fraction required to potentially neutralize the anti-R antibodies (the PCA titer of the murine reaginic anti-R sera as determined by titration in rats was 1,280). The mixtures were incubated for a period of 2-3 hrs. at room temperature prior to passive sensitization of hooded rats by the intradermal injection of 50 µl of the mixture. Twenty-four hours later the PCA reactions, if any, were elicited in these rats by intravenous (i.v.) challenge with a solution of R (1 mg dry weight) together with Evan's blue dye (final concentration 0.25% in saline). A PCA reaction was considered positive when the extent of the extravasation of the dye as measured on the underside of the skin was 5 mm in diameter or greater.

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* The reaginic anti-R sera was used to evaluate the allergenic activity not only of the focused fractions of R, but also of focused fractions of $D_{24}$ and $D_{48}$ for the following reasons: (i) the murine anti-R sera recognized all of the allergenic specificities present also in D (the combined fractions of $D_{24}$ and $D_{48}$) (Ekramouddoullah et al., 1977a; 1977b); (ii) higher PCA titers were elicited with the anti-R sera on challenge with either D or R in comparison with the titers elicited with reaginic anti-D sera; (iii) high-titered reaginic antisera were able to detect lower levels of allergenic activity as compared to the levels detectable with a low-titered reaginic antisera.

Radioallergosorbent Test (RAST)

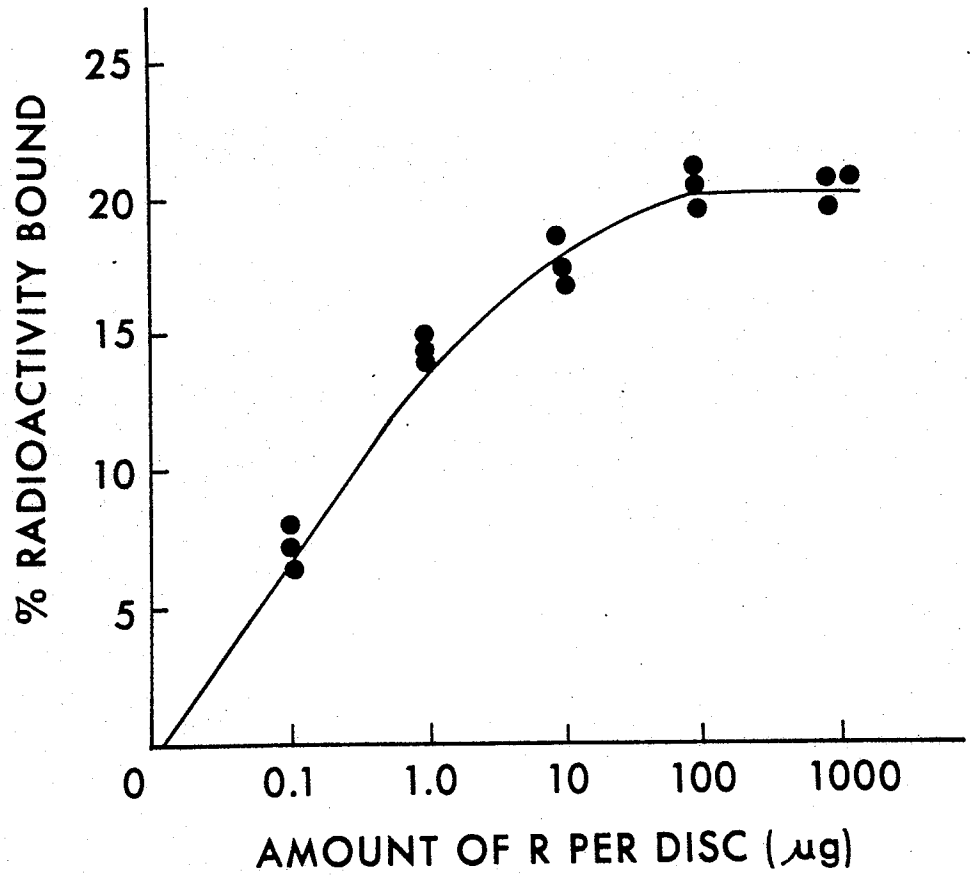
The capacity of the focused fractions to combine with human IgE antibodies present in a pool of sera obtained from individuals allergic to KBG pollen, was established by the radioimmunoassay method described by Ceska et al. (1972). R was insolubilized by attachment through covalent bonds to cellulose discs activated by cyanogen bromide. The allergosorbent discs were reacted with a solution of β-ethanolamine to block any remaining activated sites on the cellulose to which constituents of R had not become attached. The discs were incubated with aliquots of the pool of human allergic sera obtained from individuals allergic to KBG pollen. This pool of allergic sera was used for all the RAST experiments in this thesis.

The optimum amount of R used for preparation of the allergosorbent disc which would bind the maximum quantity of IgE antibodies present in a volume of 50 µl of the pool of human allergic sera, was established by varying the amount of R incubated with the activated cellulose disc. This amount was found to be 100 µg (Figure 3). The extent to which the IgE antibodies bound to the insolubilized allergens was evaluated by the addition of a preparation of $I^{125}$-labelled rabbit antibodies specific for human IgE immunoglobulin (Pharmacia, Uppsala, Sweden), such

Figure 3. Effects of increasing the amount of R for preparation of allergosorbent discs used to absorb a constant volume (50 µl) of a pool of sera from patients allergic to KBG pollen.

$$\% \text{ radioactivity bound} = \frac{\text{cpm bound to discs}}{\text{cpm added to discs}} \times 100\%$$

RAST analysis was performed in triplicate.


% RADIOACTIVITY BOUND
25
20
15
10
5
0
0.1
1.0
10
100
1000
AMOUNT OF R PER DISC (μg)

that approximately 25% of the added radioactivity was bound to the allergosorbent-antibody complexes. The levels of $I^{125}$ were measured with a Beckman Gamma 300 system counter. Since maximal binding of IgE antibodies to the allergosorbent occurred when an amount of 100 µg of R was coupled to an activated disc, this amount of R was used for the preparation of each allergosorbent disc in the RAST experiments described. Each RAST was performed in triplicate. To determine the level of nonspecific binding to the cellulose discs by the human allergic sera and/or the radiolabelled antibodies to IgE a "blank" disc was used, i.e. an activated cellulose disc was processed in an identical manner as described for the preparation and use of the allergosorbent disc except that R was omitted during the coupling reaction. The level of the radioactivity associated with the blank disc was subtracted from all the RAST values given in this thesis.

The allergenic or haptenic potency of the focused fractions was evaluated in terms of their ability to neutralize IgE antibodies present in the human allergic sera and to inhibit their binding to the allergosorbent discs. For this purpose, an amount of 100 µg of each focused fraction was incubated with a volume of 50 µl of the pool of human allergic sera for 3 hrs. at room temperature prior to use in the RAST procedure. The capacity (expressed as

a percent) of each fraction to inhibit RAST was calculated according to the formula: $\frac{A-B}{A} \times 100$, where A = RAST value (cpm) in absence of fraction, and B = RAST value (cpm) in presence of fraction.

## RESULTS

### Prep-ISO-EF of R, $D_{24}$ and $D_{48}$

The pH gradient established with ampholine in the pH range 3.5 - 10 and the profiles of the distribution of protein following Prep-ISO-EF of R, $D_{24}$ and $D_{48}$ are illustrated in Figure 4. Approximately 70% of the amount of R applied was recovered in the focused fractions. A recovery of the order of 30% and 20% of the total material of $D_{24}$ and $D_{48}$ focused respectively was achieved. This yield represents essentially a quantitative recovery of focused components of $D_{24}$ and $D_{48}$, since it had been previously determined that over 3/4 of the components of unfractionated $D_{24}$ and $D_{48}$ readily pass through Spectrapor membrane (i.e. MW. < 3,500) and are lost in the dialysis fluid.

### Anal-ISO-EF

On Anal-ISO-EF of R, the presence of over 30 components differing in their pI values could be visualized on the stained gel (Figure 5). It was observed that many focused fractions, notably 2, 3, 4 of R and 4, 5 of $D_{24}$ and $D_{48}$, possessed only 1-2 components which focused at the anodal

Figure 4. Prep-ISO-EF in Sephadex G-100 gel employing ampholine in the pH range of 3.5 - 10. The fraction number refers to 2.5 cm sections of gel eluted after focusing, with number 1 starting at the anode. The results of fractionation of R, $D_{24}$ and $D_{48}$ are given in A and D, B and E, C and F respectively. The capacity of each fraction, in an amount of 100 µg, to inhibit RAST is represented by open bars and was obtained according to the formula indicated in the text. The protein content of the fractions are represented by solid bars.

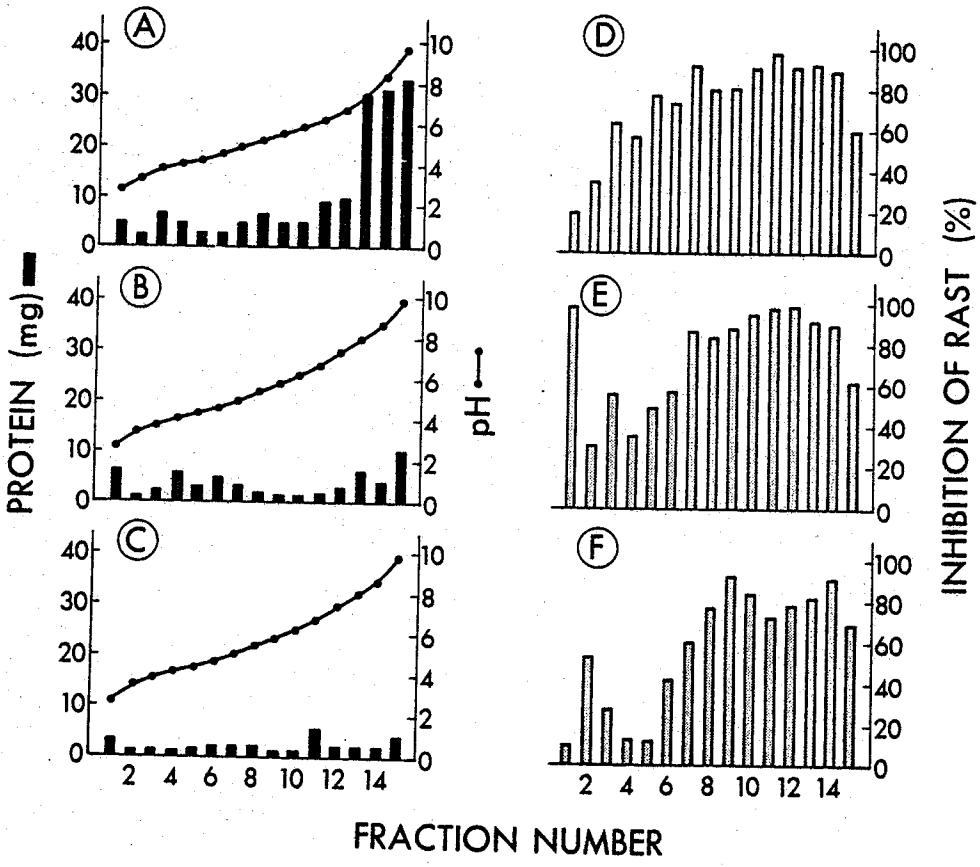
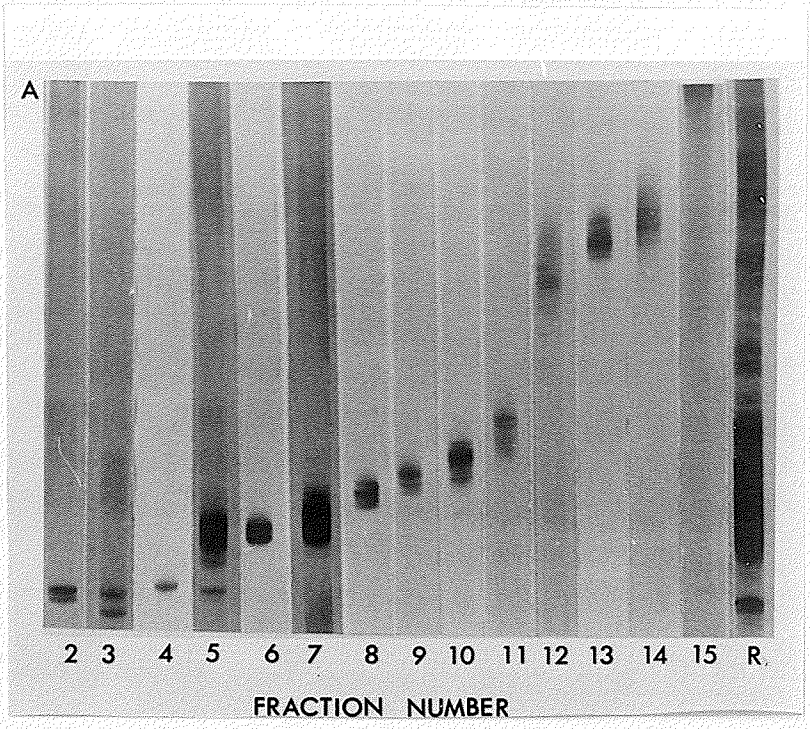
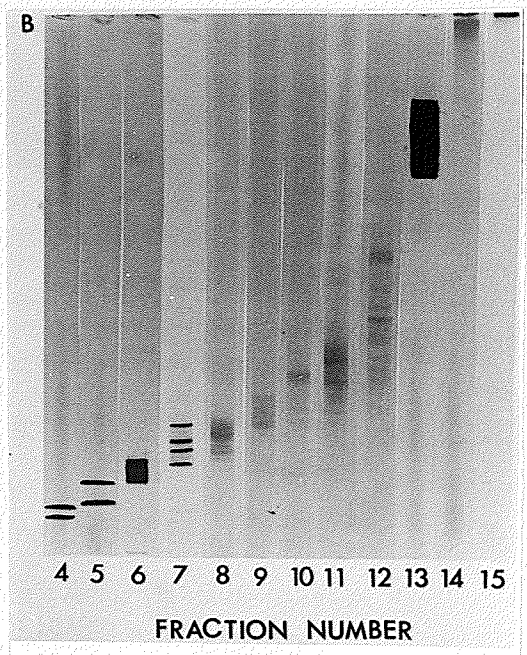
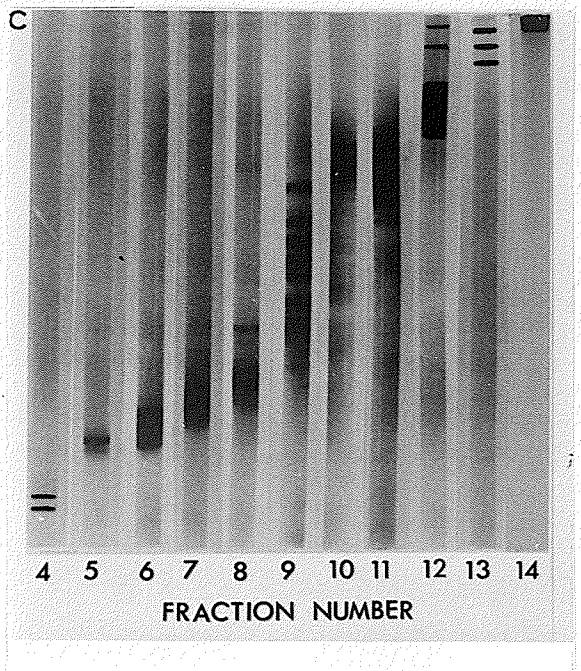

A
B
C
D
E
F
PROTEIN (mg)
pH
INHIBITION OF RAST (%)
FRACTION NUMBER
0
10
20
30
40
2
4
6
8
10
12
14
100
80
60
40
20

Figure 5. Anal-ISO-EF of focused fractions. Anal-ISO-EF on polyacrylamide gel (employing ampholine in the pH range 3.5 - 10) of focused fractions obtained by Prep-ISO-EF of R, $D_{24}$ and $D_{48}$ are illustrated in A, B and C respectively. The numbers refer to fractions eluted from 2.5 cm sections of the preparative gel, with fraction 1 starting from the anodal end. Some stained bands which did not show well were darkened on the photographic print for ease of their visualization. Gels containing fractions which did not stain with the Coomassie blue dye are not shown. The anodal end of the gels is at the bottom. The isoelectrofocused profile of R is shown in Fig. 5A.


A
2 3 4 5 6 7 8 9 10 11 12 13 14 15 R
FRACTION NUMBER


B
4 5 6 7 8 9 10 11 12 13 14 15
FRACTION NUMBER


C
4 5 6 7 8 9 10 11 12 13 14
FRACTION NUMBER

end of the gel. Focused fractions 15 of R, $D_{24}$ and $D_{48}$ possessed component(s) which focussed at the top of the cathodal end of the gel. Any components with pI values higher than 10 or lower than 2 could have migrated out of the analytical gel and consequently were not visualized. If more than one component was present in a focused fraction of R, it was observed that they focussed within a small section (i.e. narrow pH gradient) of the analytical gel. On this basis, it can be suggested that focused fractions of R possessed components with limited differences in their net charge. By comparison, many of the focused fractions of $D_{24}$ and $D_{48}$ (fractions 11, 12 of $D_{24}$ and 9, 10, 11 of $D_{48}$) had components which focused over a wider pH range (Figure 5) and therefore it appeared that they were electrophoretically more heterogeneous than the corresponding fractions of R. The focusing of components over a wider pH range for some of the focused fractions could be due to degradation during the processing of these fractions. Focused fractions that stained poorly were not illustrated. Since a protein stain was employed, poor staining could be attributed to low protein, high carbohydrate content, or low molecular size of these fractions which fixed poorly to the gels.

Evaluation of the Allergenicity of the Focused Fractions

(a) Inhibition of RAST: The capacities of the focused fractions of R, $D_{24}$ and $D_{48}$ to inhibit RAST are illustrated in Figure 4. The extent of the inhibition, evaluated with an amount of 100 µg of each focused fraction, ranged from 8 to 100%.

(b) Elicitation of PCA: Not all the focused fractions could elicit the PCA reaction in rats passively sensitized intradermally with a murine reaginic antisera to R (Table I). Focused fractions 1, 2, 3, 4 of R and $D_{48}$ and focused fractions 1, 2, 3, 4, 5, 15 of $D_{24}$ could not elicit the PCA reactions even though these focused fractions were used at a dose as high as 1 mg for the intravenous challenge. By comparison, a challenging dose of 50 µg of R was still sufficient to elicit the PCA reactions and to establish the maximum titer of the reaginic antisera to R as 640, i.e. further increasing the challenging dose of R did not result in any increase in this PCA titer. Most of the focused fractions of R and $D_{48}$ at a dose of 1 mg were capable of eliciting PCA titers comparable to that achieved with an amount of 1 mg of unfractionated R. On the other hand, many of the focused fractions of $D_{24}$ elicited PCA titers that were lower than the maximum attainable with R.

(c) Inhibition of PCA: The concentrations of the focused fractions needed to achieve complete inhibition of the PCA reactions varied (Table II). Under identical experimental conditions, R at a concentration of 50 µg/ml, completely inhibited the PCA reactions. Thus, it can be seen from the results given in Table II, that many of the focused fractions were as potent as R in neutralizing the anti-R IgE antibodies.

TABLE I

EVALUATION OF ABILITY OF FOCUSED FRACTIONS TO ELICIT PCA REACTIONS IN RATS PASSIVELY SENSITIZED WITH A MURINE REAGINIC ANTISERA TO R

| FOCUSED FRACTION NUMBER[1] | PCA TITER[2] ON CHALLENGE WITH FOCUSED FRACTIONS ISOLATED FROM | | |
|---|---|---|---|
| | R | $D_{24}$ | $D_{48}$ |
| 1 | NIL | NIL | NIL |
| 2 | NIL | NIL | NIL |
| 3 | NIL | NIL | NIL |
| 4 | NIL | NIL | NIL |
| 5 | 160 | NIL | 320 |
| 6 | 320 | 20 | 640 |
| 7 | 640 | 40 | 640 |
| 8 | 640 | 20 | 640 |
| 9 | 640 | 320 | 640 |
| 10 | 640 | 320 | 640 |
| 11 | 640 | 640 | 640 |
| 12 | 640 | 160 | 640 |
| 13 | 640 | 320 | 640 |
| 14 | 640 | 640 | 640 |
| 15 | 640 | NIL | 640 |

1 The numbers refer to fractions extracted from 2.5 cm sections of the gel following preparative isoelectrofocusing, with number 1 starting at the anodal end.

2 Hooded rats were passively sensitized with 50 µl of a serially diluted murine anti-R reaginic sera. Twenty-four hrs. later, two of the rats sensitized in an identical manner, were challenged with one of the fractions at a dose of 1 mg in 0.25% Evan's blue dye, injected intravenously. The PCA titer of the murine reaginic antisera, elicited with R at a dose of 50 µg or 1 mg (dry weight) was 640.

