Ph.D. EXPERIMENTAL MEDICINE

BRIAN JAMES UNDERDOWN

ISOLATION AND CHARACTERIZATION OF A PROTEIN ALLERGEN FROM SHORT RAGWEED POLLEN

ABSTRACT

An allergenically active protein, Antigen Ra.3, was isolated from the aqueous extract of short ragweed pollen by the combined techniques of ammonium sulphate precipitation, gel filtration and ion exchange chromatography.

Antigen Ra.3 was shown to be homogeneous by TEAE-cellulose chromatography, polyacrylamide disc electrophoresis, sedimentation velocity ultracentrifugation, gel filtration and immunodiffusion tests.

Antigen Ra.3 was shown to have a molecular weight of 15,172, an $S^0_{20,w} = 1.8 \times 10^{-13}$ sec, $D^0_{20,w} = 10.1 \times 10^{-7}$ cm^2 sec^{-1}, a total hexose + pentose content of 12.4% and an amino acid composition distinct from the major allergen of ragweed pollen, Antigen E.

By direct skin, P-K and in vitro anaphylaxis tests, Antigens Ra.3 and E differed in allergenic specificity

but shared common allergenic determinants.

Evidence was obtained by DEAE-Sephadex chromatography that reaginic antibodies to Antigen Ra.3 were relatively negatively charged compared to reagins to Antigen E.

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Department of Experimental Medicine,
McGill University,
Montreal, Canada

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PREFORATORY NOTE

Components present in short ragweed pollen are the immediate causative agents of allergic disease in hereditarily predisposed (atopic) individuals. Prior to an historical review of attempts to isolate and characterize these components, it seems appropriate to discuss those features of atopic allergy which are of particular relevance to the past and present investigations.

PART A: GENERAL INTRODUCTION

CHAPTER I

SOME FEATURES OF ATOPIC ALLERGY

Atopic allergy is induced by diverse environmental substances to which whole populations are exposed, such as airborne pollens of ragweed, grasses and trees, as well as fungi, animal danders, house dust, and foods. While harmless to most, inhalation or ingestion of these substances (the allergens) by atopic individuals rapidly causes the appearance of rhinitis, asthma, or hives (urticaria, erythema). As these symptoms are very similar (and, in some respects, essentially identical) to the manifestations of anaphylactic reactions, it is appropriate to preface a discussion of atopic allergy with a brief review of anaphylaxis (systemic, local, and in vitro) and its underlying mechanism.

1. Anaphylaxis

The term anaphylaxis was employed by Richet (1) to describe a heightened state of sensitivity to an antigenic stimulus. Portier and Richet (2) in 1902, while immunizing

dogs with a toxic substance from sea anemone tentacles, noted that the animals experienced an acute systemic reaction and died upon reinjection of an otherwise sub-toxic dose of the antigen. Subsequently, other workers (3, 4, 5) showed that this anaphylactic reaction could be obtained in other animals, although the manifestations of anaphylaxis varied from species to species.

a. Systemic Anaphylaxis

The reaction described above, termed systemic anaphylaxis, is usually accomplished by the intravenous injection of antigen into a previously sensitized animal. The symptom complex seen in systemic anaphylaxis is characterized by contraction of smooth muscle and an increase in vascular permeability, but the particular tissues affected (the shock organs) vary, depending upon the animal. Thus, in the guinea pig, contraction of the bronchi are the cause of death, while in the dog, the hepatic portal vein contracts, causing the liver to become engorged with blood, with consequent shock and death to the animal. Systemic anaphylaxis may be passively transferred from a sensitized to a non-sensitized animal by transfusion of serum. Following transfusion, a "latent" period" is required (6) for anaphylactic antibody to fix (via the Fc region of the antibody (7)) to the tissues of the host, before passive systemic anaphylaxis can be induced.

b. Local Cutaneous Anaphylaxis

Injection of antigen into the skin of a sensitized animal results in a local cutaneous anaphylactic reaction, consisting of erythema and an increase in vascular permeability leading to local edema. As with systemic anaphylaxis, this reaction can be transferred by the serum of a sensitized to a normal animal. In passive cutaneous anaphylaxis (PCA), the tissue may be sensitized by injection of antiserum intravenously followed by local cutaneous challenge with antigen, or by local cutaneous injection of antiserum and subsequent intravenous or local challenge with antigen. The reversed PCA reaction occurs when the tissue is sensitized with antigen consisting of gamma globulin.

c. In Vitro Anaphylaxis

That anaphylactic reactions may take place in vitro was first shown independently by Shultz (8) and Dale (9). Isolated pieces of uterus or ileum from a sensitized animal (usually guinea pig), when suspended in physiological media, were observed to contract on challenge with antigen. Such in vitro anaphylactic reactions may also be obtained using passively sensitized smooth muscle preparations and passive in vitro anaphylaxis has been reported for such tissues as guinea pig heart (10), rhesus monkey ileum (11), human tracheal rings (12), appendix (13), ileum and uterus (14).