TABLE II

EVALUATION OF THE ABILITY OF FOCUSED FRACTIONS OF R, $D_{24}$ and $D_{48}$ TO NEUTRALIZE REAGINIC ANTI-R ANTIBODIES[2]

| FOCUSED FRACTION NUMBER[1] | CONCENTRATION OF FOCUSED FRACTIONS OF R, $D_{24}$ and $D_{48}$ INCUBATED WITH MURINE REAGINIC ANTI-R SERA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 µg/ml | | | 50 µg/ml | | | 500 µg/ml | | | 1 mg/ml | | |
| | FOCUSED FRACTIONS OF | | | | | | | | | | | |
| | R | $D_{24}$ | $D_{48}$ | R | $D_{24}$ | $D_{48}$ | R | $D_{24}$ | $D_{48}$ | R | $D_{24}$ | $D_{48}$ |
| 1 | + | + | + | + | - | + | - | - | - | - | - | - |
| 2 | + | + | + | + | - | + | ± | - | - | - | - | - |
| 3 | + | + | + | + | - | + | ± | - | - | - | - | - |
| 4 | + | + | + | + | - | + | ± | - | - | - | - | - |
| 5 | + | + | + | + | - | + | - | - | - | - | - | - |
| 6 | + | + | + | + | - | + | - | - | - | - | - | - |
| 7 | + | + | + | - | - | - | - | - | - | - | - | - |
| 8 | + | + | + | - | - | - | - | - | - | - | - | - |
| 9 | + | + | + | - | - | - | - | - | - | - | - | - |
| 10 | + | + | + | - | - | - | - | - | - | - | - | - |
| 11 | + | + | + | - | - | - | - | - | - | - | - | - |
| 12 | + | + | + | - | - | - | - | - | - | - | - | - |
| 13 | + | + | + | - | - | - | - | - | - | - | - | - |
| 14 | + | + | + | ± | - | - | - | - | - | - | - | - |
| 15 | + | + | + | + | + | + | ± | - | - | - | - | - |

The procedure for the neutralization is described in the text.

Reactions $\geq$ 5 mm in diameter are indicated as +.
Reactions < 5 mm in diameter are indicated as ±.
Complete absence of reaction is indicated as -.

1 The numbers refer to fractions extracted from 2.5 cm sections of the gel following preparative isoelectrofocusing, with number 1 starting at the anodal end.

2 The PCA titer of the murine anti-R sera established in rats using R at a dose of 1 mg (dry weight) was 1,280. For the neutralization experiments, this antisera was used at a final dilution of 1:320.

The focused fractions, referred to in the preceding section, which were unable to elicit PCA reactions, were found to be capable of inhibiting completely the PCA reactions normally elicitable with the R-anti-R reaginic system. Therefore, it can be suggested that fractions which could not elicit PCA reactions, but which could combine with and neutralize the IgE-anti-R antibodies, possessed hapten-like activity. To examine for the specificity of the inhibition, each of these hapten-like fractions was tested for its ability to inhibit PCA reactions of allergen-IgE antibody systems unrelated to the anti-R system. It was found (Table III) that none of the hapten-like fractions had any detectable effect on the PCA reactions elicited either with the ovalbumin-anti-ovalbumin system or with the dinitrophenyl (DNP)-anti-DNP system. It was concluded that the hapten-like components isolated from both the dialyzable ($D_{24}$, $D_{48}$) and non-dialyzable (R) preparations of KBG aq. ext. were capable of specifically combining with the IgE anti-R antibodies and inhibiting the PCA reactions on subsequent challenge with R.

(d) In Vivo Neutralization of PCA by Hapten-Like Fractions: In view of the ability of haptens to block allergic reactions, the potential of administering haptens in vivo for similar purposes was investigated. The fractions with hapten-like activity were tested for their

TABLE III

SPECIFICITY OF INHIBITION OF PCA REACTIONS

BY FOCUSED FRACTIONS WITH HAPTEN-LIKE ACTIVITY

| FRACTION NUMBER[1] | ALLERGEN-REAGINIC ANTISERA SYSTEM | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | R-Anti-R | | | OA-Anti-OA | | | DNP-Anti-DNP | | |
| | FOCUSSED FRACTIONS OF | | | | | | | | |
| | R | $D_{24}$ | $D_{48}$ | R | $D_{24}$ | $D_{48}$ | R | $R_{24}$ | $R_{48}$ |
| 1 | - | - | - | + | + | + | + | + | + |
| 2 | - | - | - | + | + | + | + | + | + |
| 3 | - | - | - | + | + | + | + | + | + |
| 4 | - | - | - | + | + | + | + | + | + |
| 5 | N.A. | - | N.A. | N.A. | + | N.A. | N.A. | + | N.A. |
| 15 | N.A. | - | N.A. | N.A. | + | N.A. | N.A. | + | N.A. |
| control* | positive | | | positive | | | positive | | |

[1] The numbers refer to fractions extracted from 2.5 cm sections of the gel following preparative isoelectrofocusing, with number 1 starting at the anodal end.

The haptenic fractions were tested for their ability to inhibit murine IgE antibodies specific to (i) R; (ii) ovalbumin; (iii) dinitrophenol (DNP). PCA titers, of the three respective murine reaginic antisera established with the homologous allergens was 1,280 in each case. Each fraction at a concentration of 1 mg/ml was mixed with each of the murine reaginic antisera at a final dilution of 1:640 and incubated for 2-3 hours at room temperature. A volume of 50 µl of each of the mixtures were prepared with the anti-R and anti-OA reaginic sera was used for the passive intradermal sensitization of rats in duplicate (group 1). Another group of rats was sensitized in an identical manner with mixtures prepared with the murine anti-R and anti-DNP reaginic sera (group 2). Twenty-four hours later, the rats in group 1 were challenged intravenously with a solution (1 ml) containing 500 µg R, 500 µg OA (dry weight) and Evan's blue dye (0.25%). Group 2 was similarly challenged with a solution containing 500 µg R, 500 µg (dry weight) of the conjugate DNP-normal mouse sera and Evan's blue dye. Since the results of the inhibition experiment with the R-anti-R system performed in group 1 and 2 were identical only one set of results is given in column 1.

continued TABLE III

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+ refers to a positive PCA reaction.
- refers to an absence of PCA reaction.
N.A. not applicable.

For control, all 3 reaginic antisera were similarly incubated with saline instead of the fraction.

* Skin sites sensitized with the mixtures of the murine reaginic antisera and saline, i.e. in the absence of the haptenic fractions, all displayed PCA reactions on challenge with the homologous allergens (indicated as positive).

ability to combine with murine anti-R IgE antibodies present in skin sites of rats passively sensitized by intradermal injections of a murine reaginic antisera to R. Intravenous administration of an amount of 1 mg of the hapten-like fractions into the rats sensitized with intradermal injections of serially diluted reaginic anti-R sera prior to challenge with R, resulted in inhibition to different degrees, of the PCA reactions normally elicitable with R (Table IV). Complete in vivo inhibition of the PCA reactions was achieved only with focused fractions 2, 3 and 15 of $D_{24}$.

(e) Inhibition of RAST by Hapten-Like Fractions: The capacity of the hapten-like fractions to inhibit RAST was evaluated over a range of concentrations. The inhibition curves obtained for these hapten-like fractions are given in Figure 6. With the exception of focused fraction 1 of $D_{24}$ which inhibited RAST close to 100%, the highest inhibition level achieved with any other focused fraction was about 60 %.

Absorption of Reaginic Antibodies by Hapten Coupled to Cellulose Discs

To further test for the ability of the hapten-like fractions to combine with the IgE antibodies specific for the allergens present in KBG pollen, allergosorbent discs were prepared by coupling separately the hapten-like

TABLE IV

IN VIVO INHIBITION OF PCA REACTIONS BY HAPTEN-LIKE FRACTIONS ISOLATED FROM R, $D_{24}$ AND $D_{48}$ BY PREPARATIVE ISOELECTROFOCUSING

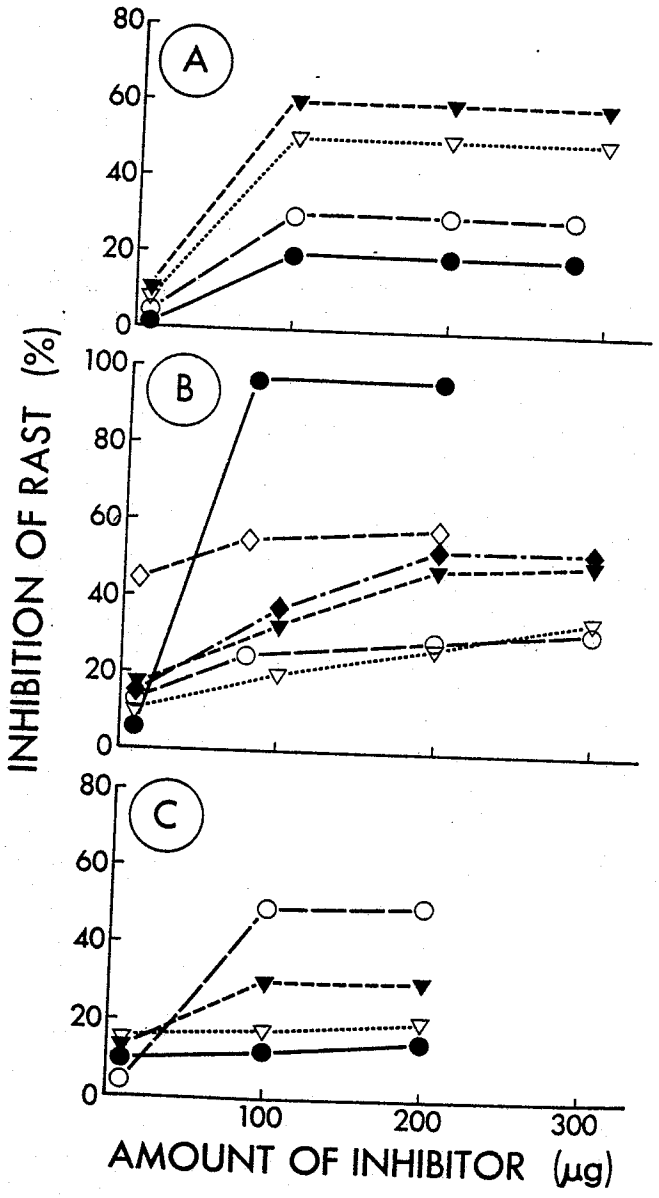
| FRACTION NUMBER* | pI | PCA TITER | % INHIBITION |
|---|---|---|---|
| R | | | |
| 1 | 3.0 | 80 | 87.5 |
| 2 | 3.4 | 80 | 87.5 |
| 3 | 3.9 | 80 | 87.5 |
| 4 | 4.1 | 40 | 93.7 |
| $D_{24}$ | | | |
| 1 | 2.7 | 160 | 75 |
| 2 | 3.5 | NIL | 100 |
| 3 | 3.8 | NIL | 100 |
| 4 | 4.1 | 160 | 75 |
| 5 | 4.3 | 160 | 75 |
| 15 | 9.6 | NIL | 100 |
| $D_{48}$ | | | |
| 1 | 2.7 | 160 | 75 |
| 2 | 3.5 | 320 | 50 |
| 3 | 3.7 | 160 | 75 |
| 4 | 4.1 | 160 | 75 |
| CONTROL (saline) | | 640 | |

*The fraction number refers to 2.5 cm wide sections cut from the gel following preparative isoelectrofocusing with number 1 starting at the anodal end.

For the in vivo inhibition experiments, hooded rats were first passively sensitized with 50 µl of serial dilutions of murine anti-R reaginic sera. Twenty-four hours later they were separately injected intravenously with a solution of 1 mg of one of the fractions indicated. For control, two of the passively sensitized animals were injected intravenously with saline instead of a haptenic fraction. Ten to fifteen minutes later all the animals were challenged intravenously with a solution of 100 µg of R (dry weight) in 0.25% Evan's blue dye.

Figure 6. Inhibition of RAST by focused fractions with hapten-like activity. The degree to which hapten-like fractions isolated from R, $D_{24}$ and $D_{48}$ could inhibit the binding of IgE antibodies, present in a pool of sera from humans allergic to KBG pollen, to allergosorbent discs prepared with R, are illustrated in A, B and C respectively.

● fraction 1; ○ fraction 2; ▼ fraction 3;
▽ fraction 4; ◆ fraction 5; ◇ fraction 15.


A
B
C
80
60
40
20
0
100
80
60
40
20
0
80
60
40
20
0
100
200
300
INHIBITION OF RAST (%)
AMOUNT OF INHIBITOR (μg)

fractions 1, 2, 3 and 4 of R to cellulose discs (see Materials and Methods under RAST). An amount of 100 µg of each fraction was incubated with an activated cellulose disc. A total of either 2 or 4 allergosorbent discs were incubated with aliquots of 200 µl of the murine reaginic anti-R sera at a dilution of 1:320 (PCA titer determined with R was 1,280). It was found that the PCA activity of the reaginic sera had been completely absorbed when 4 allergosorbent discs were employed (Table V). The specificity of the absorptions was demonstrated by the fact that the allergosorbent discs were unable to absorb IgE antibodies to ovalbumin.

Similar attempts to prepare allergosorbents by separately coupling hapten-like fractions of $D_{24}$ and $D_{48}$ to activated cellulose discs were not successful, i.e. the allergosorbent discs could not absorb murine and human IgE antibodies present in the murine reaginic and human allergic sera. This was interpreted to mean that either the hapten fractions failed to couple to the discs or that the haptens were denatured during the coupling reaction or, if the fractions had indeed become attached to the cellulose matrix, the haptenic group(s) were sterically hindered from combining with the anti-R IgE antibodies.

TABLE V

DEMONSTRATION OF THE SPECIFICITY OF ABSORPTION OF MURINE ANTI-R IgE ANTIBODIES BY ALLERGOSORBENT DISCS PREPARED WITH HAPTENIC FRACTIONS OF R

| Allergosorbent Prepared with fraction[1] | Number of allergosorbent discs used for the absorption of murine reaginic antisera to R | | Number of allergosorbent discs used for the absorption of murine reaginic antisera to OA | |
|---|---|---|---|---|
| | 2 | 4 | 2 | 4 |
| | PCA REACTIONS | | | |
| 1 | + | - | + | + |
| 2 | + | - | + | + |
| 3 | + | - | + | + |
| 4 | + | - | + | + |

[1]The fraction numbers refer to 2.5 cm sections cut from the gel following preparative isoelectrofocusing, with number 1 starting at the anodal end.

Allergosorbent discs were prepared by coupling separately the haptenic fractions of R indicated in the first column to activated cellulose discs as described for RAST in Methods. The allergosorbent discs, according to the number indicated, were incubated with a murine anti-R reaginic sera (PCA titer was 1,280 as determined with R) at a final dilution of 1:320 at room temperature for 2-3 hours after which time the supernatant was used for the intradermal sensitization of hooded rats. Twenty-four hours after their passive sensitization, the rats were challenged intravenously with a solution of 1 mg R (dry weight) in 0.25% Evan's blue dye. To test for the specificity of the absorption, the allergosorbent discs were similarly used to absorb a murine reaginic antisera to ovalbumin prior to intradermal sensitization. This antisera was used at a final dilution of 1:320. (The PCA titer of the antisera as determined with OA was 1,280). Twenty-four hours after the passive sensitization of hooded rats, the animals were challenged intravenously with 1 mg OA (dry weight) in 0.25% Evan's blue dye.

Reactions $\geq$ 2 mm in diameter are indicated as +.
Complete absence of reaction is indicated as -.

## DISCUSSION

Previous attempts by other investigators in this laboratory to demonstrate the presence of haptenic material in fractions obtained by gel-filtration and ion-exchange chromatography of the dialysate fraction of KBG aq. ext. were unsuccessful, in that all fractions possessed allergenic activity, i.e. they could elicit PCA reactions in appropriately sensitized animals (unpublished data). It can now be suggested that the haptenic components were present in at least some of the fractions examined in earlier studies, although in small quantities relative to the amount of allergenically active components. Consequently, the haptenic activity had escaped detection. In this study it was demonstrated that components possessing hapten-like properties could be isolated from the dialyzable and non-dialyzable fractions of the aqueous extract of KBG pollen by a relatively simple procedure employing isoelectrofocusing.

The isolation of hapten-like components from ragweed pollen (Attallah and Sehon, 1969; Feigen et al., 1967) and timothy pollen (Malley et al., 1967) have also been reported. In view of the general observations that haptenic activity is usually associated with substances of low molecular weight, it was somewhat surprising to find components with haptenic activity to be present in the

retentate fraction of KBG aq. ext. Nevertheless, the haptenic property of the relevant fractions isolated from the retentate, as well as those hapten-like fractions from the dialyzable fractions $D_{24}$ and $D_{48}$, was firmly established on the basis of two criteria, namely (i) they were unable to elicit PCA reactions in appropriately sensitized animals and (ii) they were able to inhibit completely and specifically the PCA reactions normally elicitable in animals sensitized with a reaginic antisera to R on challenge with R.

The specificity of the combination of the haptenic fractions with anti-R IgE antibodies was demonstrated by the finding that the PCA reactions elicitable with heterologous allergen-IgE antibody systems (DNP and OA) were unaffected by any of the haptenic fractions isolated from KBG pollen. Moreover, allergosorbent discs prepared with the haptenic fractions isolated from R, specifically adsorbed antibodies to R but were incapable of adsorbing reaginic antibodies to the unrelated antigen, ovalbumin.

The study on the immunological specificity of the hapten-like fractions was made in relation to those specificities detected by IgE antibodies. The relationship, if any, of the specificities of the hapten-like fractions relative to those present in other KBG allergens as revealed by antibodies of the IgG class of immunoglobulins will be presented in chapter IV of this thesis.

On the basis of the extent to which the haptenic fractions, following their intravenous administration, could inhibit PCA reactions normally elicitable with the R-murine reaginic anti-R system, it was established that haptenic fractions 2, 3 and 15, isolated from $D_{24}$ had the most potent haptenic activity in comparison with other haptenic fractions of $D_{24}$, $D_{48}$ or R. The studies designed to evaluate the capacity of hapten-like fractions to neutralize IgE antibodies to R also provided insights as to the allergenic composition of retentate. The observations that some of the haptenic fractions e.g. fractions 2, 3 and 4 of R which contained only a few components discernable by Anal-ISO-EF, could inhibit specifically and completely ( within the limitation of the inhibition of PCA technique) the PCA reactions normally elicitable with R, clearly indicated that such haptenic fractions might possess all of the allergenic specificities present in R.

The <u>in vitro</u> inhibition of PCA experiments employing murine reaginic sera (titer 1,280) were performed by incubating the KBG haptenic components with the reaginic sera at a final dilution of 1:320. This dilution of reaginic sera was chosen in order to minimize the amounts of purified haptenic components which would be required to neutralize completely the IgE antibodies present in the 1:320 diluted murine reaginic sera. Consequently, it's conceivable that at this high dilution of murine reaginic anti-R sera employed, some antibodies might be present in concentrations at levels below those required to elicit PCA reactions. As a result, the possibility arose that the presence of IgE antibodies

of some specificities other than those capable of combining with the haptenic components in the undiluted sera might have not been detected in the PCA reactions. To exclude this possibility, undiluted reaginic sera should be employed in the inhibition of PCA experiments. These experiments were not performed because of the unavailability of large amounts of haptenic components that would have been required to neutralize all the IgE antibodies present in the undiluted reaginic sera.

Therefore, it can be suggested that (i) the specificity of the murine IgE antibodies was directed to (allergenic) determinants which were common to either allergenic or haptenic fractions; (ii) the heterogeneity of the allergenic components observed in the various fractions of KBG aq. ext. may be due primarily to differences in their net charge, i.e. allergens possessing the same allergenic specificity exist in several forms differing in pI value; and (iii) the pool of murine reaginic anti-

sera to R probably recognized only a limited number of different allergenic determinants (if indeed more than one). If on the other hand, it is suggested that the retentate contained several different allergens or alternatively an allergen with several different allergenic determinants and each was capable of eliciting the formation of a specific IgE antibody, it would be reasonable to expect that in order to achieve complete neutralization of the IgE antibodies to R, the haptenic fractions would be comparably more complex than that which was actually found. Thus, in spite of the observation that R could be resolved by ISO-EF into at least 30 focusable components, and allergenic activity could be detected in fractions which focused over a broad range of pI values, these findings cannot be interpreted to mean that there was a corresponding number of different allergenic specificities. In this connection it is worth mentioning that antigen $D_3$ isolated from timothy pollen by Malley et al. (1975) was a low molecular weight allergen (M.W. 1,000) with a simple structure which consisted of a flavonoid pigment and a disaccharide of cellobiose linked to threonine. In spite of its simplicity relative to the complex and heterogeneous components present in the aqueous extract of timothy pollen, antigen $D_3$ was able to inhibit to the extent of 78% of the induced

histamine released from monkey lung tissue sensitized with human allergic serum on challenge with the whole water soluble timothy pollen extract.

It is important to note that a somewhat different interpretation regarding the specificities of the haptens can be made on the basis of results of the inhibition of RAST experiments in which a pool of human sera from individuals allergic to KBG pollen was employed. Using allergosorbent discs prepared with R, the maximum inhibition of the binding of human IgE antibodies achieved by any of the haptenic fractions (with one exception*) was of the order of 60%. Since complete inhibition of RAST could not be attained, it would appear, therefore, that the human IgE antibodies recognized some allergenic specificities in R which were absent in the majority of the haptenic fractions. A corollary of this conclusion is that the haptenic fractions (with the one exception noted) lacked some of the specificities associated with allergenic determinants on components of R.