Challenge with antigen of cellular and chopped tissue preparations (actively or passively sensitized) leads to the release of pharmacologically active mediators. The anaphylactic release of such mediators has been observed with chopped tissue preparations of guinea pig lung (15, 16), rhesus monkey lung (17), and skin (18), and with rat mast cells (19), rabbit platelets (20), and human leukocytes (21).

d. Mechanism

The biochemical events following combination of antigen with tissue-fixed antibody depends to a degree on the shock organ affected.

Present evidence indicated that the cell-bound antibody-antigen complex initiates a series of reactions leading to the release of pharmacologically active chemical mediators which react directly on the surrounding tissue to produce contraction of smooth muscle and an increase in vascular permeability. Histamine is the chemical mediator most frequently implicated in anaphylaxis. Thus, the release of histamine has been directly observed in vivo and in vitro following challenge with antigen (22, 23, 24). Furthermore, a reduction in the extent of anaphylactic shock has been noted upon pre-treatment of the sensitized animal with antihistamines (25). The failure of antihistamines to completely inhibit anaphylaxis (26) suggests that other mediators, such as slow reacting substance (SRS-A), serotonin, and

several plasma kinins, also participate. The role that a given mediator plays in a particular anaphylactic reaction depends on the concentration at which it is present in the shock organ and the degree of sensitivity of the organ to the mediator.

The pathway leading from the antigen-antibody reaction to the release of chemical mediators has not been fully elaborated. In vitro studies with chopped guinea pig lung (27) and rat mast cells (28) indicate that the reaction is relatively rapid, two-thirds of the total histamine release occurring within 1 - 2 minutes. Maximum local cutaneous anaphylaxis occurs in human skin in about 20 minutes (29). The release of chemical mediators appears to involve a chymotrypsin-like enzyme since inclusion of diisopropylfluorophosphate, an inactivator of chymotrypsin, inhibits their release subsequent to antigen-antibody combination (30). Calcium ion, required in several anaphylactic systems (31, 32, 33), may be involved in the activation of such an enzyme (34). Complement is not necessary for in vitro anaphylaxis (17, 33); however, some evidence has been reported which implicates complement in PCA reactions in the rat (35).

A number of physiological conditions and components such as pH, salt concentration, glucose, and dibasic acids required for optimum in vitro anaphylaxis have been determined, particularly for guinea pig lung suspensions (31, 36) and human leukocytes (37).

Preformed antigen-antibody complexes have been shown (38, 39, 40, 41) to induce anaphylactic reactions, indicating that antigen-antibody combination itself is not necessary to initiate the sequence of reactions leading to anaphylaxis: rather, the complexes as a whole possess this property. While complexes prepared in antigen excess having the molecular formula Ag_3Ab_2 were the most efficient, complexes formed at the equivalence point and in antibody excess with non-precipitating antibody could also induce anaphylaxis (42).

Studies by Ishizaka and Campbell (43) showed that the formation of antigen-antibody complexes was accompanied by an increase in levorotation. These workers postulated that, following combination with antigen, structural changes occur in the antibody molecule which initiate the anaphylactic reaction (44).

It should be mentioned that in order for an antigen to participate in anaphylactic reactions it must be minimally divalent (45). This condition would appear to be related to the inability of univalent antigens to form complexes of suitable molecular composition.

Investigations into the nature of the antibody in anaphylaxis have indicated a species variation. In general, the antibodies responsible for passive transfer of anaphylaxis within a species are of the $gamma_1$ type, while those responsible for passive transfer to a different species are of the $gamma_2$ type. This has been shown in the mouse (46), dog (47, 48),

rabbit (47, 49), and man (50, 51). The guinea pig has a $gamma_1$ antibody which produces both types of anaphylactic reaction (52, 53); however, it has been reported that guinea pig $gamma_2$ antibodies can sensitize for homologous PCA (54) and for heterologous PCA in the rat (53). In the guinea pig (53) and mouse (55) it has been shown that the antibody responsible for complement fixation is of the $gamma_2$ type. Austen (56) has suggested that PCA reactions with guinea pig $gamma_2$ antibodies in the rat may proceed by a complement dependent cytolytic pathway similar to that seen in immune hemolysis.

The $gamma_1$ homologous anaphylactic antibodies are readily inactivated by heating at 56°C, whereas the $gamma_2$ heterologous anaphylactic antibodies are heat stable (49, 57, 58). In some cases, it has been established that the antibodies responsible for homologous PCA have a higher sedimentation coefficient than the antibodies which mediate anaphylaxis across the species barrier (49, 50, 59).

2. Anaphylaxis and Atopy

The similarities between anaphylaxis and atopy led Noon (60) to postulate that atopy was a form of anaphylaxis. Some allergic reactions which occur in the human, such as drug hypersensitivity (e.g., to penicillin (61)), are anaphylactic

in nature but are not considered to be atopic because of the absence of a familial tendency in these disorders (62).

Sherman (63) and Arbesman (64) have stressed the differences between anaphylaxis in animals and atopy in man. They point out that anaphylaxis in animals is usually associated with precipitating antibody which the sera of atopic patients do not usually contain. In addition, they note that the frequency of induction of anaphylaxis in the experimental animal is considerably higher than the incidence of atopy in man. On the other hand, Patterson and Sparks (65) have shown that serum from ragweed atopic dogs which contained no demonstrable precipitating antibody passively sensitized normal dogs for systemic anaphylaxis. Thus, the association of precipitating antibody with anaphylaxis is probably fortuitous.

Kabat et al (66) have shown that the majority of the human population is able to produce anaphylactic antibody to dextran. A similar observation has been reported for ascaris antigen (67). However, the occurrence of atopy in only a fraction of the population exposed to the atopic allergens (such as pollen spores) indicates a predisposition on the part of some to develop the disease. Studies in the rat (68) and rabbit (49) have revealed that the induction of homologous anaphylactic antibody could be accomplished only in a fraction of the animals tested. Thus, it is possible that most individuals of a species can manufacture homologous anaphylactic antibody depending on

the intensity of immunization, while atopic individuals have an increased propensity to produce such antibodies compared to the non-atopics.

The higher frequency of occurrence of atopy in certain individuals may be connected to the route of sensitization. Salvaggio and his associates (69, 70) have reported that groups of non-atopic and atopic individuals exhibited the same tendency to become sensitized after intradermal injections of antigen; the atopic group on the other hand, had a greater tendency to become sensitized by nasal insufflation of antigen. These workers postulated that, compared with non-atopic, the atopic individuals have a different method (apparently genetically determined) of processing the allergen on contact with the nasal mucosa.

3. Antibodies in Human Atopic Serum

a. Skin Sensitizing Antibodies (Reagins)

The passive transfer of cutaneous activity from an atopic to a normal individual was first accomplished by Prausnitz and Kustner (71) in 1921. Injection of fish extract into a skin site previously sensitized with the serum of a fish sensitive individual induced a cutaneous reaction similar to that seen on direct challenge of a fish sensitive subject with the same extract.

Characteristically, the sera of atopic subjects can regularly sensitize the skin of almost all normal persons to cutaneous reactions on challenge with specific allergens. The Prausnitz-Kustner (P-K) reaction is identical to that obtained on direct skin testing of the atopic patient with allergen, namely: the development of a pale, elevated, irregular wheal surrounded by a zone of erythema due to local edema and arteriolar dilation, respectively. As found for passive cutaneous anaphylaxis in the guinea pig (72), a latent period is required between the time of injection and challenge, presumably in order for the antibody to become fixed to receptor sites of tissue mast cells and probably also platelets (73). Combination of allergen with cell fixed antibodies, likely changes the permeability of the mast cell membrane (possibly due to changes in the configuration of the antibody molecule (43)) and leads to the leakage of pharmacologically active mediators, such as histamine.

The anaphylactic release of such mediators probably accounts for the various manifestations of atopic disease in man. The anaphylactic antibodies in the serum of atopic individuals are termed skin sensitizing antibodies or reagins.

Considerable research has been directed toward the characterization of reaginic antibodies. There is a striking similarity between reagins and the homologous antibodies mediating anaphylactic reactions in animal species in general. Thus,

reagins are capable of transferring cutaneous activity only within the human or sub-human primate species such as the monkey (74). Reaginic antibodies are of the gamma$_1$ type as judged by their behavior on ion-exchange chromatography (75), electrophoresis (76), and ammonium sulfate precipitation (77). Their molecular weight is slightly greater than IgG judging by their behavior on gel filtration (78) and by their sedimentation coefficient, variously reported as having values between 8 and 11 S (79, 80).

Goodfriend and his associates (81, 82) provided evidence from column chromatographic and immunoabsorption studies that reaginic antibodies (in the main, at least) do not belong to the IgG, IgA, IgM, or IgD classes and suggested that they belong to an as yet uncharacterized immunoglobulin class. At the same time, Ishizaka and co-workers (83, 84) reported direct evidence that reagins belong to a new immunoglobulin class, IgE, with light chain determinants in common with the other four immunoglobulin classes but with distinct heavy chain determinants. The IgE reagins are electrophoretically heterogeneous (85), and show a sedimentation constant of 8 S on sucrose density gradient centrifugation (84). The heat lability of IgE reaginic antibody appears to be due to alteration of its skin fixing moiety and not to its antigen binding sites (86).

b. Blocking Antibodies

Sera from atopic individuals also contain immune type antibodies produced by hyposensitization of the atopic patient (see below). Such antibodies are able to block the reaction between allergen and reagin and are referred to as blocking antibodies (87). To demonstrate such antibodies, allergen is incubated with the atopic serum in vitro and the mixture is tested for its content of allergenic activity by P-K test. The quantity of blocking antibody is measured in terms of a dilution (titer) of the serum just capable of blocking the P-K reaction.