It would appear that there were two divergent conclusions as to the number of different allergenic specificities present in R or the haptenic fractions. In view of the fact that a murine reaginic antisera was used in one set of experiments (within the limitation of the inhibition of PCA technique discussed above) and a pool of sera from human

* Only focused fraction 1 of $D_{24}$ was able to completely inhibit RAST.

allergic to KBG pollen was used in another, the divergent results and conclusions were not unexpected. Differences in the ability of different strains of mice to mount an IgE antibody response to various allergens has been reported by other investigators (Levine and Vaz, 1970). The IgE antibody response of the A/HeJ strain of mice appeared to be limited to the immunodominant allergenic determinant(s) of R recognized by this strain. On the other hand, the source of the human IgE antibodies present in a pool of sera from humans allergic to KBG pollen, must be regarded as being obtained from an outbred population. As a result, the IgE antibodies collectively present in the pool of human antisera recognized a greater number of allergenic determinants than did the murine IgE antibodies. It would be logical to assume that the immunization of several different responding strains of mice with R, would result in antisera which on pooling could recognize a similar number of different allergenic determinants as was recognized by the pool of human antisera. These observations serve to emphasize one of the shortcomings of employing one inbred strain of mice for examining the diversity of allergenic specificities to which humans may respond by forming IgE antibodies.

# CHAPTER III

# ISOLATION AND CHARACTERIZATION OF AN ALLERGEN FROM KENTUCKY BLUE GRASS POLLEN

## INTRODUCTION

In this chapter the isolation and characterization of Allergen C from one of the focused fractions of R is described. Focused fraction 15 of R was found to contain pink colored material. This fraction was allergenically active and elicited PCA reactions in rats sensitized with a murine anti-R reaginic sera (Table I). It appeared to possess all the allergenic activities (detectable with the murine reaginic and human allergic sera) present in the unfractionated R as revealed by inhibition of PCA (Table II) and inhibition of RAST studies (Figure 13). Anal-ISO-EF of focused fraction 15 revealed the presence of components that focused only over a narrow segment of the gel (Figure 5). Therefore, it was decided to further purify focused fraction 15 of R in an attempt to isolate an active allergenic component. Since focused fraction 15 of R was originally purified according to charge (Prep-ISO-EF), it was decided to further purify this fraction according to molecular size by gel filtration.

## METHODS AND MATERIALS

### Gel filtration on Bio-Gel P-60

Bio-Gel P-60 (Bio Rad Laboratories, Richmond, Calif.) was rehydrated and equilibrated with phosphate buffered saline (PBS). Focused fraction 15 of R was dissolved in a volume of 2 ml of PBS, applied to a column (85 x 1.7 cm) and eluted at 4-5°C with PBS. Since Allergen C was subsequently found to be stable towards denaturing conditions, dissociating agent such as urea was employed to determine if better resolution could be achieved in the gel filtration experiments. For this purpose, the sample was dissolved in a volume of 2 ml of 10 M urea, applied to the column and eluted with an aqueous solution containing 0.1 M urea. The flow rate was adjusted to 12 ml per hour and fractions were collected over 15 minute intervals. The absorbance of the eluates at 280 nm was monitored continuously with a Uvicord II Ultraviolet Absorptionmeter type 8303A (LKB, Bromma, Sweden). Fractions containing the separated protein peaks were pooled and dialyzed against distilled water overnight using Spectrapor membrane with a molecular size cut-off range of 6,000 - 8,000. The non-dialyzable components remaining inside the membrane were concentrated by lyophilization.

### Evaluation of the Allergenicity of Chromatographic Fractions

The allergenicity of the fractions (before pooling) was

established in terms of their ability to elicit PCA reactions according to procedures described in Chapter II. An amount of 200 µg of a fraction in a solution of Evan's blue dye (0.25%) was used for the intravenous challenge of hooded rats which were sensitized intradermally with a serial dilutions of a murine anti-R reaginic sera.

Production of Sheep Precipitating Antiserum to R

Sheep (North American Laboratory Supplies, Gunton, Manitoba, Canada) were hyperimmunized by repeated immunization with a dose of R (0.5 mg dry weight) emulsified with Freund's complete adjuvant. A series of five injections were given intradermally over a period of 138 days. Antiserum employed in the crossed immunoelectrophoresis was obtained at the end of this period.

Production of Rabbit Precipitating Antisera to Allergen C

New Zealand white rabbits (Canadian Breeding Laboratories, Montreal, Quebec) were immunized by intradermal injections into multiple sites; each animal receiving a dose of 500 µg Allergen C emulsified with complete Freund's adjuvant. Two weeks later antibodies to Allergen C were detected in the sera by immunodiffusion using R or Allergen C as antigen. Blood samples were collected by cardiac puncture into centrifuge tubes. Sera were harvested by allowing the blood to coagulate overnight at $4^{o}C$ and the clot removed by centrifugation.

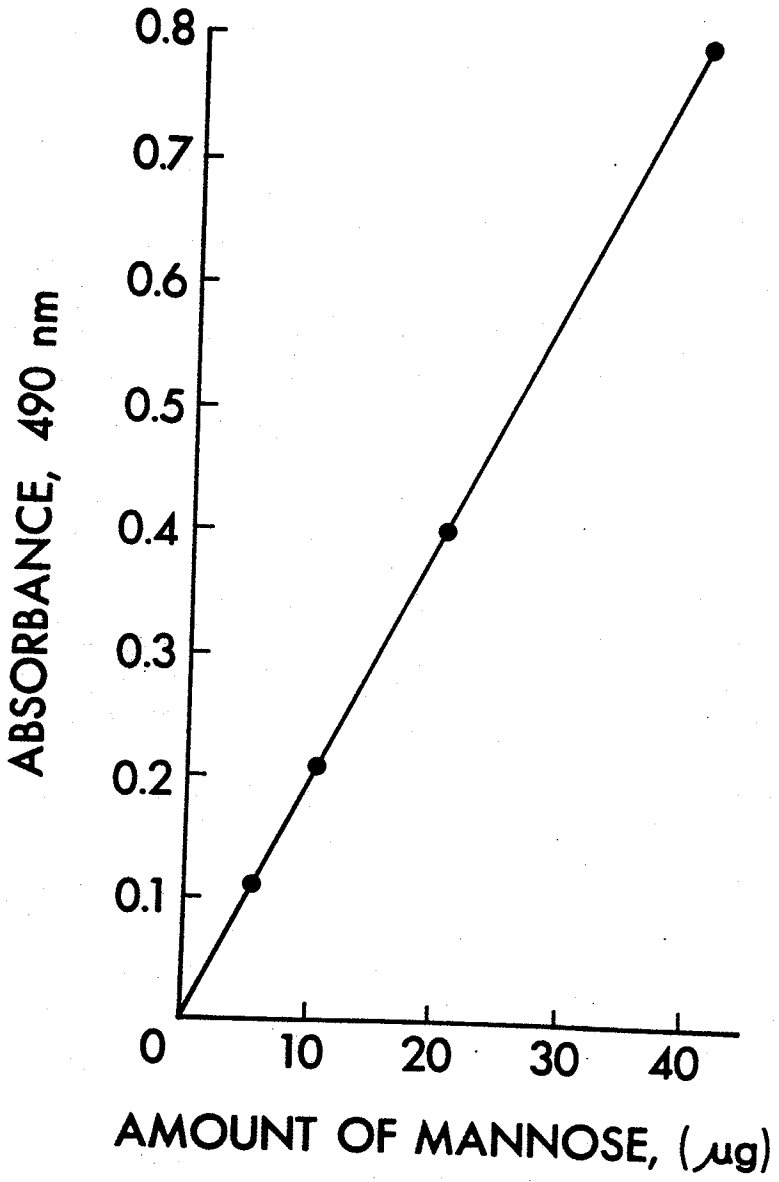
## Determination of Carbohydrate Content

The Phenol-sulfuric acid method (Montgomery, 1961) was employed to determine the carbohydrate content of Allergen C. An aqueous solution of 80% phenol (0.1 ml) was added to a volume of 2 ml of a solution of Allergen C. Five ml of concentrated $H_2SO_4$ was added to the mixture with constant agitation. The temperature rose spontaneously to approximately 110°C and the heat so generated led to complete development of chromogen after standing at room temperature for 30 minutes. The absorbance of the solution at 490 nm was measured in a Coleman UV-VIS spectrophotometer. A reference blank solution was similarly prepared by substituting water for the solution of Allergen C. A standard curve was established with mannose in the concentrations ranging from 5 - 40 µg (Figure 7).

## Estimation of pI Value

Since allergenic activity was subsequently found to be associated with the pigmented material, the pI value of Allergen C was directly determined from the preparative gel following Prep-ISO-EF of R. To determine the pH gradient of the area of gel corresponding to focused fraction 15 of R, this segment of the gel was divided into five 0.5 cm sections and these were removed separately into small test tubes. One of these sections contained the pink colored

Figure 7. Reference curve for the determination of carbohydrate by the method of Montgomery (1961). Mannose was used as the standard solution.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
ABSORBANCE, 490 nm
AMOUNT OF MANNOSE, (μg)

Allergen C. 0.5 ml of distilled water was added to each tube and the pH of the gel suspension was measured by a pH meter.

Amino Acid Analysis

Allergen C was hydrolyzed with 6N HCl at 110-112°C for 24 hours and analyzed* with a Beckman automatic amino acid analyzer, Model 120-139 (Beckman Instruments Inc., Palo Alta, Calif.).

Determination of Molecular Weight

Molecular weight of Allergen C was determined by gel filtration on a Bio-Gel P-60 column calibrated with standard proteins of known molecular weight: Ovalbumin, 45,000; Chymotrypsinogen A, 25,000 (Pharmacia, Sweden) and Cytochrome C, 11,700 (Sigma, St. Louis, Mo., U.S.A.).

Antigenic Analysis by Immunodiffusion

Ouchterlony (1948; 1949) double diffusion method was carried out in 1% agar in PBS, containing 0.001% sodium azide in plastic petri dishes. Antiserum was allowed to diffuse in the antibody well for a period of 3 hours prior to the addition of antigens. In this manner, the immunoprecipitates formed approximately equidistant between antibody and antigen wells.

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* Amino acid analysis was kindly performed by Dr. F. Stevens of the Department of Biochemistry, University of Manitoba.

## Rocket Immunoelectrophoresis and Crossed Immunoelectrophoresis

Rocket immunoelectrophoresis (Axelsen et al., 1973) was used to determine the amount of Allergen C in R. A solution of agarose (Indubiose A-37, L'Industrie Biologie Francaise, Gennevilliens, France) was made by heating 0.2 gm agarose in 18 ml Tris-barbital-lactate buffer* at pH 8.6 in a boiling water bath until the agarose dissolved. The agarose solution was cooled to 55°C and 2 ml of rabbit anti-Allergen C serum was added and mixed throughly before layering onto a glass plate (10.1 x 8.2 cm) placed on a level surface. After congelation of the agarose (2-5 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 solutions containing 1 - 10 µg of Allergen C were added. This plate containing known amounts of Allergen C was used to construct a reference curve (Figure 8) for the determination of the amount of Allergen C present in R. Another plate containing constant volumes (40 µl) of solutions of R (2 - 20 µg) was similarly prepared for electrophoresis into gel containing anti-Allergen C serum.

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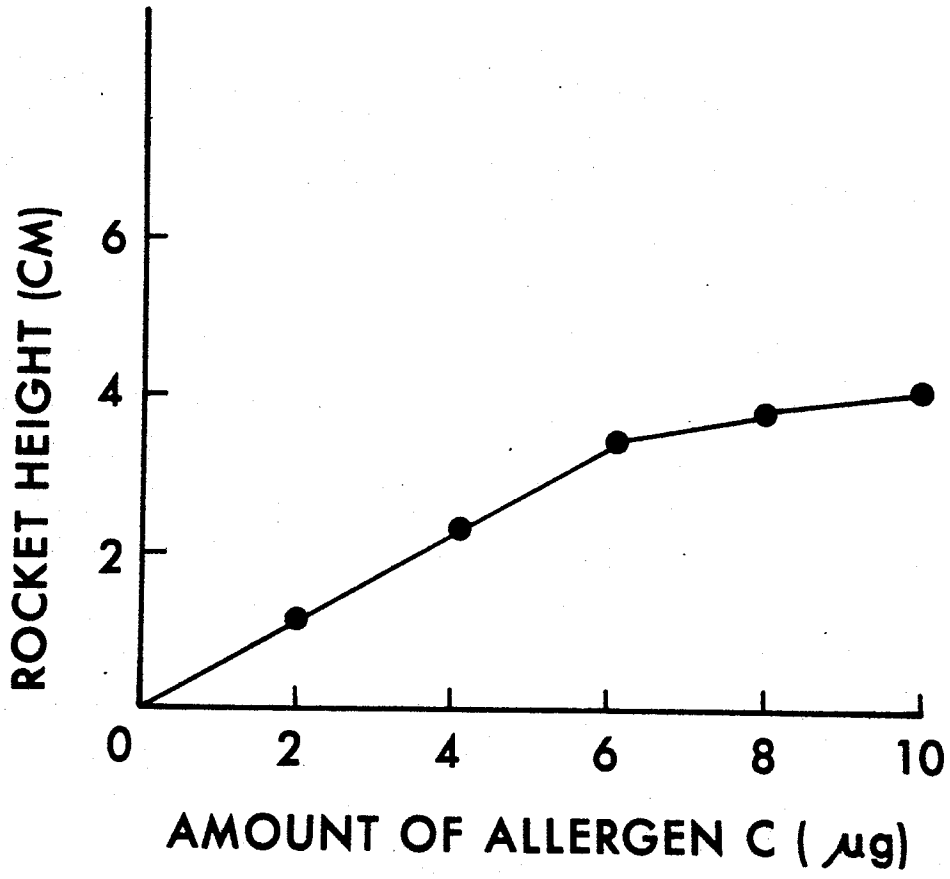
* Tris-barbital-lactate buffer at pH 8.6 was prepared by dissolving amounts of 8.843 g Tris, 4.42 g barbital, 1.848 g calcium lactate and 0.0195 g sodium azide in distilled water to a final volume of 1 liter.

Figure 8. Standardization of rocket immunoelectrophoresis for the quantitation of Allergen C. Allergen C was electrophoresed into agarose gel containing a rabbit precipitating anti-Allergen C serum.


ROCKET HEIGHT (CM)
6
4
2
0
2
4
6
8
10
AMOUNT OF ALLERGEN C ( μg)

The plates were placed on the Gelman electrophoresis apparatus (Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.) and connected to the Tris-barbital-lactate buffer electrode vessels by means of paper wicks. Electrophoresis was carried out at a potential of 2.5 volts per cm for a period of 20 hours employing water cooling (9 - 12$^{o}$C). After the completion of the electrophoresis, 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 to dryness with several changes of layers of cellulose paper. The dried plates were stained for 5 minutes with a solution of 0.5% Coomassie blue dissolved in a mixture of methanol, acetic acid and water (4.5:1:4.5). After staining, the plate was destained with the same mixture in the absence of Coomassie blue. The concentration of Allergen C in R was estimated from the reference curve by measuring the rocket heights following electrophoresis of R into gel containing rabbit anti-Allergen C serum.

Crossed immunoelectrophoresis (Axelsen et al., 1973) was used to analyze the antigenic complexity in R and Allergen C. Crossed immunoelectrophoresis was performed on glass plates (10.1 x 8.2 cm) which were overlaid with a volume of 20 ml of the 1% agarose solution in Tris-barbital-lactate buffer at pH 8.6. 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 (containing 200 µg R or Allergen C) was applied into the well. The electrophoresis of antigen in the first dimension was carried out at a potential of 10 volts per cm for a period of 2 hours.

After that time, a thin strip of agarose (1 x 8.2 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; 2 ml sheep anti-R or rabbit anti-C serum 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 aparatus 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 per cm at 9-12°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.

Stability of Allergen C

The procedures described below were carried out to test the stability of Allergen C to acid, base, denaturing agent, moderate heat treatments and proteolytic digestion.

500 µg of Allergen C was separately dissolved in an aqueous solution of 0.1M $H_2SO_4$ and 0.1N NaOH and incubated at 4°C for 5-6 hours. For treatment with denaturing agent, the sample was dissolved in an aqueous solution of 6M guanidine hydrochloride and incubated at 37°C for 25 minutes. After this time, all samples were separately dialyzed using Spectrapor membrane with a molecular cut-off size of 3,500 against distilled water at 4°C overnight. The Allergen C remaining inside the membrane was recovered by lyophilization. To test for the stability of Allergen C to moderate heat, an aqueous solution of Allergen C (1 mg/ml) was incubated at 56°C for 5-6 hours.

Proteolytic digestion of Allergen C was carried out with insolubilized protease (from Streptomyces griseus, attached to carboxymethyl cellulose; Sigma, St. Louis, Mo.). 50 mg (dry weight) of insoluble protease was suspended in 1 ml of PBS and washed several times with PBS. 1 mg of Allergen C was added to the enzyme suspension and incubated at 30°C for 30 minutes with continuous shaking. The insoluble enzyme

was removed by centrifugation at the end of the incubation period. Following the various treatments described, the allergenic activity of Allergen C was assessed by the method of inhibition of RAST.

Trypsin, αChymotrypsin and Papain Treatments of Allergen C

These enzymes were employed in the degradation studies of Allergen C in order to gain some information on the allergenic and antigenic moiety of the molecule. Insolubilized trypsin (from bovine pancreas, attached to carboxymethyl cellulose), αchymotrypsin (from bovine pancreas, attached to polyacrylamide), and papain (from papaya latex, attached to carboxymethyl cellulose) were purchased from Sigma (Sigma, St. Louis, Mo.). 20 mg of each insolubilized enzyme was weighed into separate small test tubes and washed several times with PBS. An amount of 1,000 µg of Allergen C in a total volume of 0.5 ml was added to each of the insoluble enzyme preparations. The suspensions were then incubated at 30°C for 5-6 hours in a water bath with continuous shaking. After incubation, the enzymes were removed by centrifugation. For control, a solution of Allergen C was incubated in the same manner in the absence of enzymes.

Since the inhibition of RAST method was unable to distinguish between multivalent allergenic and monovalent hapten-like components, the allergenic activity of Allergen C after enzyme treatments was tested by evaluating their ability to elicit and inhibit PCA reactions in rats employing a murine

anti-R reaginic sera. Antigenic analysis after enzyme treatment was performed by Ouchterlony double diffusion in agar employing a rabbit precipitating anti-Allergen C serum.

## RESULTS

### Isolation of Allergen C by Gel Filtration on Bio-Gel P-60

The chromatographic profile of focused fraction 15 of R eluted with PBS is illustrated in Figure 9. Two protein fractions were obtained. The first fraction could not elicit PCA reactions in rats sensitized with the murine anti-R reaginic sera. The second fraction was considered to be allergenically active since it elicited PCA reactions in appropriately sensitized animals. This allergenically active fraction designated as Allergen C was still found to be associated with pink colored material. A better resolution of these two fractions was obtained by dissolving fraction 15 of R in 10M urea before applying to the column which was equilibrated with 0.1M urea and eluted with the same (Figure 10). Since allergenic activity was associated with the colored material, the isolation of Allergen C was modified. Instead of using the whole focused fraction 15 of R for subsequent chromatography on Bio-Gel P-60, the pink focused band was cut from the Prep-ISO-EF plate, and processed as described under Methods for Prep-ISO-EF in Chapter 1. Ampholine was removed by dialysis through Spectrapor membrane with a molecular weight cut-off range of 6,000-8,000. Figure 11 illustrates the elution profile of Allergen C on Bio-Gel P-60

Figure 9. Elution profile of focused fraction 15 of R on Bio-Gel P-60. Focused fraction 15 of R was dissolved in 2 ml of PBS, applied to the column and eluted with PBS. + refers to fractions that were capable of eliciting PCA reactions and - refers to fractions that were unable to elicit PCA reactions in rats sensitized with murine reaginic antisera to R. The shaded area represents the pink colored fractions, which on pooling, constitute the allergenic material designated as Allergen C.