Various studies have shown that the antibody responsible for blocking activity differs from reaginic antibody and in fact behaves like a $gamma_2$ antibody on ammonium sulphate precipitation (88), ion-exchange chromatography (75), electrophoresis (89) and ultracentrifugation (90). In contrast to reagins, blocking antibodies give positive heterologous PCA reactions (51).

Reaginic antibody may be further distinguished from blocking antibody by several other properties. In contrast to blocking antibody, reaginic antibody is heat labile (86), unstable at pH 3 (91), and sensitive to reducing agents (92).

c. In Vitro Assay of Reaginic and Blocking Antibodies

Detection of antibodies in the serum of atopic patients may be accomplished by the passive hemagglutination technique,

using either the tanned cell (93) or bisdiazotized benzidine method (94). The close relationship between the physicochemical properties of blocking and hemagglutinating antibodies (75, 95) suggests that the blocking antibodies account for the bulk of the hemagglutinating activity of allergic serum. However, the possibilities have not been excluded that reaginic antibodies participate in passive hemagglutination and that the passive hemagglutination test measures antibody other than blocking and skin sensitizing types.

The passive transfer or P-K test has provided the principal method for the assay of reaginic activity. This test, along with the direct skin testing of atopic subjects, has also been used to assay the cutaneous activity of various allergenic preparations.

Recently, in vitro model systems of human atopic reactions have been developed which utilize leukocytes from allergic (37) or normal (96) individuals, rhesus monkey ileum (11) and suspensions of rhesus monkey lung (17) or skin (18).

d. Hyposensitization with Allergens

The similarities between anaphylaxis and atopy led Noon (60) to introduce the procedure of desensitization, which he hoped would lead to a refractory state, as in anaphylaxis (97). The procedure of injecting small quantities of allergen in increasing doses, termed hyposensitization, has been shown

to be effective in reducing symptoms in several atopic diseases, notably ragweed atopy. The exact mechanism by which the reduction is achieved is poorly understood. Various studies have tried to relate the improved clinical status with a decrease in reagin titer (98) or an increase in blocking antibody (99, 100). There seems to be general agreement that changes in serum levels of the two antibody types do occur but their quantitative relationship to clinical improvement is unclear.

4. Chemical Nature of the Allergens in Atopy

a. The Theory of Berrens:

Berrens (101) advanced an hypothesis relating the capacity of substances to function as atopic allergens with their chemical structure. The hypothesis was based on the findings that purified allergenic preparations are amber in color, polydisperse in the ultracentrifuge, and are often not digested by trypsin. Berrens attributed these properties to the presence of N-glycosidic linkages which exist as 1-amino-1-deoxy-2-ketose structures. He postulated that these moieties were formed by the conjugation of sugars to proteins through the epsilon-NH_2 of lysine and consequent rearrangement via the Maillard reaction (102). Experimental evidence for the existence of these structures was obtained in spectroscopic (103) and fluorescence emission spectra (104) studies. Thus, synthetic protein-sugar

conjugates were found to have a characteristic absorption maximum at 305 mμ, and to exhibit blue fluoresence when excited at 365 mμ. Partially purified allergens such as kapok, ipecacuanha, and tomato exhibited similar behavior to these synthetic products.

In a subsequent paper, Bleumink and Berrens (105) reported that the cutaneous activity of beta-lactoglobulin increased with the addition of N-glycosidic linkages when tested in milk sensitive patients. However, no explanation was offered for the fact that native beta-lactoglobulin, while allergenic, did not contain N-glycosidic linkages detectable by spectroscopy (103), fluorometry (104) or with specific antisera (106).

In another study, Berrens et al (107) correlated the increased allergenic activity of extracts of ripe, compared to unripe tomato, with the "browning" which takes place during the ripening process via the Maillard reaction mechanism. The increased allergenic activity of ripe tomato is not surprising since tomato sensitive patients are usually exposed to the ripe fruit.

The results of Berrens and co-workers have undoubtedly demonstrated the presence of N-glycosidic linkages in allergenic extracts, but the lack of homogeneity of the allergens tested makes it difficult to assess the relation of such linkages to allergenicity.

b. The Theory of Stanworth

In a discussion on the chemical basis of allergenicity, Stanworth (77) pointed to the chemical similarity of several purified atopic allergen preparations. A highly purified glycoprotein preparation, of molecular weight 34,000 and containing 9% hexose, was isolated by Stanworth (108) from horse dander extract. Berrens and associates provided evidence that the allergens of house dust (109) and ipecacuanha (110) were glycoprotein in nature. The molecular weight of the house dust allergen preparation was calculated to be approximately 23,500. The glycoprotein nature of a purified allergenic preparation from rye pollen was reported by Johnson and Thorne (111). The allergen contained some 40% carbohydrate and had a molecular weight of approximately 19,000. Purified allergenic preparations isolated from short ragweed pollen by Goldfarb et al (112) and King et al (113) were reported to contain carbohydrate and to have molecular weights in the range of 30,000.

Based on the evidence cited, Stanworth proposed (77) that the atopic allergens are glycoproteins with sedimentation coefficients in the range of 2-4 S, and that the carbohydrate moieties of these proteins determine their allergenic specificity. He proposed a scheme for the development of atopy in which the allergen would stimulate the production of reaginic antibody specific for the glycopeptide moiety of the molecule.

He also suggested that, since the surface membrane of epithelial tissue cells is known to possess glycopeptide components, a dual-specific antibody would be produced capable of binding to glycopeptides of epithelial cells. According to Stanworth, this would be tantamount to breaking the atopic patient's tolerance of his own tissue components. Subsequently, Stanworth (114) revised this model and suggested reagin to be a gamma G - gamma A dimer. The antibody site of gamma A was considered to bind to the glycopeptide determinants of the epithelial cells, and the antibody active site of the gamma G to bind to the allergen.

While recent studies on the immunoglobulin nature of reaginic antibodies (cited earlier) appear to make the Stanworth model improbable, the possibility could not, on these grounds, be excluded that allergenic determinants may be glycopeptide in nature. However, recent studies on cutaneously active proteins isolated from rye and ragweed pollens suggest that carbohydrate is not an essential structural feature of atopic allergens.

c. Purified Allergens of Rye and Ragweed Pollen

Johnson and Marsh reported (115) the isolation of three allergens from rye pollen: I-B, I-C, and II-B. Allergens I-B and I-C were immunochemically identical and were highly purified judging by their behavior on starch gel electrophoresis at acid and alkaline pH. The molecular weights of I-B and I-C were identical (34,000) and both contained 5% carbohydrate. The authors

suggested (116) that allergens I-B and I-C represent an isoallergen system, with the two allergens differing only in their amide content. Allergen II-B was allergenically active in a small proportion of rye sensitive patients. It had a molecular weight of approximately 10,000 and contained one-third the carbohydrate content of allergens I-B and I-C.

Studies by Johnson and Marsh (117) on allergen I-B strongly suggest that carbohydrate is not involved as allergenic determinant. Thus, enzymatic digestion of the carbohydrate moiety was without effect on the activity of the allergen. As no evidence was obtained that digestion had been complete, implication of carbohydrate in the allergenic determinant could not be completely ruled out. However, the results of chemical modification studies done on I-B suggest involvement of tertiary polypeptide structures in the activity of the allergen. Thus, reduction, oxidation, alkylation by iodoacetic acid, digestion with trypsin and chymotrypsin, and alkaline hydrolysis of allergen I-B separately caused a loss of activity (118). Partial dansylation of amino groups resulting in the substitution of 2 groups per mole was without effect, but a reduction of activity was observed on substitution of 5 dansyl groups per mole.

In a study of major significance, culminating years of research by numerous investigators, King et al (113, 119) isolated a highly allergenically active protein, Antigen E,

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In a study of major significance, culminating years of research by numerous investigators, King et al (113, 119) isolated a highly allergenically active protein, Antigen E,

from short ragweed pollen. Antigen E was found to exist in at least 4 different electrophoretic forms, each with a molecular weight of 37,500. The carbohydrate (arabinose) content was found to decrease with increasing purification of the allergen, reaching a "final" level of 0.7%. King et al (119) considered the presence of this low level of carbohydrate to be due to contamination. No absorption peak was observed at 305 mμ, indicating the absence of N-glycosidic linkages of the type predicted by the Berrens theory of allergen structure. Furthermore, chemical modification studies (118) strongly implicated the involvement tertiary protein structures in the allergenically active sites.

As the present studies are concerned with the isolation and characterization of ragweed pollen allergens, a more detailed discussion of Antigen E will be deferred to the historical review of studies on the allergens of ragweed pollen.

CHAPTER II

STUDIES ON THE ALLERGENS OF RAGWEED POLLEN: AN HISTORICAL REVIEW

Ragweed pollen is the most important cause of hay fever in North America. Ragweed plants depend on wind pollination for reproduction and, as a result of their occurrence from the Atlantic seaboard to the Rocky Mountains, over 90% of the North American population is annually exposed to the pollen during a period from late summer to autumn. There are two principal species of ragweed plant: short or common ragweed (Ambrosia elatior) and giant ragweed (Ambrosia trifida). The pollen grains of both plant types have a similar appearance, being approximately 20 microns in diameter and bright yellow in color. Each grain consists of an exine or outer covering from which blunt spicules protrude, and an inner cavity containing two nuclei and cytoplasm.

The pollen grains contain most substances found in living cells, such as enzymes, nucleoproteins, carbohydrate, proteins, lipids, amino acids and pigment.