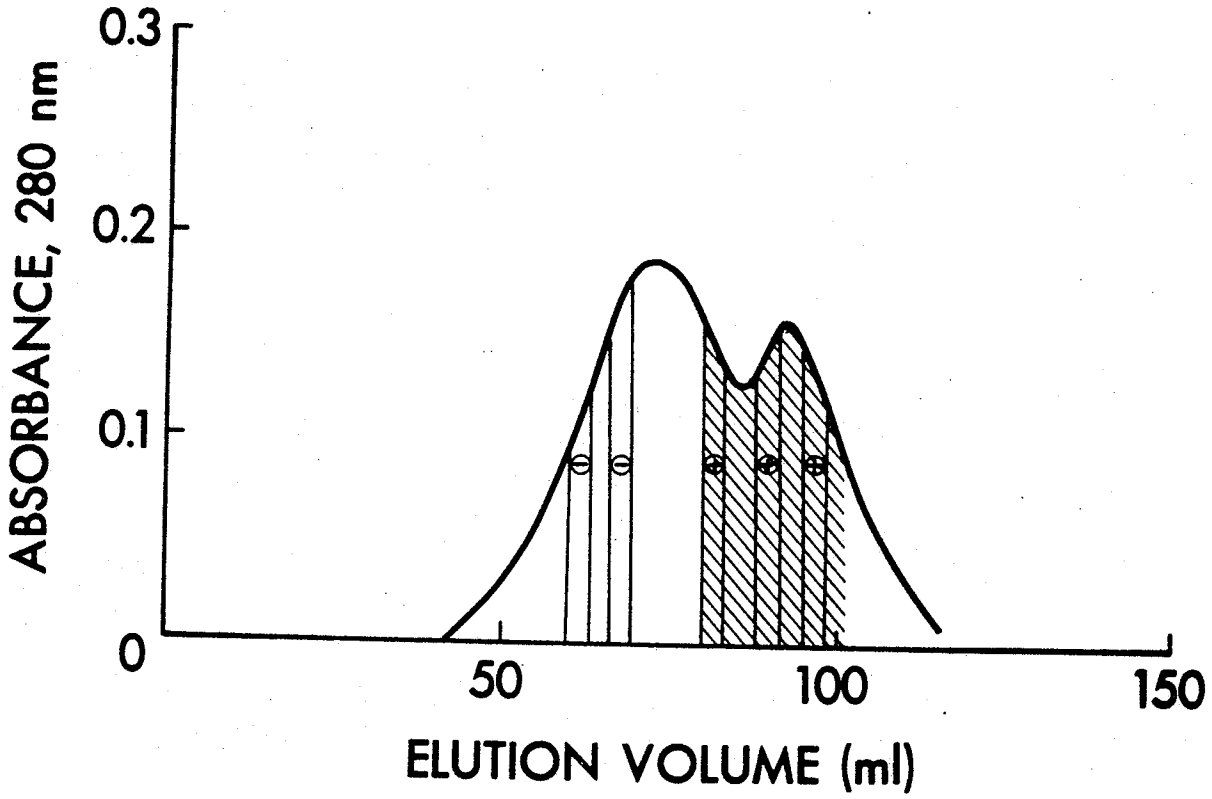

0.3
0.2
0.1
0
ABSORBANCE, 280 nm
50
100
150
ELUTION VOLUME (ml)

Figure 10. Elution profile of focused fraction 15 of R on Bio-Gel P-60 equilibrated with 0.1 M urea. Focused fraction 15 of R was dissolved in 2 ml 10 M urea, applied to the column and eluted with 0.1 M urea. + refers to the recovered protein fraction (Allergen C) that was capable of eliciting PCA reactions in rats sensitized with a murine reaginic anti-R sera and - refers to the recovered protein fraction that was unable to elicit the PCA reactions.

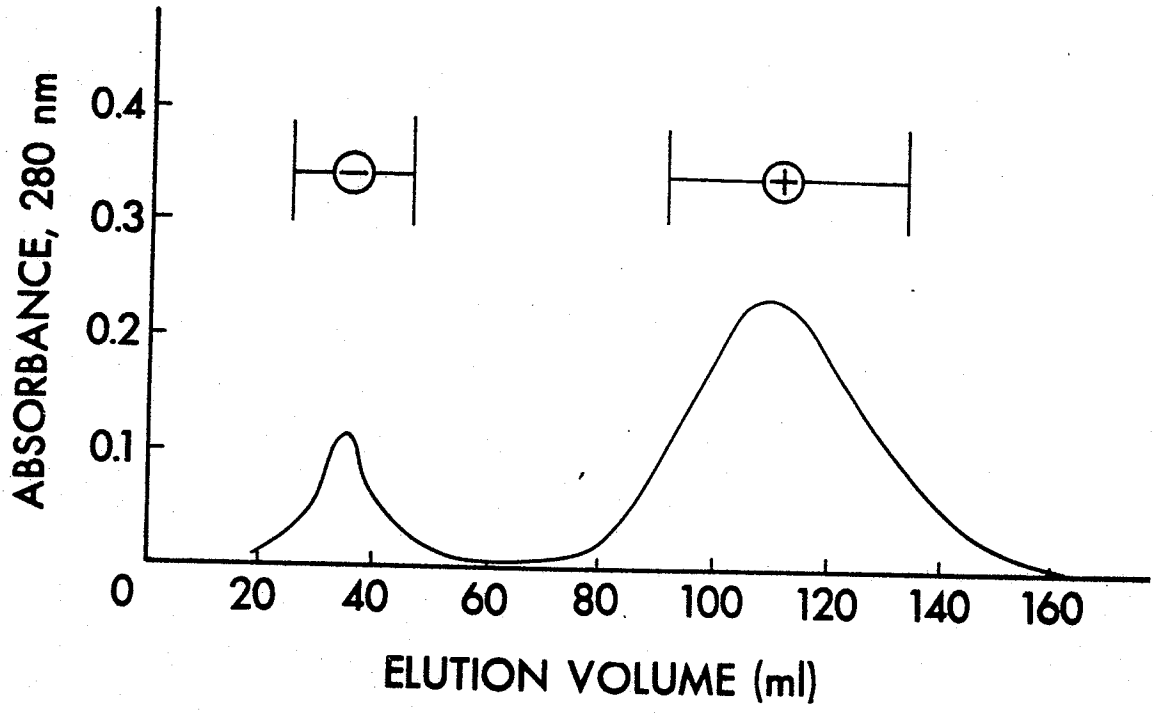
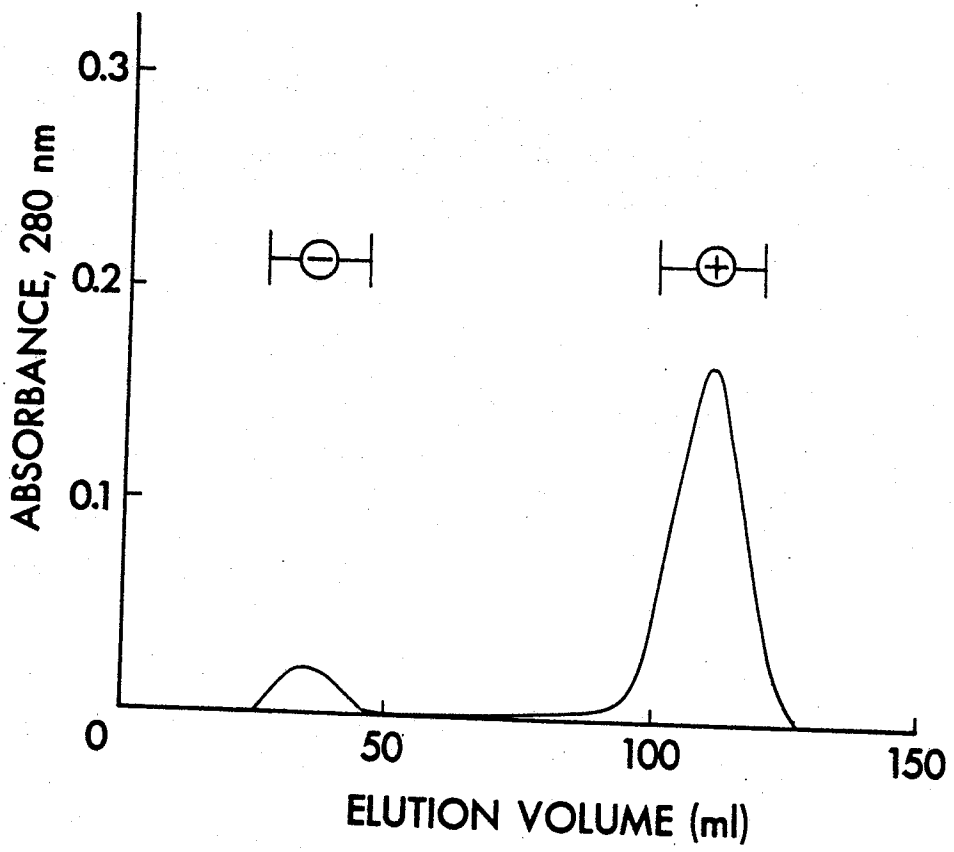

ABSORBANCE, 280 nm
0.4
0.3
0.2
0.1
0
20
40
60
80
100
120
140
160
ELUTION VOLUME (ml)

Figure 11. Elution profile of the pink focused band, containing Allergen C, obtained from Prep-ISO-EF of R on Bio-Gel P-60. The pink focused band was excised from the preparative plate following Prep-ISO-EF of R. It was dissolved in 2 ml 10 M urea after the removal of ampholine; applied to the column and eluted with 0.1 M urea. + refers to the recovered protein peak (Allergen C) that was capable of eliciting PCA reactions in rats sensitized with a murine reaginic anti-R sera and - refers to the recovered protein peak that was unable to elicit the PCA reactions.


ABSORBANCE, 280 nm
0.3
0.2
0.1
0
50
100
150
ELUTION VOLUME (ml)
⊖
⊕

using the pink band as starting material. The protein peaks were sharper than those seen in Figures 9 and 10.

Molecular Weight of Allergen C

The molecular weight of Allergen C was of the order of 11,000 daltons as determined by gel filtration on a calibrated column of Bio-Gel P-60 (Figure 12).

Allergenicity of Allergen C

The allergenicity of Allergen C was established in terms of its ability to (i) elicit PCA reactions in rats passively sensitized with a murine reaginic anti-R sera; (ii) neutralize the PCA reactions elicitable with R; and (iii) inhibit RAST. It was found that Allergen C at a dose of 200 µg could elicit PCA reactions specifically with the murine reaginic anti-R sera, which corresponded to a titer of 640 (TableVI ). In animals sensitized in an identical manner, R at a dose of 50 µg elicited a PCA titer of 1,280. Allergen C, employed at a dose of 500 µg/ml, was found to be able to inhibit completely and specifically the PCA reactions in rats normally elicitable with R and the murine reaginic anti-R sera system (Table VII ). By comparison R could inhibit the PCA reactions in this system at a dose of 50 µg/ml. Allergen C could inhibit RAST to the extent of 78% (Figure 13). By comparison, both R and focused fraction 15 of R could achieve 100% inhibition.

Figure 12. Estimation of the molecular weight of Allergen C. Molecular weight was estimated by gel filtration on a Bio-Gel P-60 column calibrated with standard proteins of known molecular weight. Ve = elution volume. Vo = void volume.

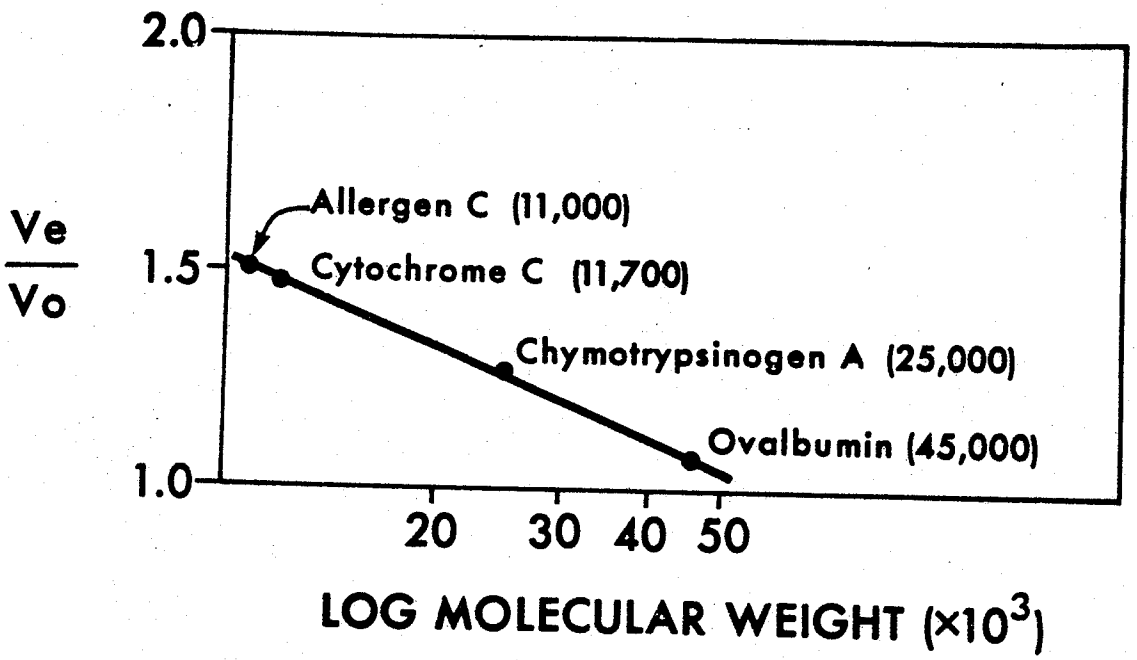

2.0
1.5
1.0
Ve
Vo
Allergen C (11,000)
Cytochrome C (11,700)
Chymotrypsinogen A (25,000)
Ovalbumin (45,000)
20
30
40
50
LOG MOLECULAR WEIGHT (×10³)

TABLE VI

ELICITATION OF PCA BY ALLERGEN C

| DOSE OF CHALLENGING ALLERGEN C | ALLERGEN-REAGINIC ANTISERA SYSTEM | |
|---|---|---|
| | PCA TITER | |
| | R-anti-R | OA-Anti-OA |
| 100 µg | 320 | - |
| 200 µg | 640 | - |
| 500 µg | 640 | - |
| R 50 µg | 1,280 | |

Hooded rats were passively sensitized with 50 µl of a serially diluted murine anti-R and anti-OA reaginic sera. Twenty-four hrs. later, two of the rats sensitized in an identical manner, were challenged with one of the doses of Allergen C in 0.25% Evan's blue dye, injected intravenously. The PCA titer of the murine reaginic anti-R and anti-OA sera determined with R and OA (1 mg dry weight) separately were 1,280.

TABLE VII

INHIBITION OF PCA BY ALLERGEN C

| CONCENTRATION OF ALLERGEN C INCUBATED WITH MURINE REAGINIC ANTISERA* | | | | | |
|---|---|---|---|---|---|
| ANTI-R | | | ANTI-OA | | |
| 100 µg/ml | 500 µg/ml | 1 mg/ml | 100 µg/ml | 500 µg/ml | 1 mg/ml |
| + | - | - | + | + | + |

* Each reaginic antisera at a final dilution of 1:500 was incubated with the appropriate amount of Allergen C for 2-3 hrs. before intradermal sensitization of rats. Twenty-four hrs. later the rats were challenged intravenously with a solution containing 500 µg R and 500 µg OA (dry weight) in 0.25% Evan's blue dye.

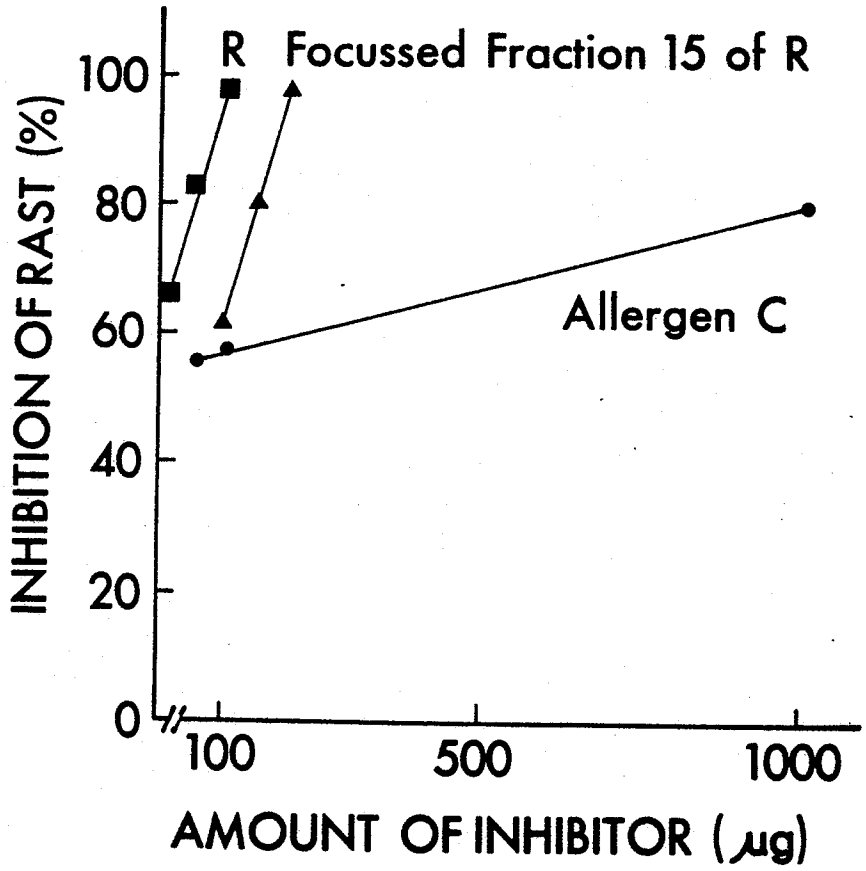
50 µg R was sufficient to neutralize this murine reaginic anti-R sera (titer 1,280 as determined with R).

\+ refers to a positive PCA reaction.

\- refers to an absence of PCA reaction.

The titer of the murine anti-OA reaginic sera was 1,280 as determined with 500 µg (dry weight) of OA.

Figure 13. Measurement of the allergenic potency of R, focused fractions 15 of R and Allergen C by inhibition of RAST. Allergosorbent discs were prepared by coupling R to cellulose discs. The potency was measured in terms of the ability of these allergens to inhibit the binding of the human IgE antibodies present in the pool of allergic sera to the allergosorbent discs.


R
Focussed Fraction 15 of R
Allergen C
INHIBITION OF RAST (%)
100
80
60
40
20
0
100
500
1000
AMOUNT OF INHIBITOR (μg)

## Anal-ISO-EF of Allergen C

Anal-ISO-EF of Allergen C revealed the presence of basic component(s) that focused at the cathodal end of the gel (Figure 14). Any components that focused at a higher pH than the maximum obtained in the gel could have migrated out of the gel into the cathodal electrolyte solution. By comparison, R was resolved into at least 30 components that focused over the entire gel.

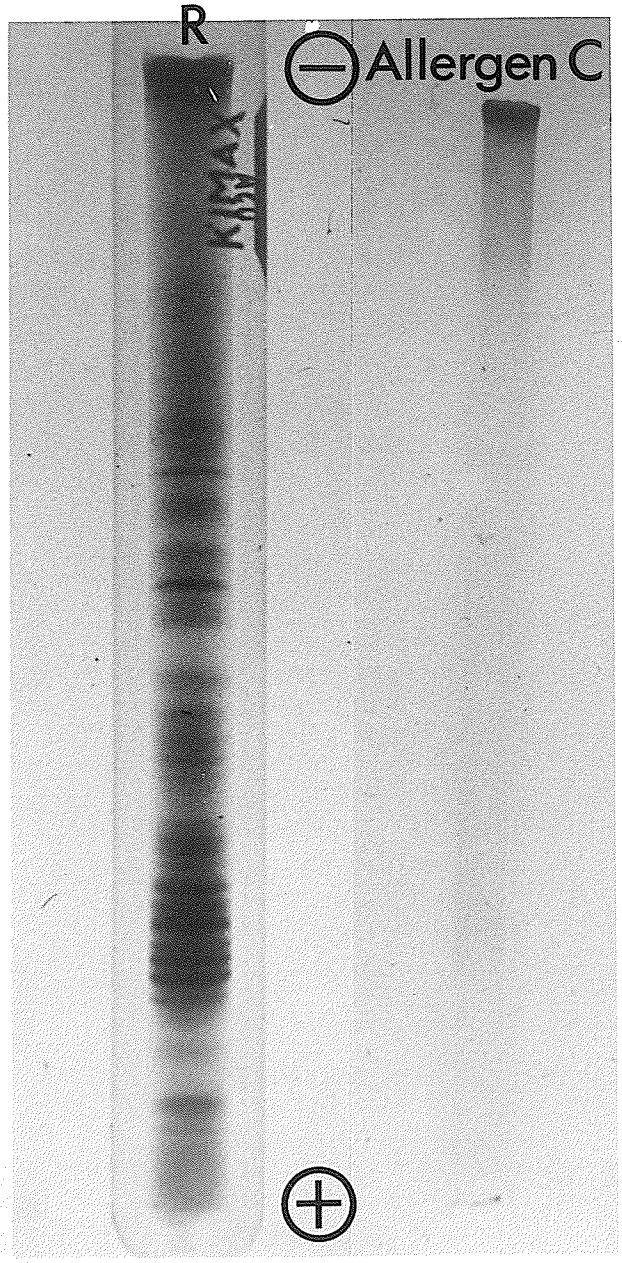
## Amino Acid Analysis and Carbohydrate Determination

Allergen C was found to contain all the natural occurring amino acids with the notable exception of cysteine (Table VIII). It contained 500 µg of carbohydrate per mg of protein.

## Analysis of Allergen C by Crossed Immunoelectrophoresis

Crossed immunoelectrophoretic analysis of Allergen C employing a sheep precipitating antiserum to R revealed one cathodally migrating component (Figure 15). By comparison R contained 12-15 antigenic components; the majority of these components migrated in the anodal direction. Analysis of Allergen C employing a rabbit precipitating antiserum to Allergen C revealed the presence of one cathodally migrating antigenic component (Figure 16). Analysis of R employing this rabbit antiserum also revealed a predominant cathodally migrating antigenic component with the possibility of another component detected in the center of the gel.