Heyl, in a series of papers (120, 121, 122, 123, 124) between 1917 and 1922, reported quantitative determinations of moisture, phosphorous, crude fiber, carbohydrate, ash, protein and alcohol-soluble and ether-soluble material of ragweed

pollen. He demonstrated the presence of seven aliphatic acids, several amino acids, peptones, and two classes of proteins based on heat coagulability. He also isolated the pigments isoquercitin and isorhamnetin.

At the same time, Koessler (125) independently confirmed the existence of ether-soluble, alcohol-soluble and water-soluble substances, findings which were used by later investigators in attempts to isolate the allergenic principles.

Such attempts began soon after the reports by Heyl and Koessler. The central questions posed in these studies pertained to the number and nature of the allergenic components in short ragweed pollen. The voluminous literature on the subject prior to 1960 has been the subject of several reviews (126, 127, 128, 129) and shows wide disagreement on the nature of ragweed allergens.

1. Early Studies

a. The Exine

In an early study on the allergenicity of the outer covering of ragweed pollen grains, Campbell and Sussdorf (130) reported that rabbit antisera produced against a dialysed suspension of pollen grains contained a factor absent from normal rabbit serum, which would adsorb to the washed pollen grains.

The factor was localized by means of a fluorescent anti-rabbit globulin serum, suggesting that the exine adsorbed ragweed specific antibody. Campbell and Sussdorf explored the efficacy of this technique to detect antibodies in human ragweed allergic sera. While both ragweed allergic and normal sera showed positive adsorption, the normal sera showed less.

In a more recent study, Wicher et al (131) reported that ragweed pollen grains were agglutinated by rabbit anti-sera, but an indirect Coombs test was necessary to demonstrate agglutination with human allergic sera. Positive agglutination, however, was also obtained with normal human sera.

Clearly, no conclusion regarding the allergenicity of the exine can be drawn from the studies cited.

b. Ether and Methanol Soluble Material

Milford (132) reported that ether soluble material, a dark greenish-brown oil, elicited positive skin tests in approximately two-thirds of the ragweed sensitive patients tested. However, no attempt was made to purify the pigmented fraction. Moore et al (133) and Johnson and Rappaport (134) confirmed Milford's finding but reported that the oil contained nitrogen of unknown origin. Thus, the possibility could not be excluded that the cutaneous activity of the ether-soluble material was due to contamination with peptidyl allergen.

Goldfarb et al (135) removed an appreciable

quantity of yellow-colored material from defatted pollen by methanol extraction prior to aqueous extraction. As the aqueous extracts prepared in this way were as allergenically active as those prepared without methanol extraction, these workers concluded that the methanol extract contained little allergenic activity, although no attempt was made to measure this directly.

c. Molecular Size and Dialysability of Allergenic Material

In early studies, sedimentation coefficients were determined in the analytical ultracentrifuge to obtain an approximate estimate of the molecular weight of the allergens in partially purified fractions. Sanigar (136) reported a sedimentation coefficient of 1.05 S for an aqueous extract of ragweed pollen. Stevens et al (137) found a value of 1.35 S for a dialysed residue of the aqueous extract. Abrahamson and associates (138) studied an electrophoretically purified product and found a value of 1.5 S. Richter et al (139) calculated values of 1.81 and 4.51 S for two components of a purified dialysed fraction. It should be noted that crude preparations were employed in these studies and the assumption was made that the peak(s) observed in the ultracentrifuge were due to the allergenic principle(s).

Considerable controversy developed concerning the dialysability of allergenic components of aqueous ragweed

extract. The data obtained by different investigators conflicted, partly due to lack of standardization of dialysis membranes and, occasionally, lack of quantitation. Unger et al (140) observed that active material could pass through dialysing membranes. The latter were not standardized, however, and Coca (141) reported that egg albumin could pass through similar membranes. Johnson and Rappaport (134) also found some dialysable activity but attributed this to protein leakage. Stone et al (142) reported that 50% of the allergenic activity was dialysable, but the membranes used were not standardized. Dankner et al (143), using similar tubing, demonstrated allergenic activity with concentrations of dialysate of 2 mg/ml, but the bulk of the activity was non-dialysable. Loveless (144) reported similar results: she estimated the dialysate to be 500 times less active than the retentate. The studies of Grove and Coca (145), Black (146), Stull and Sherman (147), and Rockwell (148) indicated that most of the allergenic activity was non-dialysable.

Richter et al (149) found that dialysate was allergenic only in those ragweed patients who had received hyposensitization therapy. In non-treated patients, the bulk of the allergenic activity remained in the retentate. Treated individuals reacted to dialysate both by direct skin tests and by passive transfer tests with their sera.