Figure 14. Anal-ISO-EF of Allergen C and R on polyacrylamide gel employing ampholine in the pH range of 3.5 - 10. + refers to the anodal end of the gel and - refers to the cathodal end of the gel. The protein bands were stained with Coomassie blue as described in Methods.


R
⊖ Allergen C
⊕

## TABLE VIII

### AMINO ACID COMPOSITION OF ALLERGEN C

| AMINO ACID | RESIDUE/MOLE |
|---|---|
| Lysine | 5 |
| Histidine | 2 |
| Arginine | 2 |
| Aspartic Acid | 10 |
| Threonine | 7 |
| Serine | 9 |
| Glutamic Acid | 11 |
| Proline | 8 |
| Glycine | 14 |
| Alanine | 10 |
| Cysteine | 0 |
| Valine | 7 |
| Methionine | 1 |
| Isoleucine | 4 |
| Leucine | 7 |
| Tyrosine | 4 |
| Phenylalanine | 4 |
| Tryptophan | N.D. |

Amino acid composition was determined with a Beckman automatic amino acid analyzer following acid hydrolysis of Allergen C at 110-112$^{o}$C for 24 hours.

N.D. not determined.

Figure 15. Crossed immunoelectrophoretic analysis of Allergen C and R in agarose gel employing a sheep precipitating antiserum to R. In the top plate Allergen C was employed as the antigen and in the bottom plate R was employed as the antigen. - refers to the cathodal ends of the gel and + refers to the anodal ends in the 2 dimension electrophoresis as described under Methods.

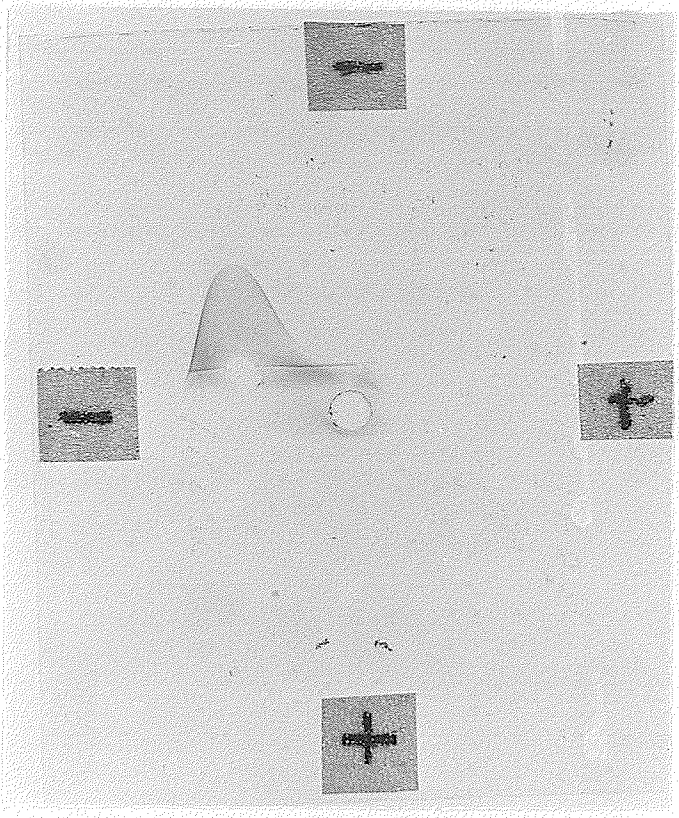
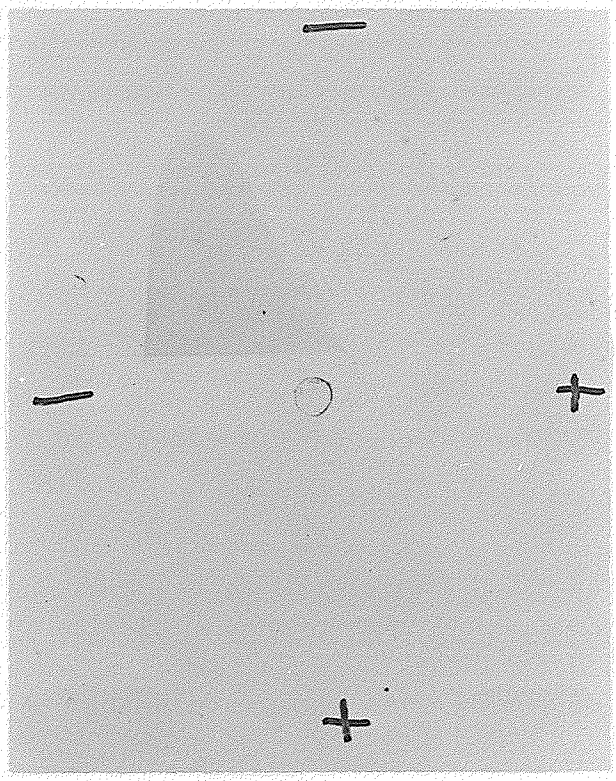
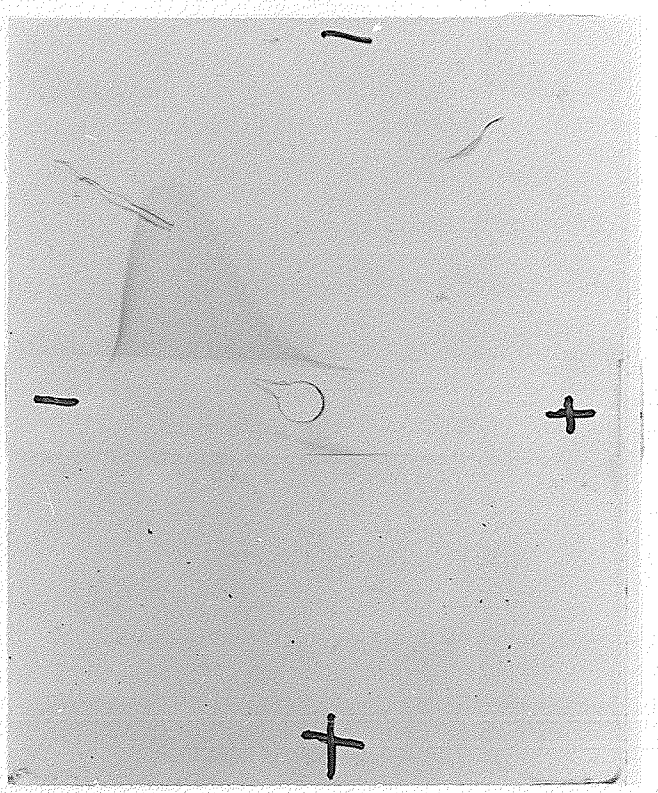


Figure 16. Crossed immunoelectrophoretic analysis of Allergen C and R in agarose gel employing a rabbit precipitating antiserum to Allergen C. In the top plate Allergen C was employed as the antigen and in the bottom plate R was employed as the antigen. - refers to the cathodal ends of the gel and + refers to the anodal ends in the 2 dimension electrophoresis described under Methods.





pI Value of Allergen C

Allergen C was found to possess a pI value of 9.7 as estimated from the Prep-ISO-EF gel (Figure 17). The pI values of the whole focused fraction 15 of R ranged from 8.7 to 10.6.

Amount of Allergen C in R

By means of rocket immunoelectrophoresis employing a rabbit precipating antiserum to Allergen C, it was determined that Allergen C was present in R to the extent of 10%.

Stability of Allergen C

Allergen C was found to be stable towards moderate heat (56°C), acid, base and guanidine hydrochloride treatments. The % inhibition of RAST was virtually unchanged after $H_2SO_4$ and guanidine hydrochloride treatments, (Table IX). There appeared to be a slight loss (a drop from 76 to 69 per cent) of RAST inhibitory activity after treatment with NaOH and incubation at 56°C. Protease treatment of Allergen C, however, resulted in complete loss of inhibition of RAST activity.

Trypsin, αChymotrypsin and Papain Treatments of Allergen C

The ability of Allergen C to elicit PCA reactions in rats sensitized with a murine reaginic anti-R sera after treatments with the various insolubilized enzyme preparations is illustrated by Table X. Treatment with trypsin eliminated completely the ability of Allergen C to elicit the PCA reactions. The PCA titer, elicited with Allergen C following

Figure 17. Determination of the pI value for Allergen C on the preparative plate following Prep-ISO-EF of R. The area indicated by the arrow represents the 0.5 cm segment of gel in which the pigmented Allergen C focused. The pH of this gel segment was found to be 9.7.

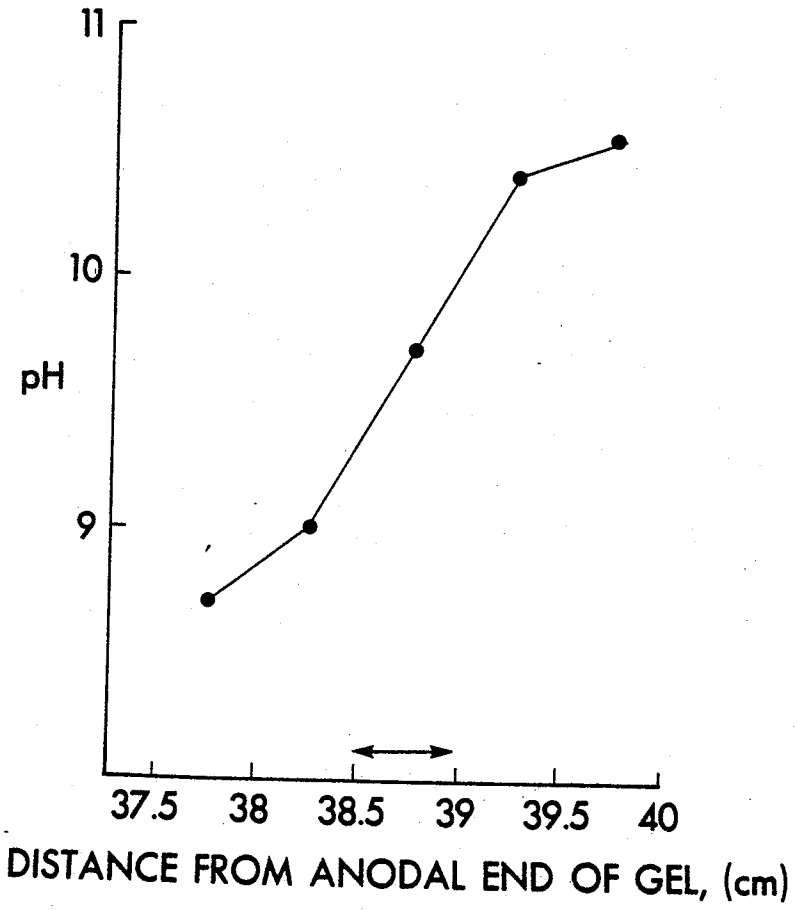

11
10
9
pH
37.5
38
38.5
39
39.5
40
DISTANCE FROM ANODAL END OF GEL, (cm)

TABLE IX

INHIBITION OF RAST BY ALLERGEN C

AFTER VARIOUS TREATMENTS

| TREATMENTS* | % INHIBITION OF RAST |
|---|---|
| NONE | 76 |
| 56°C | 69 |
| $H_2SO_4$ | 70 |
| NaOH | 69 |
| GUANIDINE HCl | 76 |
| PROTEASE | 0 |

An amount of 50 µg of Allergen C (both native and treated) was used in the inhibition of RAST.

* Details of the treatment procedures are described under Methods.

TABLE X

EFFECTS OF ENZYME TREATMENT OF ALLERGEN C ON ITS ABILITY TO ELICIT PCA REACTIONS

| TREATMENT | ALLERGEN-REAGINIC ANTISERA SYSTEM | |
|---|---|---|
| | PCA TITER | |
| | R-Anti-R | OA-Anti-OA |
| Trypsin | - | - |
| α Chymotrypsin | 40 | - |
| Papain | 320 | - |
| CONTROL* | 320 | - |

Hooded rats were passively sensitized by injecting intradermally volumes of 50 µl of a serially diluted murine anti-R and anti-OA reaginic sera. Twenty-four hrs later, two of the rats, sensitized in an identical manner, were challenged by an intravenous injection of one of the enzyme treated Allergen C at a dose of 200 µg per rat in 0.25% Evan's blue dye. The PCA titer of the murine reaginic anti-R and anti-OA sera as determined separately on challenge with a dose of 500 µg of R or OA (dry weight) was 640 and 1,280 respectively.

* For control Allergen C was incubated at 30°C for 5-6 hrs in the absence of enzyme. A dose of 200 µg was used to challenge sensitized animals.

- refers to an absence of PCA reactions.

treatment with αchymotrypsin, was several dilutions lower as compared with the PCA titer elicited with the native Allergen C. Treatment with papain had no detectable effect on the ability of Allergen C to elicit PCA reactions. Allergen C, after treatment with each of these enzymes, was capable of combining with the murine IgE antibodies and inhibiting the PCA reactions elicitable with R and the murine reaginic anti-R sera (Table XI).

Antigenic analysis of enzyme treated Allergen C, by immunodiffusion employing a rabbit precipitating antiserum to Allergen C, revealed that the ability of trypsin and αchymotrypsin treated Allergen C to form precipitin bands with rabbit anti-Allergen C was unaffected; reactions of identity were formed with native Allergen C (Figure 18). On the other hand, papain treated Allergen C did not form a detectable precipitin band with the antiserum.

## DISCUSSION

Allergen C was isolated from the retentate of KBG aq. ext. by a combination of techniques involving Prep-ISO-EF and gel filtration. Allergen C was found to possess a pI value of 9.7 and a molecular size* of 11,000 daltons. Its molecular size is similar to Ra3 but its pI value is higher than the basic allergens of ragweed Ra3, Ra4 and Ra5 (pIs range, 8.0 - 9.6). Unlike the ragweed basic allergens (Ra3 and Ra4 may contain a very small amount of carbohydrate), Allergen C is a glycoprotein containing 500 µg of carbohydrate per mg of protein. It possessed all the natural occurring amino acids

* The molecular size of Allergen C was at best a rough estimate due to its high carbohydrate content and the fact that protein standards were employed in the estimation of molecular size.

TABLE XI

INHIBITION OF PCA BY ENZYME TREATED ALLERGEN C

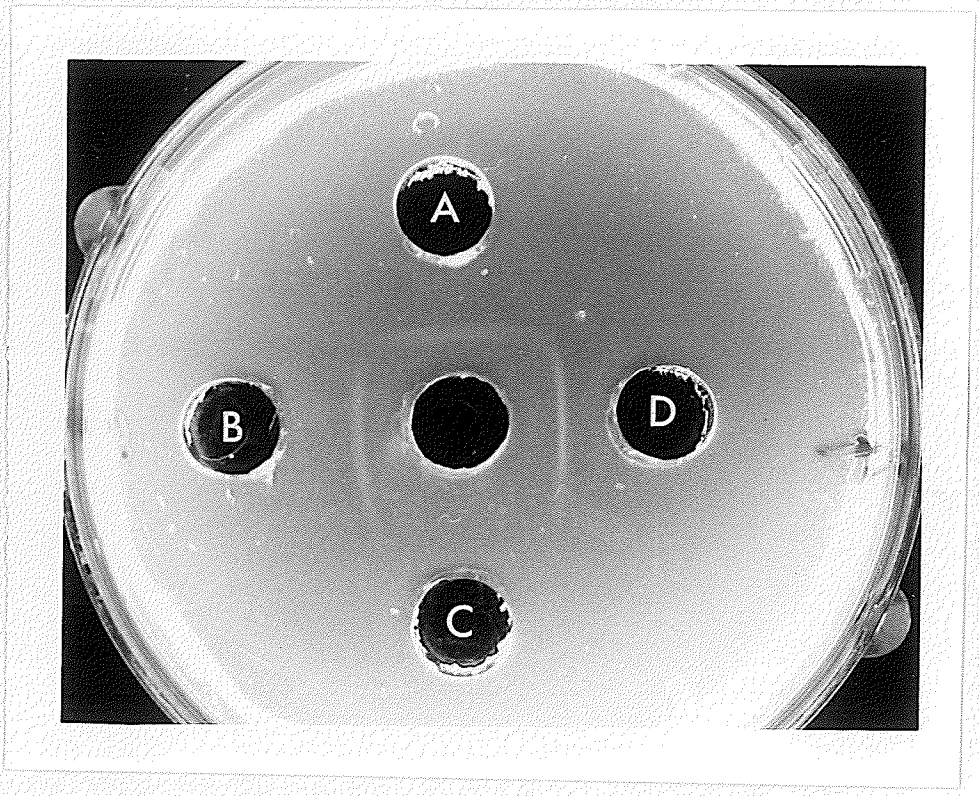
| TREATMENT | CONCENTRATION OF ENZYME TREATED ALLERGEN C* INCUBATED WITH MURINE ANTI-R REAGINIC SERA | | |
|---|---|---|---|
| | 100 µg/ml | 500 µg/ml | 1 mg/ml |
| Trypsin | + | + | - |
| α Chymotrypsin | + | + | - |
| Papain | + | + | - |

For this study the murine anti-R reaginic sera at a final dilution of 1:500 was incubated with the indicated amounts of Allergen C for 2-3 hrs. before intradermal sensitization of rats. Twenty-four hrs. later the rats were challenged intravenously with a solution containing 1 mg R in 0.25% Evan's blue dye.

+ refers to a positive PCA reaction.
- refers to an absence of PCA reaction.

* The titer of the murine anti-R reaginic sera evaluated with R at a dose of 1 mg (dry weight) was 1,280.

Figure 18. Immunodiffusion analysis of enzyme treated Allergen C. The center well contained rabbit precipitating anti-Allergen C serum.
Well A contained trypsin treated Allergen C.
Well B contained α chymotrypsin treated Allergen C.
Well C contained papain treated Allergen C.
Well D contained native Allergen C.


A
B
D
C

with the exception of cysteine which was not detectable. A high proportion of asparctic acid and glutamic acid was found to be present. This observation would suggest that the pI value of Allergen C would have been lower than actually found. However, it must be emphasized that the natural form of glutamic acid and aspartic acid i.e. glutamine and asparagine, if indeed present in Allergen C, would during hydrolysis be converted to the acidic forms. It can be suggested, because of the high pI value obtained for Allergen C, that the neutral amino acids were present in higher proportion than the acidic forms. Since Allergen C was found to possess a high carbohydrate content (the nature of the carbohydrate at present has not been determined), the presence of basic amino sugars could also contribute to its high pI value.

It was demonstrated that better resolution of the eluted protein fractions was obtained when focused fraction 15 of R was chromatographed on Bio-Gel P-60 in the presence of a dissociating agent such as urea. The reason for better resolution in the presence of urea is not known; urea probably improved the resolution by dissociating non-covalent forces holding molecules together.

The allergenicity of Allergen C was established by its ability to elicit PCA reactions in rats sensitized with a murine reaginic anti-R sera and by its ability to neutralize completely (within the limitation of the technique) and specifically the PCA reactions elicitable with R and the murine anti-R sera. On this basis it was

concluded that Allergen C possessed all the allergenic specificities present in the original R that were recognized by the murine sera used in this study. This lends further support to the evidence presented in Chapter II that the actual number of different allergenic specificities present in R detectable by the murine reaginic anti-R sera may be quite limited. Thus, the IgE antibody response of the A/HeJ strain of mice was limited to the immunodominant allergenic determinant(s) of R recognized by this strain. The exact chemical nature of this immunodominant determinant(s) at present is not known. Evidence to suggest that an immunodominant determinant can possess a relatively simple structure was provided by the studies of Malley et al. (1975) who reported that allergen $D_3$, isolated from the aq. ext. of

timothy pollen, contained at least 78% of the allergenic specificities present in the whole water soluble extract of timothy pollen. $D_3$ was found to be composed of flavonoid pigment, a sugar moiety of cellobiose and at least one amino acid threonine.

Allergen C, at a dose of 500 µg, elicited a PCA titer of 640. By comparison, a titer of 1,280 was elicited with R at a dose of 500 µg. Nevertheless, Allergen C was capable of completely ( within the limitation of the technique discussed in chapter II ) and specifically inhibiting the PCA reactions elicitable with R and murine reaginic anti-R sera at a concentration of 500 µg/ml. By comparison, R at a concentration of 50 µg/ml (Chapter II) was capable of inhibiting the PCA reactions. These results suggested that Allergen C possessed all the qualitative allergenic specificities in R; it probably possessed quantitatively fewer allergenic determinants since 10 times more Allergen C was required to neutralize the murine anti-R IgE antibodies. With respect to the pool of human allergic sera. Allergen C did not possess all the allergenic specificities present in R; the maximum degree to which Allergen C could inhibit RAST was of the order of 78%. An examination of the results presented in Figure 13 indicated that the slope of the inhibition curve obtained with Allergen C was lower than that obtained with either R or focused fraction 15 of R. To account for this decrease in allergenic potency in terms of the inhibition of RAST, it can be suggested that during the isolation of Allergen C from

focused fraction 15 of R, the isolation procedures had resulted in the removal of some allergenic and/or haptenic components. Focused fraction 15 of R was found to contain additional components that could not elicit PCA reactions in rats sensitized with the murine reaginic anti-R sera; nevertheless, these components could inhibit the PCA reactions elicitable with the R and anti-R reaginic system(results not shown in this thesis). Since these components could combine with murine IgE antibodies, it's conceivable that they could also combine with human IgE antibodies. Although Allergen C was shown to be immunochemically pure(see following pages), it was clear that it lacked some of the allergenic determinant(s) present in the components collectively present in focused fraction 15 of R and R. Thus, it would appear that there were two divergent conclusions as to the allergenic specificities present in Allergen C which were detectable by the murine reaginic sera on one hand and the human allergic sera on the other. The reasons for this difference in the allergenic specificities detected by these two sera have been discussed in Chapter II of this thesis.

The basic ragweed allergens (Ra3, Ra4 and Ra5) are minor allergens i.e. only a small percentage of ragweed sensitive individuals are sensitive to these allergens. At present the proportion of KBG sensitive individuals that are sensitive to Allergen C has not been determined. Using the pool of human allergic sera in the inhibition of RAST studies, it can be concluded that Allergen C possessed 78% of the allergenic specificities present on R with respect to this pool of human allergic sera.

The criteria of purity for Allergen C were established on the basis of isoelectrofocusing and crossed immunoelectrophoresis. Anal-ISO-EF revealed the presence of only cathodally focusing component(s). Other components, if any, that possessed a higher pI value than the maximum pH established on the pH gradient of the gel would have migrated out of the gel into the cathodal electrolyte solution and consequently not visualized. This possibility, however, was unlikely because Allergen C was found to possess a pI value of 9.7; this value was determined directly on the preparative gel. The maximum pH obtained on the pH gradient on the preparative gel was 10.7, and the maximum pH obtained on the analytical gel was 10.

Crossed immunoelectrophoretic analysis of Allergen C employing a hyperimmune sheep precipitating antiserum to R revealed the presence of one cathodally migrating antigenic component. By comparison, 12-15 antigens can be detected

on R by this method. Crossed immunoelectrophoretic analysis of R employing a rabbit precipitating antiserum to Allergen C revealed one major cathodally migrating antigen with the possibility of one or two additional immunoprecipitin bands localized at the center of the gel. These immunoprecipitin bands may represent some antigens which share cross-reacting determinants with Allergen C. Crossed immunoelectrophoretic analysis of Allergen C employing the rabbit precipitating antiserum to Allergen C revealed as expected, one antigenic component. This rabbit anti-Allergen C serum was obtained only after 2 weeks after one immunization of the animal with Allergen C; therefore, it does not provide much useful information regarding purity. The most stringent test for purity is to employ hyperimmune serum, in the crossed immunoelectrophoretic analysis, obtained by repeatedly immunizing animals over a course of 1-2 years (Løwenstein, 1979). Repeated immunization would lead to production of antibodies to any impurities that might be present in a purified antigenic preparation. In this way other antigenic components, if present, in addition to the major antigen, can be detected by employing the hyperimmune serum in the crossed immunoelectrophoretic analysis.

In spite of many attempts to isolate pure allergens, absolute purity has rarely been attained. The conclusions as to the degree of purity of an allergen is only as good

as the method used in the analysis. The fact that impurities have not been detected by a certain method does not necessarily mean that no impurities are present. What is considered to be pure by one method of analysis may not be considered as pure by another method depending on the resolving capability of the method employed. With the development of more sophisticated techniques, what is considered to be pure today may not be considered as pure tomorrow. Thus, with this consideration in mind, it may be said that Allergen C is pure in terms of its homogeneity of net charge as revealed by Anal-ISO-EF, it consists of one antigen as detected by CIE analysis employing a hyperimmune sheep precipitating antiserum to R; and it was homogeneous with respect to its molecular size as determined by gel filtration on Bio-Gel P-60.

Allergen C may contain some of the immunodominant constituents, with respect to the induction of precipitating antibodies in rabbits, present in R. This conclusion was arrived at on the basis of the short time interval required in the induction of precipitating antibodies in these animals. By comparison, other allergens isolated from KBG pollen (Kum, 1979; Ekramouddoulah et al., 1978) required repeated immunization in rabbits over a period of 2-3 months before antibodies to these allergens can be detected.

Protease treatment of Allergen C resulted in complete

loss of allergenic activity. However, Allergen C was remarkably stable to acid, base, moderate heat, and guanidine hydrochloride treatment. This suggests that the allergenic activity is associated with the stable protein moiety of the molecule. This is consistent with observations on the stability of most grass pollen allergens. On the other hand, the allergenic activity of ragweed antigen E, is labile to denaturation apparently because of its structure of 2 polypeptide chains.

Trypsin, αchymotrypsin and papain treatments of Allergen C have provided some insight into the allergenic and antigenic properties of the allergen. Treatment of Allergen C with trypsin resulted in the generation of hapten-like components i.e. products of the trypsin digest could not elicit PCA reactions in rats passively sensitized with a murine reaginic anti-R sera but they could inhibit this PCA reaction normally elicitable with R. However, the antigenic determinants recognized by the rabbit precipitating anti-Allergen C serum did not appear to be affected. Thus in Allergen C, those peptide linkages involving the carbonyl groups of arginine and lysine may be important in the elicitation of PCA reactions. Chymotrypsin treatment of Allergen C led to a reduced ability of Allergen C to elicit PCA reactions, but did not appear to affect the antigenic determinants suggesting that the peptide

linkages involving the carbonyl groups of aromatic amino acid residues may also play a role in the elicitation of PCA reactions. Treatment of Allergen C with papain had no detectable effect on its allergenic activity; however, its antigenic determinants were affected as indicated by its inability to form precipitin reaction with rabbit anti-Allergen C serum.

At present the exact chemical composition of Allergen C has not been elucidated. The effect of these enzyme treatments on the conformational and or sequential determinants of Allergen C is not known. It appears that the allergenic determinants recognized by the murine IgE antibodies are different from the antigenic determinants recognized by the rabbit IgG antibodies. Allergen C was employed in further studies designed to investigate the relationship between allergenicity and antigenicity. The results of this study are presented in Chapter IV of this thesis.

# CHAPTER IV

# ALLERGENIC AND ANTIGENIC RELATIONSHIPS OF TWO ALLERGENS ISOLATED FROM KENTUCKY BLUE GRASS POLLEN

## INTRODUCTION

Allergens are defined by their capability to provoke allergic type I reactions in allergic individuals. Apart from this distinction in the biological property, there are no obvious or unique chemical features that can be used to distinguish an allergen from an antigen. Most allergens isolated fall within certain range of molecular size (20,000 - 40,000); the lower limit for the molecular weight of allergens is determined by the molecular capability to be structurally complex enough to be immunogenic and the upper limit of molecular weight is determined by their ability to penetrate mucosal membranes (Crumpton, 1974). The ability of a substance to act as an allergen is modulated by the genetic predisposition of an individual to form IgE antibodies following stimulation with low doses of the substance (Marsh, 1975). Although allergens have been regarded as "special" antigens which induce the formation of IgE antibodies, there is no evidence to suggest that

in a natural course of induction of an allergic condition, the determinants which are responsible for the induction of IgE antibodies are different from those determinants which are responsible for the induction of IgG antibodies. In fact there is some evidence to suggest that the IgG and IgE antibodies in ragweed sensitive individuals may be recognizing the same determinants in the ragweed pollen aqueous extract (Leiferman et al., 1979). However, in experimental conditions when immunizations are given using different adjuvants or different species of animals, the determinants that are responsible for the induction of IgE antibodies in one species may be different from the determinants that are responsible for the induction of IgG antibodies in another. In the present study (Chapter III), the determinants of Allergen C recognized by murine IgE antibodies were found to be different from the determinants recognized by the rabbit IgG antibodies.

With the availability of two purified KBG allergens, Allergens C (Chapter III) and KBG-1* (Ekramoddoulah et al., 1978), the relationship between the allergenic and antigenic properties of these two allergens was investigated employing murine reaginic antisera and rabbit precipitating antisera produced separately to each of these allergens; and a pool of human allergic sera from individuals sensitive to KBG pollen. Allergen KBG-1, isolated from the dialysate of KBG aq. ext. by a combination of methods

*KBG-I consisted of glycoprotein with molecular size corresponding to 10,000 daltons and of two components with closely related pI values (4.5-4.9). Its protein moiety contained all the amino acids except cysteine. Crossed Immunoelectrophoretic analysis employing a hyperimmune sheep precipitating anti-R serum revealed that KBG-I shared 2 common antigenic components with R.

involving ion-exchange chromatography, gel filtration, and Prep-ISO-EF is an anodally migrating allergen (pI value 4.7). A comparison of the Anal-ISO-EF and CIE analysis of these two allergens are illustrated by Figures 19 and 20 respectively. The Anal-ISO-EF and CIE analysis of KBG-I had already been reported (Ekramoddoullah et al., 1978).

## METHODS AND MATERIALS

### Production of Murine Reaginic Antisera to Allergens C and KBG-1

Murine reaginic antisera to Allergens C and KBG-1 were produced in A/HeJ mice using exactly the same procedures as described for the production of murine reaginic antisera to R (see Methods, Chapter II). 100 µg of each allergen adsorbed onto aluminum hydroxide gel was used to immunize the animals.

### Production of Rabbit Precipitating Antisera to Allergens C and KBG-1

Rabbit precipitating antisera to Allergen C were produced as described (see Methods, Chapter III). Rabbit precipitating antisera to Allergen KBG-1 was obtained after multiple (spaced over a period of approximately 3 months) intradermal immunization. A dose of 100-500 µg of Allergen KBG-I (per injection) emulsified with Freund's complete adjuvant was given to each rabbit.

### Analysis of Allergenic Cross Reactivity

The allergenic cross reactivity of Allergens C and KBG-1 was evaluated (i) in terms of their ability to elicit

Figure 19. Anal-ISO-EF of Allergens C and KBG-1 in polyacrylamide gel employing ampholine in the pH range of 3.5 - 10. (-) refers to the cathodal end of the gel and (+) refers to the anodal end.

ANALYTICAL ISOELECTROFOCUSING IN
POLYACRYLAMIDE GEL, pH 3.5-10

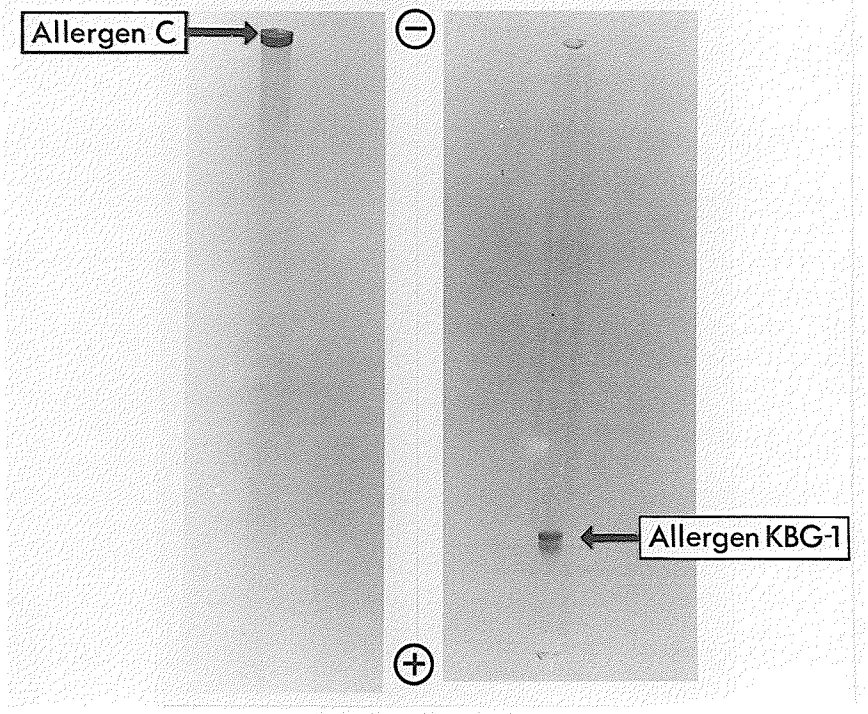
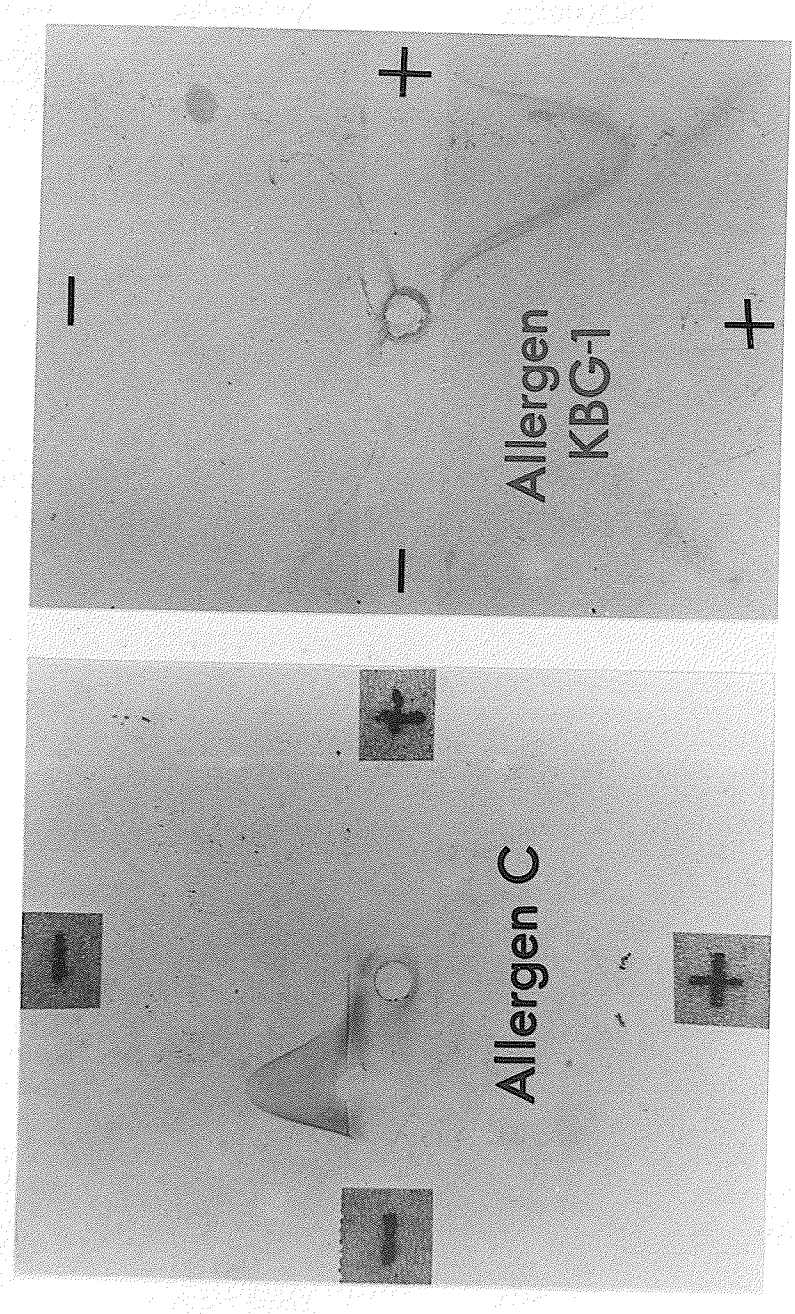


Figure 20. Crossed immunoelectrophoretic analysis of Allergens C and KBG-1 employing a hyperimmune sheep precipitating antiserum to R of KBG pollen. The - and + signs refer to the cathodal and anodal ends of the gel in the 2 dimensional electrophoresis as described in Methods.


Allergen
KBG-1
Allergen C

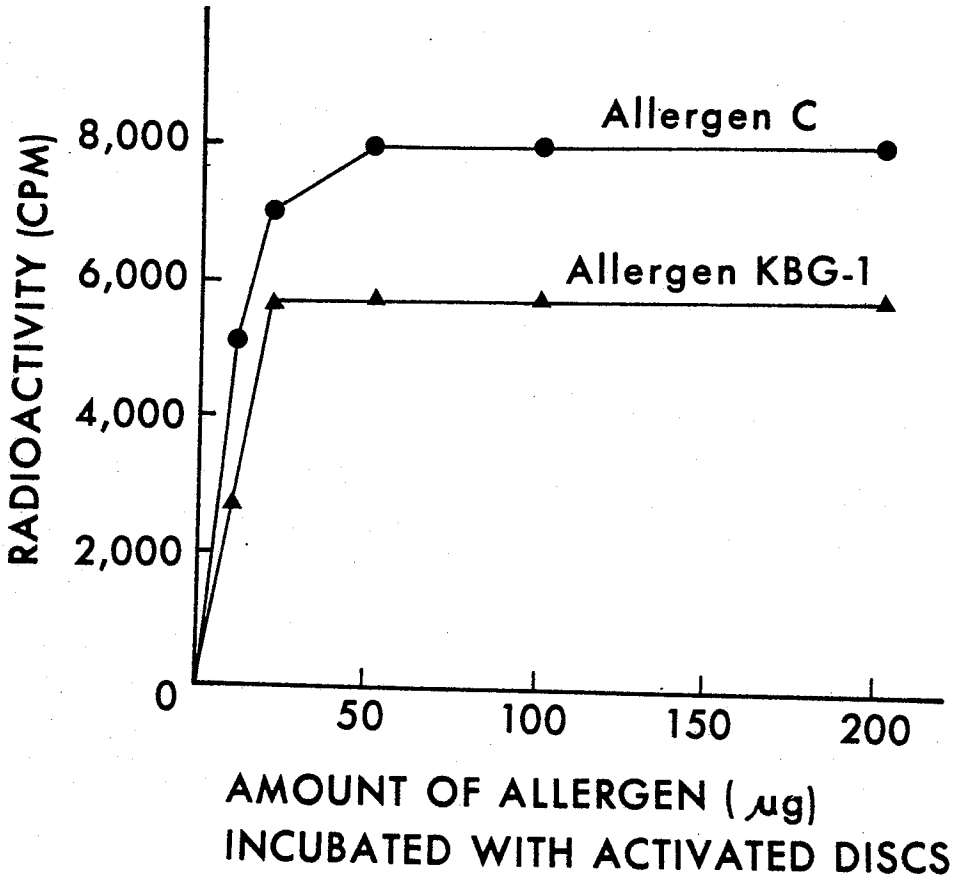
PCA reactions in rats sensitized with a murine reaginic sera to R, Allergens C and KBG-1 (ii) in terms of their ability to neutralize the PCA reactions employing these murine reaginic sera and (iii) in terms of their ability to neutralize human IgE antibodies to these allergens in the inhibition of RAST method. The PCA and inhibition of PCA were performed according to procedures described in Methods, Chapter II of this thesis.

Allergosorbent discs, to be employed in the inhibition of RAST, were prepared by coupling Allergen C or Allergen KBG-1 to cyanogen bromide activated cellulose discs and the RAST was performed essentially as described in Chapter II. The optimum amount of Allergens C or KBG-1 used for the preparation of the allergosorbent disc (solid phase allergen) which would bind the maximum quantity of IgE antibodies present in a volume of 50 µl of the pool of human allergic sera, was established by varying the amount of each allergen incubated with the activated cellulose disc. This amount was found to be 50 µg (Figure 21). For the preparation of solid phase Allergen C and Allergen KBG-1, an excess of each allergen in the amount of 100 µg was incubated with each activated cellulose disc. Homologous and heterologous allergens were then used to inhibit the binding of IgE antibodies to each of the solid phase allergen.

Analysis of Antigenic Cross Reactivity by Immunodiffusion

Immunodiffusion analysis of antigenic cross reactivity

Figure 21. Determination of the amount of Allergens C or KBG-1 required to prepare allergosorbent discs capable of binding the maximum amount of IgE antibodies present in a pool of human allergic sera. Activated cellulose discs were incubated with various amounts of Allegens C or KBG-1. The amount of IgE antibodies bound to the allergosorbent discs, after incubation of these discs with the human allergic sera, was evaluated by the addition of $I^{125}$ labelled rabbit anti-human IgE antibodies.


Allergen C
Allergen KBG-1
RADIOACTIVITY (CPM)
8,000
6,000
4,000
2,000
0
50
100
150
200
AMOUNT OF ALLERGEN (μg)
INCUBATED WITH ACTIVATED DISCS

on agar employing rabbit precipitating antisera was performed according to procedures described in Chapter III.

Radio-Labelling of Allergens C and KBG-1 with $I^{125}$

Allergens C and KBG-1 were labelled with $I^{125}$ according to the procedures of Hunter and Greenwood (1962). 1 mC of carrier free $NaI^{125}$ (Amersham Corp., Oakville, Ontario) was diluted with PBS to a final volume of 0.1 ml and added to a 0.1 ml solution of PBS containing 200 µg of Allergen C or Allergen KBG-1. Next, a volume of 0.1 ml of a solution of chloramine T (1 mg/ml in 0.1 M phosphate buffer, pH 7.4) was added followed by immediate mixing. The reaction mixtures were allowed to stand at room temperature for 2 1/2 minutes after which time a volume of 0.1 ml of a solution of sodium metabisulphite (2 mg/ml in 0.1 M phosphate buffer pH 7.4) was added to stop the reaction. To remove free $I^{125}$ the mixtures were then dialyzed against distilled water extensively through Spectrapor membrane (molecular cut off size of 3,500).