Meacock et al (150) repeated the studies of

Richter and co-workers, and concluded that the differences observed between treated and non-treated patients resulted from the technique used to sterilize the solutions for testing. Thus, dialysate sterilized by millipore filtration was equally allergenic in both groups of patients; sterilization by Seitz filtration (previously employed by Richter et al) removed material allergenic in non-treated patients. The relative activity of the dialysate and dialysed residue was not reported, but presumably the bulk of the activity remained in the dialysis sac, as shown for non-treated patients in the previous study by Richter et al (149).

d. Carbohydrate Constituents

In 1924, a brief account (145) of studies by Grove and Coca appeared which indicated that the allergen in a ragweed pollen extract was not of a protein nature. In a more detailed paper (151) published a year later, Grove and Coca reported that incubation of the crude pollen extract with a pancreatic protease and subsequent dialysis resulted in a loss of some 90% of the nitrogen content with no decrease in activity. This was taken as evidence against the then current view that the allergenic components were protein in nature. However, Grove and Coca did not dialyse their control, which consisted of crude extract in saline, and their failure to do so must be viewed in light of

the fact that 95% of crude aqueous ragweed extract is dialysable (152).

Following Grove and Coca's report, numerous investigators focussed their attention on carbohydrate constituents of ragweed pollen. Black (153) isolated a fraction by successive alcohol and acid precipitation which contained 55% carbohydrate as reducing sugar, and 6% nitrogen. The fraction was allergenically active, but no evidence of homogeneity was reported. Caulfeild et al (154) isolated an allergenically active, carbohydrate-containing material, but no account was taken of a small amount of nitrogen present in the preparation.

While the above studies demonstrate that fractions rich in carbohydrate may be allergenic, no definitive conclusion is possible regarding the allergenic activity of the constituent carbohydrate owing to the lack of purity of the preparations.

Other studies tended to rule out the role of carbohydrate in allergenic activity. Thus, Baldwin and associates (155) found a decrease in carbohydrate concomittant with purification of an allergenic fraction. Stull et al (156) isolated a carbohydrate fraction and subsequently showed that its allergenic activity was due to contaminating protein. Similar conclusions were reached by Johnson and Rappaport (134) and Unger et al (157). However, these studies excluded some, but not all, of the carbohydrate as structural elements or as specificity determinants of

the allergenically active proteins of ragweed pollen.

e. Proteinaceous Material

The hypothesis that the allergenic activity in pollen was due to protein was made by Wolf-Eisner (158) in 1906, following the then newly discovered phenomenon of sensitivity to protein (anaphylaxis) by Portier and Richet (2). Meltzer (159) reported a patient with asthma and hay fever which was termed a case of pollen-toxalbumin sensitivity. Reference to the protein toxin nature of the allergen is found in the papers by Noon (60) and Koessler (160). Cooke (161), in conjunction with the standardization of ragweed extract used for hyposensitization, redefined the Noon unit (60) in terms of protein nitrogen, agreeing with the theory of the day that the excitant of hay fever was a protein.

Experimental evidence for the allergenic activity of protein fractions from ragweed pollen was reported by Caulfeild et al (162) in 1926. Adopting the chemical procedures of Heyl (122), these workers isolated albumin-proteose, proteose, and glutenin fractions. The albumin-proteose was the most active and the glutenin fraction the least. Unger et al (156), confirming the earlier studies of Stull (155), carried this work further by employing fractional ammonium sulphate precipitation combined with freezing and thawing. They reported that all fractions were

allergenically active. Johnson and Rappaport (163) isolated three protein-containing fractions by ammonium sulphate precipitation and showed all three to possess allergenic activity.

Goldfarb (164) isolated a highly purified, allergenically-active fraction from giant ragweed pollen by methanol precipitation. This fraction, called Trifidin A, was judged to be homogeneous by antigen analysis and isoelectric electrophoresis. Chemical analysis revealed that the allergen contained both protein and carbohydrate.

Some investigators considered that the allergens of ragweed pollen were either small proteins or polypeptides. The evidence for this hypothesis was obtained from studies which entailed heating the aqueous extract to precipitate protein. The derivative supernatant, thought to contain low molecular weight protein or polypeptides, was found to be as allergenically active as the original extract. Hecht et al (165) heated the whole extract at acid pH to precipitate high molecular weight protein which they considered responsible for precipitin reactions with rabbit anti-ragweed antiserum. The supernatant lost its ability to precipitate with rabbit anti-sera, but retained its allergenicity. The validity of this finding is questionable, however, owing to the absence in this study of exact quantitation. Thus, a loss of 50% of the allergenic activity could not have been detected by the method employed to assay cutaneous activity. Robbins et al (166) attempted to

isolate the allergen from the supernatant of a heated aqueous extract. An active fraction was obtained which contained both protein and carbohydrate, but no determination of its size was reported.

Sehon et al (152) isolated several fractions by paper electrophoresis from aqueous extract of short ragweed pollen. The fractions were allergenically active and contained both protein and carbohydrate. In a subsequent study (167), the same workers "de-proteinated" the aqueous extract by heating and fractionated the resulting supernatant by paper electrophoresis. By this procedure an allergenically active component was isolated containing both peptide and carbohydrate material. The authors concluded the allergen was a glycopeptide with a sedimentation constant of 1.2 S.