Radioimmunoassay (RIA) Analysis of Antigenic Cross Reactivity

Antigenic cross reactivity between Allergens C and KBG-1 was tested on the basis of the ability of one allergen to inhibit the combination of the other $I^{125}$ labelled allergen with its homologous rabbit precipitating antiserum i.e. to test if one allergen would combine with precipitating

antibodies raised against another allergen. For this purpose, a titration curve was first established for each $I^{125}$ labelled allergen with its homologous rabbit antiserum. The antigen-antibody complexes formed were precipitated by 33% saturated $(NH_4)_2SO_4$. 10 µl of $I^{125}$ labelled Allergens C or KBG-1 (approximately 100,000 cpm) was added separately to a serial dilutions of its homologous antiserum prepared in normal rabbit serum diluted 1:10 in PBS. This normal rabbit serum did not contain any antibody activity to Allergen C or KBG-1 as detected by immunodiffusion and neither of these labelled allergens were precipitated by the $(NH_4)_2SO_4$ solution when the normal rabbit serum was employed in the RIA procedures. The final volume of each dilution before the addition of labelled allergen was 200 µl. The tubes were shaken at 200 RPM on a continuous horizontal shaker at room temperature for 2 hours after which time 2 10 µl of 66% saturated solution of $(NH_4)_2SO_4$ was added to each tube. The tubes were allowed to stand at $4^{o}C$ overnight. The precipitates formed were washed twice with 33% saturated $(NH_4)_2SO_4$ and recovered by centrifugation at 1,800 RPM for 15 minutes (International Centrifuge, Head no. 259, Model PR-J). The cpm precipitated were determined with a Beckman gamma counter. A titration curve was established for each allergen and its homologous antiserum by plotting the cpm in the precipitates versus the dilution of the antisera.

For the antigenic cross reactivity studies, 10 µl of each labelled allergen (approximately 100,000 cpm) was added to its homologous antiserum at one dilution (mid-point of the titration curves). The cpm precipitated by the $(NH_4)_2SO_4$ solution was taken as the maximum cpm precipitable under these conditions. Unlabelled allergens (homologous and heterologous) were then employed to inhibit the cpm precipitable by $(NH_4)_2SO_4$ of the complexes formed between labelled allergen and its homologous antiserum. For this purpose, unlabelled allergens in various amounts were preincubated with the antiserum at a fixed dilution (1:100) at room temperature for 2 hours at 200 RPM on a continuous horizontal shaker before the addition of labelled allergen (10 µl , approximately 100,000 cpm). The reduction in cpm from the maximum cpm precipitable in this system was expressed as per cent inhibition of the precipitation by unlabelled allergen.

## RESULTS

### Analysis of Allergenic Cross Reactivity

(i) Elicitation of PCA: R elicited the highest PCA titers obtained with all three murine reaginic sera (Table XII). Allergen C and Allergen KBG-1 could elicit the PCA reactions regardless of the murine antisera used to sensitize rats; however, the titers elicited were lower than the titers elicited with R. Neither R, nor Allergen C and

Table XII

PCA TITERS ELICITED IN RATS PASSIVELY SENSITIZED INTRADERMALLY WITH MURINE REAGINIC ANTISERA TO R, ALLERGENS C AND KBG-1 ON CHALLENGE WITH R, ALLERGENS C AND KBG-1

| MURINE REAGINIC SERA | PCA TITERS ON CHALLENGE WITH R | ALLERGEN C | ALLERGEN KBG-1 |
|---|---|---|---|
| ANTI-R | 1,280 | 320 | 640 |
| ANTI-ALLERGEN C | 1,280 | 640 | 640 |
| ANTI-KBG-1 | 1,280 | 320 | 640 |
| ANTI-OA | NIL | NIL | NIL |

Hooded rats were passively sensitized by intradermal injections of a serially diluted murine: (a) anti-R, (b) anti-Allergen C, (c) anti-KBG-1 and (d) anti-OA reaginic sera. Twenty-four hours later, two of the rats sensitized in an identical manner were challenged with one of the allergenic preparations at a dose of 500 µg in 0.25% Evan's blue dye, injected intravenously. The PCA titer of the murine reaginic anti-OA sera determined with 1 mg OA (dry weight) was 1,280.

Allergen KBG-1 could elicit PCA reactions with an unrelated murine reaginic serum such as anti-OA.

(ii) Neutralization of PCA: Each Allergen could neutralize the PCA reactions elicitable with its homologous murine reaginic antisera at concentration of 500 µg/ml (Table XIII). In the cross neutralization of PCA experiments employing murine reaginic sera to Allergen C and Allergen KBG-1, Allergen C at a dose of 500 µg/ml could completely neutralize the PCA reactions in rats sensitized with the anti-KBG-1 murine reaginic sera upon subsequent challenge with R. Allergen KBG-1 at a dose of 200 µg/ml could completely neutralize the PCA reactions in rats sensitized with the anti-Allergen C murine reaginic sera upon subsequent challenge with R. R was used as the challenging allergen because it was readily available and always elicited a higher PCA titers with the anti-Allergen C or anti-Allergen KBG-1 reaginic sera than the titers obtained with the homologous allergens. Allergen C and Allergen KBG-1 had no effect on the PCA reactions of a non-related murine reaginic system of OA and anti-OA.

(iii) Inhibition of RAST: Analysis of allergenic cross-reactivity by the inhibition of RAST procedure employing a pool of human sera from individuals sensitive to KBG pollen revealed that Allergens C and KBG-1 shared common allergenic determinant(s). Allergen KBG-1 could inhibit the binding of human IgE antibodies to solid phase Allergen C

Table XIII

EVALUATION OF THE ABILITY OF ALLERGENS C AND KGB-1 TO CROSS NEUTRALIZE MURINE REAGINIC SERA PRODUCED TO THESE TWO ALLERGENS

| ALLERGEN MURINE REAGINIC ANTISERA SYSTEM | CONCENTRATION OF ALLERGEN INCUBATED WITH MURINE REAGINIC ANTISERA | | | | |
|---|---|---|---|---|---|
| | 5 µg/ml | 50 µg/ml | 200 µg/ml | 500 µg/ml | 1 mg/ml |
| ALLERGEN C-ANTI-C | + | + | + | - | - |
| ALLERGEN KBG-1-ANTI-KBG-1 | + | + | + | - | - |
| ALLERGEN C-ANTI-KBG-1 | + | + | + | - | - |
| ALLERGEN KBG-1-ANTI-C | + | + | - | - | - |
| ALLERGEN C-ANTI-OA | + | + | + | + | + |
| ALLERGEN KBG-1-ANTI-OA | + | + | + | + | + |

The procedure for the neutralization is described in Methods (Chapter II). The PCA of the murine anti-Allergen C and the murine anti-KBG-1 antisera established in rats with R at a dose of 1 mg (dry weight) was 1,280. For the neutralization experiments, these antisera were used at a final dilution of 1:400. For positive control, the reaginic antisera at a final dilution of 1:400 in the absence of allergens were used for the intradermal sensitization of rats. The PCA reactions, if any, were elicited by challenging the animals with 1 mg R (dry weight) in 0.25% Evan's blue dye. All the controls exhibited positive PCA reactions.

The titer of the murine anti-OA reaginic sera was 1,280 as determined with a challenging dose of 1 mg OA (dry weight). This antisera was used at a final dilution of 1:400 in the test for specificity of PCA neutralization with Allergens C and KBG-1. The PCA reactions were elicited in these animals with 1 mg OA (dry weight) in 0.25% Evan's blue dye.

\+ refers to a positive PCA reaction.
\- refers to an absence of PCA reaction.

to the extent of 20%; and Allergen C could inhibit the binding of human IgE antibodies to solid phase Allergen KBG-1 to the extent of 16 % (Figure 22).

Analysis of Antigenic Cross Reactivity

(i) Immunodiffusion Analysis: The results of the immunodiffusion analysis of Allergens C and KBG-1 employing rabbit precipitating antisera produced separately to each of these allergens are illustrated in Figure 23. When anti-Allergen C and anti-Allergen KBG-1 were placed in separate antiserum wells and diffused against R*, a reaction of non-identity was observed. When a mixture of the rabbit antisera to Allergens C and KBG-1 was placed in a central antiserum well surrounded by antigen wells containing R, the presence of 2 distinct antigen-antibody systems was observed.

(ii) RIA Analysis: A RIA procedure was employed to further investigate the apparent antigenic non-identity of Allergens C and KBG-1. The titration curves for $I^{125}$ labelled Allergens C and KBG-1 with their homologous rabbit antisera are illustrated in Figure 24. Each antiserum at a final dilution of 1:100 was employed for the inhibition studies i.e. to evaluate the degree to which the addition of one of the unlabelled allergens would inhibit the binding

---

* R was used in the antigen well because R was readily available and contained antigenic determinants in common with both Allergens C and KBG-1.

Figure 22. Inhibition of RAST by Allergens C and KBG-1 employing (a) solid phase Allergen C and (b) solid phase Allergen KBG-1. A pool of human sera from individuals allergic to KBG pollen was employed in these studies.

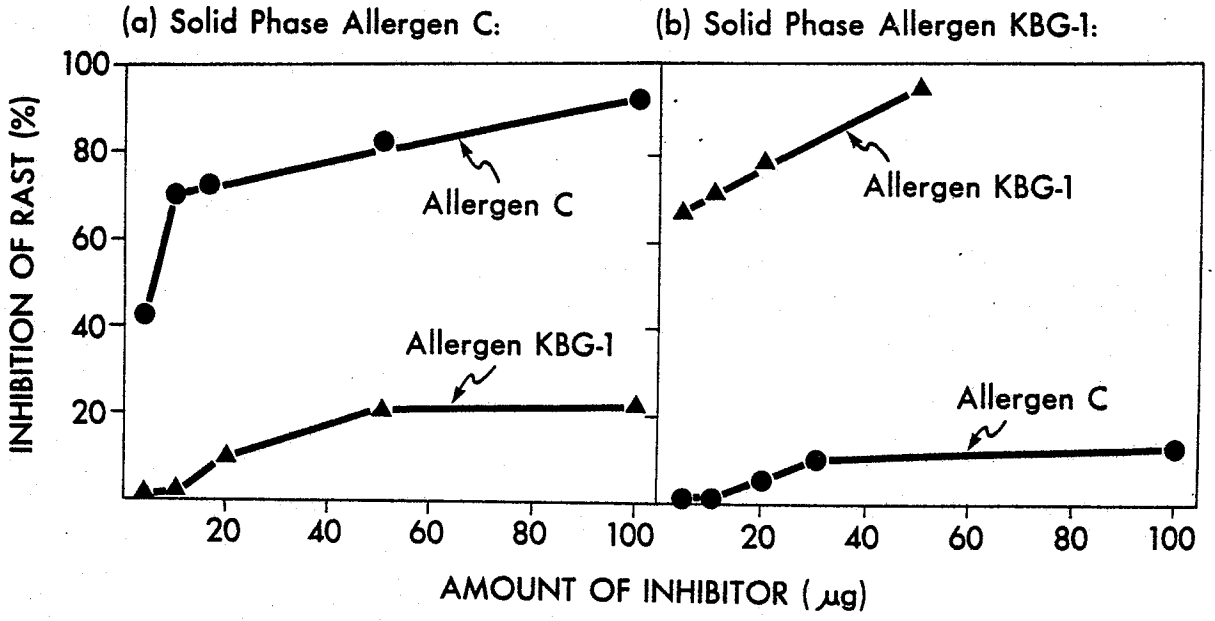

(a) Solid Phase Allergen C:
(b) Solid Phase Allergen KBG-1:
INHIBITION OF RAST (%)
100
80
60
40
20
Allergen C
Allergen KBG-1
Allergen KBG-1
Allergen C
20
40
60
80
100
20
40
60
80
100
AMOUNT OF INHIBITOR (μg)

Figure 23. Immunodiffusion analysis of antigenic cross-reactivity in agar gel employing rabbit precipitating antisera to Allergens C and KBG-1. R at a concentration of 5 mg/ml was employed in the antigen wells.

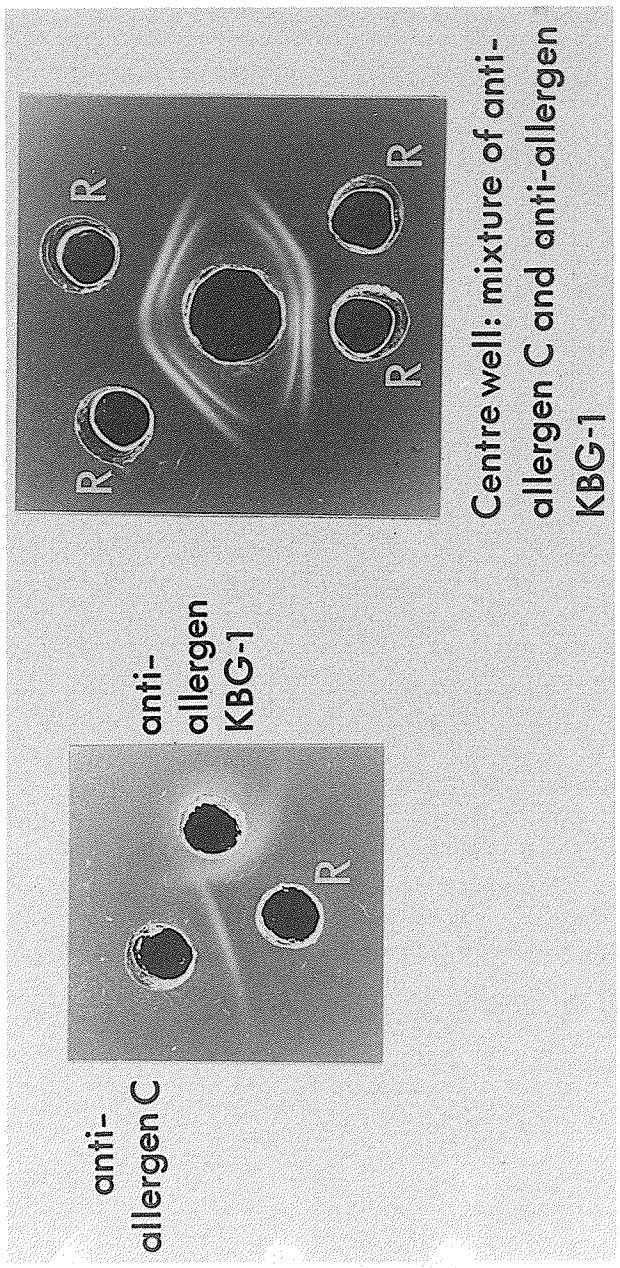
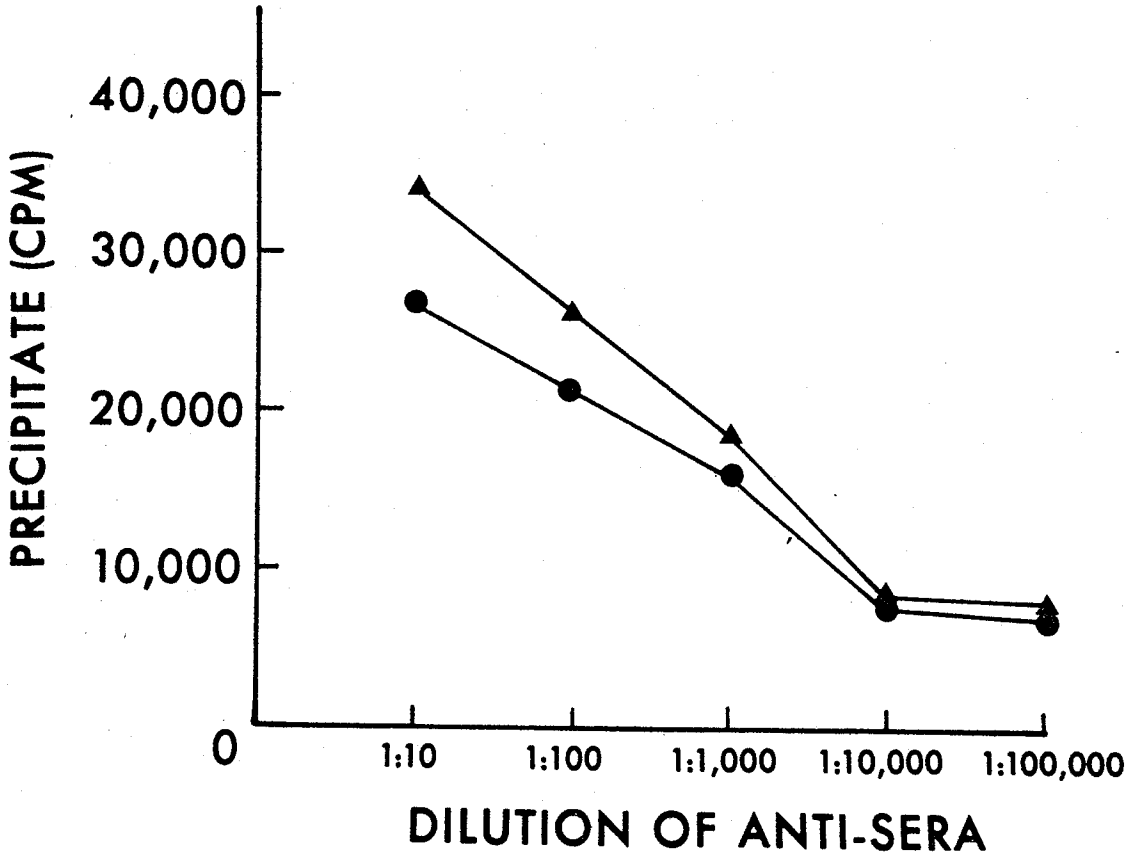

anti-
allergen C
anti-
allergen
KBG-1
R
R
R
R
R
Centre well: mixture of anti-
allergen C and anti-allergen
KBG-1

Figure 24. Titration curves established for rabbit anti-Allergens C and KBG-1 with their homologous allergens. $I^{125}$ labelled Allergens C and KBG-1 (approximately 100,000 cpm) were separately incubated with the homologous rabbit antisera at various dilutions. The antigen-antibody complexes were precipitated by addition of a solution of $(NH_4)_2SO_4$ (final concentration 33% saturated) and the radioactivity (cpm) in the precipitates was measured. Solid circles represent the titration curve for anti-Allergen C and Allergen C system. Solid triangles represent the titration curve for anti-Allergen KBG-1 and Allergen KBG-1 system.

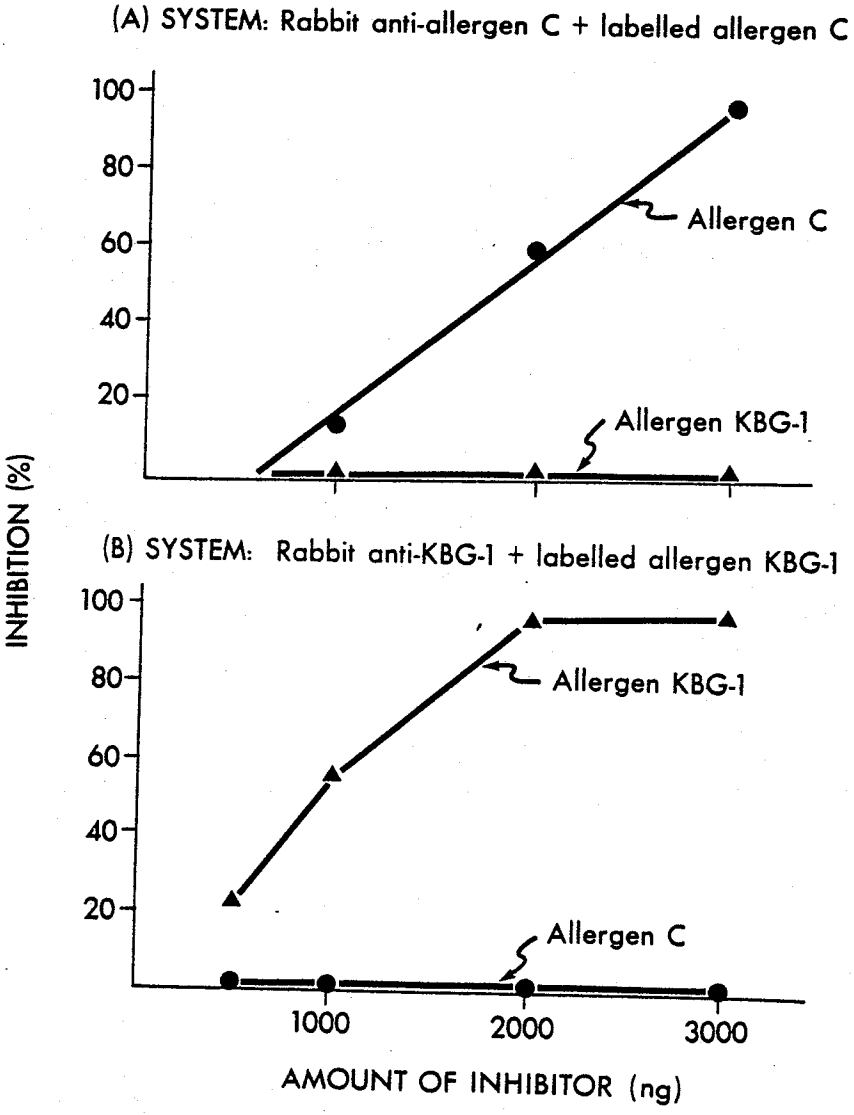

40,000
30,000
20,000
10,000
0
PRECIPITATE (CPM)
1:10
1:100
1:1,000
1:10,000
1:100,000
DILUTION OF ANTI-SERA

of labelled allergens to the homologous antisera. Unlabelled Allergen C inhibited the precipitation of $I^{125}$ labelled Allergen C by the rabbit anti-Allergen C serum to an extent of 100% in a linear fashion (Figure 25). Allergen KBG-1 was completely ineffective in inhibiting the precipitation of Allergen C in this system.

With the $I^{125}$ labelled Allergen KBG-1 and rabbit anti-KBG-1 serum system, Unlabelled Allergen KBG-1 could inhibit the precipitation of labelled Allergen KBG-1 by the rabbit anti-KBG-1 serum to an extent of 100% (Figure 25). A linear inhibition curve was obtained. Allergen C could not inhibit the precipitation of labelled Allergen KBG-1 in this system. Thus on the basis of these observations, it can be concluded that Allergens C and KBG-1 did not possess cross-reactive antigenic determinants with respect to these 2 rabbit antisera.

In Chapter II, the studies on the immunological specificity of the hapten-like components was made in relation to those specificities detected by IgE antibodies. Attempts to inhibit the precipitation of R with the sheep anti-R serum by haptenic components isolated from KBG aq. ext. were unsuccessful. Pooled haptenic fractions of $D_{24}$ and R in various amounts were incubated with the anti-R serum at room temperature (with continuous shaking) for 2-3 hours prior to adding the mixtures to the antiserum well in the immunodiffusion plate. Immunodiffusion in agar was

Figure 25. RIA analysis of antigenic cross-reactivity. Increasing amounts of unlabelled allergens was employed to inhibit the precipitation obtainable with a fixed amount of $I^{125}$ labelled allergen with its homologous rabbit antiserum (final dilution 1:100).


(A) SYSTEM: Rabbit anti-allergen C + labelled allergen C
100
80
60
40
20
Allergen C
Allergen KBG-1
INHIBITION (%)
(B) SYSTEM: Rabbit anti-KBG-1 + labelled allergen KBG-1
100
80
60
40
20
Allergen KBG-1
Allergen C
1000
2000
3000
AMOUNT OF INHIBITOR (ng)

carried out as described (see Methods, Chapter III) with R in the antigen wells. Inhibition of precipitation of R with the anti-R serum was not detected. With the availability of $I^{125}$ labelled Allergens C and KBG-1, it was decided to investigate the antigenic relationship between these haptenic components and the two allergens. Attempts were made to inhibit the precipitation of labelled Allergens C and KBG-1 with their homologous antisera by pools of haptenic components isolated from $D_{24}$ and R. Maximum inhibition of the precipitation of $I^{125}$ labelled Allergen KBG-1 with its homologous antiserum achieved by haptenic components of $D_{24}$ and R was of the order of 30%and 20% respectively (Figure 26). Haptenic components of $D_{24}$ and R were completely ineffective in inhibiting the precipitation of $I^{125}$ labelled Allergen C with its homologous antiserum (Figure 27).

## DISCUSSION

Allergens C and KBG-1 appeared to be allergenically identical with respect to the specificities detected with murine IgE antibodies produced in separate groups of mice following immunization with the allergens in aluminum hydroxide. This conclusion was made on the basis of observations that Allergen C could completely neutralize the IgE antibodies produced to Allergen KBG-1 and Allergen KBG-1 could completely neutralize the IgE antibodies produced to Allergen C. The results of these experiments

Figure 26. Inhibition of precipitation of $I^{125}$ labelled Allergen KBG-1 with rabbit anti-KBG-1 serum by haptenic components of D24 and R of KBG pollen. Solid circles represent the inhibition curve obtained by employing non-labelled Allergen KBG-1. Open circles and squares represent the inhibition curves obtained by haptenic components of D24 and R respectively.

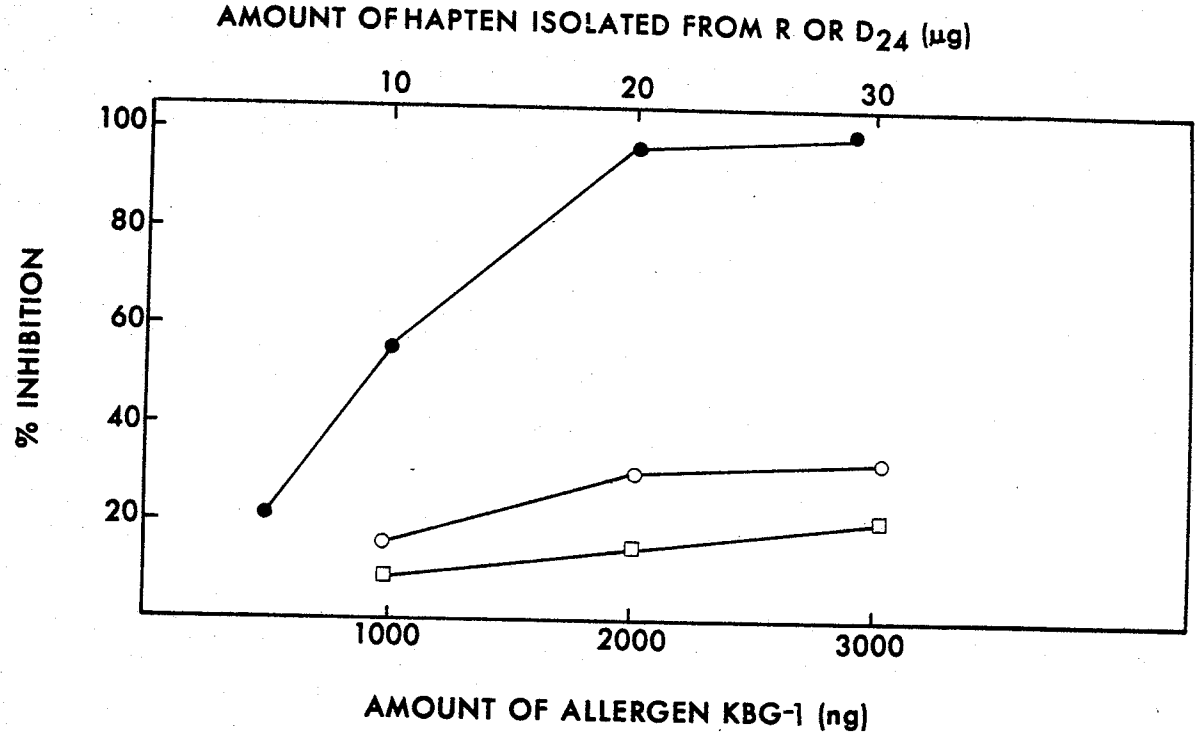
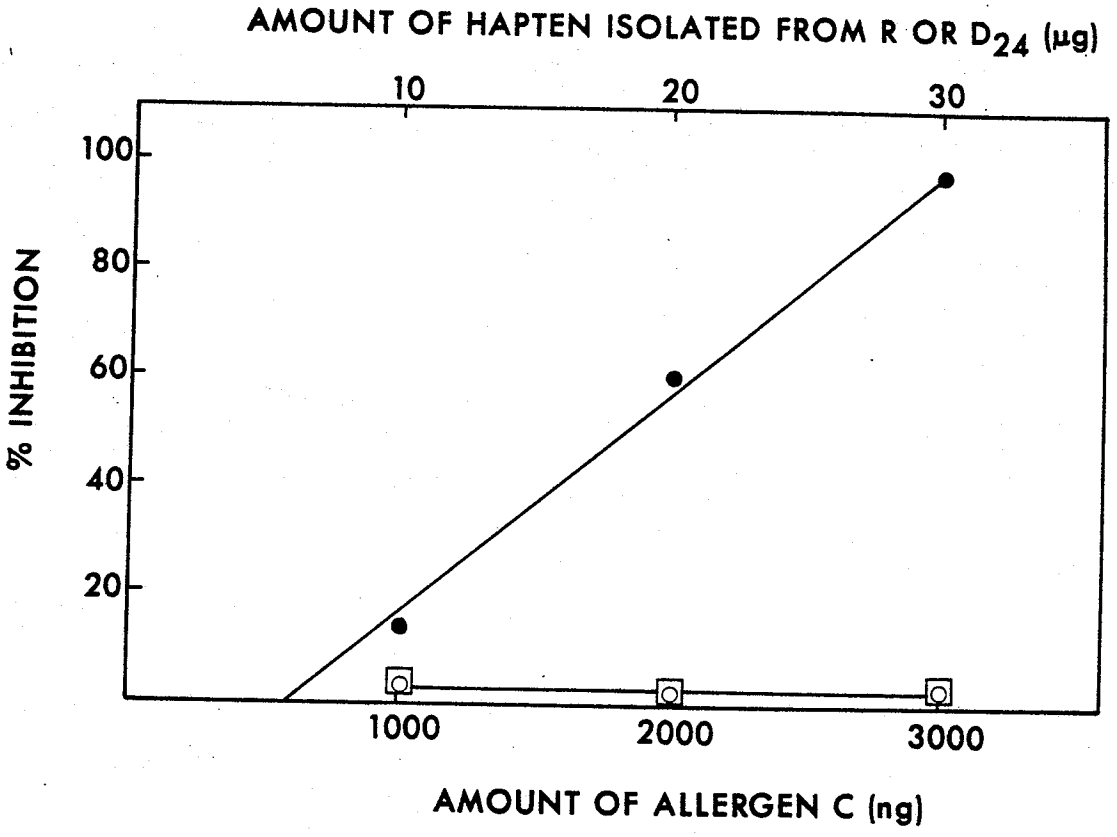

AMOUNT OF HAPTEN ISOLATED FROM R OR $D_{24}$ (μg)
10
20
30
100
80
60
40
20
% INHIBITION
1000
2000
3000
AMOUNT OF ALLERGEN KBG-1 (ng)

Figure 27. Inhibition of precipitation of $I^{125}$ labelled Allergen C with rabbit anti-Allergen C serum by haptenic components of D24 and R of KBG pollen. Solid circles represent the inhibition curve obtained by employing non-labelled Allergen C. Open circles and squares represent the inhibition curves for haptenic components of D24 and R respectively.


AMOUNT OF HAPTEN ISOLATED FROM R OR $D_{24}$ (μg)
10
20
30
100
80
60
40
20
% INHIBITION
1000
2000
3000
AMOUNT OF ALLERGEN C (ng)

further substantiated the conclusions made in Chapter II that one or two discrete components (detected by Anal-ISO-EF) isolated from R could completely neutralize the PCA reactions elicitable with the R anti-R murine system. It appears that the IgE antibody response in A/HeJ mice is induced by an allergenic determinant(s) common to many of the components present in KBG aq. ext. Thus,even though Allergens C and KBG-1 differed widely in their pI values,they appeared to possess an allergenic determinant(s) in common with each other which was immunodominant with respect to IgE production in this inbred strain of mice.

R was found always to be able to elicit higher PCA titers regardless of the murine reaginic antisera (produced to R, Allergens C or KGB-1) employed to sensitize rats. This suggested that R was more efficient than purified allergens for cross-linking IgE antibodies on mast cells; this may have been due to a larger number of components present in R which collectively possessed more of the common allergenic determinants than either Allergen C or Allergen KBG-1.

By contrast the allergenicity of Allergens C and KBG-1 evaluated with respect to the human IgE antibodies appeared to cross react only to a limited degree with each other. This pool of human allergic sera is of necessity obtained from an outbred population, and consequently it is reasonable

to expect that the human antisera recognized a greater number of allergenic determinants of Allergens C and KBG-1, in comparison with a restricted number of determinant(s) recognized by the murine sera. The results of these studies serve to illustrate the pitfalls of using an animal model system in the standardization of allergens if the animals' IgE antibodies recognize allergenic determinants which are different from the determinants recognized by human IgE antibodies.

Immunodiffusion analysis of Allergens C and KBG-1 employing rabbit precipitating antisera produced separately to each of these allergens revealed that these two allergens were antigenically unique. Using a more sensitive RIA method, it was confirmed that Allergens C and KBG-1 possessed unique antigenic determinants. The results of these studies support the conclusions made in Chapter III which suggested that the allergenic determinants recognized by the murine IgE antibodies in Allergen C were different from the antigenic determinants recognized by the rabbit IgG antibodies. Similar results have been reported for Ascaris suum allergens from which two proteins were isolated and found to be allergenically similar when tested with murine IgE antibodies; however, there was no evidence for antigenic cross reactivity when tested with a rabbit precipitating antiserum against the whole extract (Dandeu and Lux, 1978). It appears that the specificity of the IgE antibodies

elicited in one species of animal was different from the specificity of the IgG antibodies elicited in another species.

With the availability of the unique antigens, Allergens C and KBG-1, the antigenic relationship of haptenic components of $D_{24}$ and R to Allergens C and KBG-1 was examined using a sensitive RIA method. In view of the large number of antigenic components detectable in R with the sheep antiserum to R, the inhibition of precipitin formation of one or a few antigen-antibody system, if it did occur, would have been obscured by the many precipitin bands visualized by the immunodiffusion technique. Obviously, it would be simpler to demonstrate the inhibition of precipitin formation of a discrete antigen-antibody system. It was found that haptenic components of $D_{24}$ and R could not inhibit the precipitation of labelled Allergen C with rabbit anti-Allergen C serum. This observation suggested that these haptenic components did not share any antigenic determinants in common with Allergen C. However, the haptenic components of $D_{24}$ and R were found to inhibit the precipitation normally obtained with Allergen KBG-1 and rabbit anti-KBG-1 serum, to the maximum extent of 30%and 20% respectively. Therefore, it was suggested that these haptenic components shared some antigenic determinants in common with Allergen KBG-1.

## CONCLUDING REMARKS

It was demonstrated in this study that the complex and heterogeneous mixture of components present in the aqueous extract of KBG pollen can be readily fractionated by Prep-ISO-EF in Sephadex gel employing ampholine in the pH range of 3.5 - 10.

Components with hapten-like activities were isolated from both the retentate and dialysate fractions of KBG aq. ext. by the method of Prep-ISO-EF. The haptenic properties of these components were firmly established on the basis of two criteria, namely (i) they were unable to elicit PCA reactions in appropriately sensitized animals and (ii) they were able to inhibit completely* and specifically the PCA reactions normally elicitable in animals passively sensitized with a pool of murine reaginic antisera to R, upon challenge with R. Studies designed to evaluate the capacity of hapten-like fractions to neutralize IgE antibodies to R provided an insight into the nature of the allergenic composition of the retentate. Some of the haptenic fractions which contained only a few components, discernable by Anal-ISO-EF, could inhibit completely and specifically the PCA reactions normally elicitable with R. These results clearly indicated that such haptenic fractions possessed all the

* Within the limitation of the inhibition of PCA technique as discussed in Chapter II.

allergenic specificities present in R which were recognized by the IgE antibodies present in the murine anti-R reaginic sera.

It was suggested that the components in R probably contained common allergenic determinant(s); and the pool of murine reaginic antisera* to R recognized only a limited number of different allergenic determinants (if indeed more than one). Thus, the number of different allergenic specificities recognized by the murine anti-R sera appeared to be very limited. In view of the fact that KBG aq. ext. possessed a large number of components with pI values ranging from 3-10, it was not unreasonable to have expected, at the onset of this investigation, that a number of different allergenic specificities would have been found to be collectively present in R. Since the murine IgE antibody response was directed to a limited number of different allergenic determinants, the IgE antibody response, which is under immune response gene control, recognized primarily an immunodominant allergenic determinant(s) present in KBG aq. ext.

The number of allergenic specificities recognized by the pool of human allergic sera appeared to be greater than the murine reaginic sera.* This conclusion was arrived at on the basis of results of the inhibition of RAST experiments in which a pool of human sera from individuals allergic to KBG pollen was employed. Using allergosorbent discs

* Within the limitation of the inhibition of PCA technique as discussed in Chapter II

prepared with R, the maximum inhibition of the binding of human IgE antibodies achieved by most of the haptenic fractions was of the order of 60%. Since complete inhibition of RAST could not be attained, it would appear, therefore, that the human IgE antibodies recognized some allergenic specificities in R which were absent in the majority of the haptenic fractions. A corollary of this conclusion is that the haptenic fractions lacked some of the specificities associated with allergenic determinants on components present in R. In view of the fact that murine reaginic antisera were used in one set of experiments and human allergic sera used in the other, the divergent results and conclusions were not surprising. Differences in the ability of different strains of mice to mount an IgE antibody response to various allergens have been reported by other investigators (Levine and Vaz, 1970). Thus, the IgE antibody response of the A/HeJ strain of mice was limited to the immunodominant allergenic determinent(s) of R recognized by this strain. On the other hand, the source of the human IgE antibodies present in a pool of sera from humans allergic to KBG pollen, must be regarded as being obtained from an outbred population. As a result, the IgE antibodies collectively present in the pool of human antisera recognized a greater number of allergenic determinants than did the murine IgE antibodies.

The conclusions regarding the allergenic specificities recognized by the murine reaginic and human allergic sera were confirmed by studies designed to establish the relationship of allergenic specificities of purified allergens C and KBG-1. Allergen C (isolated from KBG aq. ext. by a combination of techniques involving Prep-ISO-EF and gel filtration), with a pI value of 9.7, could inhibit completely the PCA reactions* with R upon passive sensitization of rats with the murine anti-R sera. Likewise, Allergen KBG-1 (isolated from KBG aq. ext. by a combination of techniques involving gel filtration, ion-exchange chromatography and Prep-ISO-EF), with a pI value of 4.7, could also completely* inhibit the PCA reactions elicitable in rats with R and the murine anti-R reaginic sera (Ekramouddoullah et al., 1979). In addition, each allergen also neutralized completely* and specifically the murine IgE antibodies present in the murine reaginic sera produced separately to Allergens C and KBG-1. However, Allergens C and KBG-1 apparently lacked some allergenic specificities present in R which were recognized by the IgE antibodies in the pool of human allergic sera, since the maximum inhibition of RAST achieved with Allergen C was 78% and with Allergen KBG-1 was 49% (Ekramouddoullah et al., 1979).

At present the exact chemical nature of the immunodominant determinant(s) of KBG pollen (recognized by the

*Within the limitation of the inhibition of PCA technique as discussed in ChapterII.

murine anti-R reaginic sera) is not known. Allergen $D_3$ of timothy pollen which contained at least 78% of the allergenic specificities present in the water soluble extract was found to be composed of flavonoid pigment, a sugar moiety of cellobiose and at least one amino acid, threonine (Malley et al., 1975). Thus, an immunodominant determinant could possess a relatively simple structure. If a simple structural moiety was present on components which differed in their net charge, it would account for the observations that most of the components of R which had been separated by Prep-ISO-EF possessed a similar allergenic specificity.

The purity of Allergen C was established on the basis of Anal-ISO-EF, crossed immunoelectrophoresis employing a hyperimmune sheep precipitating antiserum to R and gel filtration on Bio-Gel P-60. It was found to have a molecular size of 11,000 daltons and possessed 500 µg of carbohydrate per mg of protein. Allergen C was found to be stable on exposure to moderate heat, extremes of pH and to the denaturing agent guanidine HCl. However, its allergenic activity was completely eliminated following protease treatment suggesting that allergenic activity was associated with the stable protein moiety of the molecule. Analysis of allergenic and antigenic properties of Allergen C following treatments with trypsin and papain suggested that the determinants that were recognized by the murine

IgE antibodies were quite different from the determinants that were recognized by the rabbit IgG antibodies. The treatment with trypsin of Allergen C completely eliminated its ability to elicit PCA reactions in rats sensitized with murine reaginic anti-R sera; however, its ability to form a precipitin band with the rabbit anti-Allergen C serum was not affected. Papain treatment, on the other hand, had no effect on its ability to elicit the PCA reactions but destroyed its ability to form a precipitin band with the rabbit antiserum.

Allergens C and KBG-1 (although appearing to be allergenically identical with respect to the murine reaginic sera) were found to be antigenically distinct from one another when their antigenic cross-reactivity was examined by immunodiffusion and RIA techniques employing rabbit precipitating antisera produced separately to each of these allergens. However, Allergens C and KBG-1 cross-reacted allergenically to a limited degree with respect to the human allergic sera. Dandeu and Lux (1978) have reported similar findings with regard to Ascaris suum allergens. Two proteins isolated from Ascaris suum were found to be allergenically identical with respect to murine IgE antibodies, but they were antigenically distinct with respect to rabbit IgG antibodies. It is conceivable that different determinants on a molecule may be involved in the induction

of antibodies when different species of animals and different adjuvants are employed in the production of these antibodies.

The antigenic relationship of the haptenic components of R and $D_{24}$ to Allergens C and KBG-1 was investigated employing sensitive RIA procedures. It was found that these haptenic components shared no common antigenic specificities with Allergen C but shared some antigenic specificities with Allergen KBG-1. The maximum inhibition of the combinations of $I^{125}$ labelled Allergen KBG-1 with its homologous rabbit antibodies achieved with haptenic components of R and $D_{24}$ was of the order of 20 and 30 per cent respectively.

Thus, this investigation has resulted in the isolation of haptenic and allergenic components from KBG pollen and the discovery of some unique characteristics of the allergenic and antigenic relationships of these components.

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