In a subsequent study, Sehon and his associates (168) found that the procedure of heating gave rise to irreproducibility due to the variable amounts of protein precipitated. A different procedure was therefore developed which involved paper electrophoretic separation of the exhaustively dialysed aqueous extract. An allergenically active fraction, Delta, was isolated which was 100 times more active than the "Dialysed Residue". Chromatography of Delta fraction on Sephadex G-75 revealed size heterogeneity. No electrophoretic analysis of the Delta fraction was reported. Studies by Arbesman and co-workers (169) showed Delta to contain several antigens.

Richter et al (170) isolated a partially purified fraction from the dialysate of an aqueous extract of ragweed pollen by phosphotungstic acid precipitation. On an equal weight basis, the purified fraction was 100 times more allergenically active than the whole dialysate and contained predominantly peptidyl material.

It may be concluded from a review of early studies that the most allergenically active components in aqueous ragweed extract are likely of macromolecular dimension. However, the chemical composition of these macromolecules, i.e., their protein or carbohydrate nature, could not be deduced from these studies, since the purified fractions obtained were heterogeneous. Recently, more homogeneous allergen preparations have been prepared by the use of gel filtration and ion exchange chromatography in the isolation procedures.

2. Recent Studies

The studies to be discussed in this section have been grouped together for several reasons. All were executed in a manner which would yield the most active allergen in short ragweed pollen, used the same starting material and similar techniques, and achieved essentially identical results. The

degree of homogeneity of the purified allergen preparations obtained was higher than had ever been previously demonstrated.

Robbins et al (171) fractionated the aqueous extract of short ragweed pollen by precipitating the allergens with ammonium sulphate at full saturation. Subsequent ion exchange chromatography (DEAE-Sephadex)and gel filtration (Sephadex G-75 and G-200) yielded a fraction, A.la_2, which had the highest allergenic activity of all the separated fractions. By immunoelectrophoresis and immunodiffusion, fraction A.la_2 contained at least 4 antigens; however, the homogeneity of this preparation was not examined by zone electrophoresis or analytical ultracentrifugation. Robbins et al (171) reported that fraction A.la_2 was 1000 times more allergenically active than the dialysed extract, indicating a considerable degree of purification of the most active allergen in the aqueous extract. They did not exclude the possibility that the aqueous extract contained allergens other than those of fraction A.la_2. Chemical analysis of fraction A.la_2 revealed a carbohydrate content of 20%, calculated as arabinose.

Goldfarb and his associates (172) isolated a highly purified allergenic preparation from short ragweed pollen, Pool Cc, by DEAE-cellulose chromatography and ammonium sulphate precipitation. Pool Cc contained a single antigen on immunodiffusion with an antiserum to the whole extract. However,

immunoelectrophoresis using the same antiserum demonstrated the presence of several antigens in Pool Cc. Cellulose acetate electrophoresis at pH 8.6 showed only one component. Pool Cc contained approximately 12% nitrogen and its acid hydrolysate revealed all of the common amino acids. Ultracentrifugation of a cruder preparation, Pool C, demonstrated a single peak with a sedimentation coefficient of 3.75 S. Pool C contained approximately 12% carbohydrate calculated as arabinose. Pool C and Trifidin A, the major allergen of giant ragweed pollen, contained both common and different antigenic components. By direct skin tests of ragweed allergic patients, Pool Cc was shown to have specific activity similar to that reported for Robbins' fraction $A.1a_2$ (171).

King and co-workers (113, 119), using the technique of gel filtration and ion exchange chromatography, isolated an allergenically active preparation containing a single antigen when tested by immunodiffusion with rabbit antisera to whole extract. The specific activity of Antigen E was similar to that reported for Goldfarb's Pool Cc and Robbins' $A.1a_2$. Antigen E was shown to exist in 4 electrophoretically distinct forms: IV-A, B, C and D. Fractions IV-B and IV-C were immunochemically identical and had similar amino acid compositions.

Chemical analysis of Antigen E (IV-C) gave 17.1% nitrogen and 0.5% arabinose which the authors judged to be a contaminant. Antigen E (IV-C) was judged to be homogeneous when

when tested by starch gel electrophoresis at pH 7.9 and 6.3, and by analytical ultracentrifugation. The allergen was determined to have a molecular weight of 37,000± 2,000 by equilibrium ultracentrifugation.

Antisera produced by immunization of rabbits with purified AgE (IV-C) were highly specific, since antibodies to other antigens could be demonstrated only at high concentrations of the whole aqueous ragweed extract. The allergenic activity of AgE was stable between pH 5.8 to 7.9 but decreased at pH 4.1 and 10.1, with a parallel reduction in antigenic content of the fraction.

King et al (119) determined the contribution of Antigen E to the allergenic activity of the whole extract by measuring the decrease in activity of the whole extract after precipitation of Antigen E with specific antisera. Testing of ragweed sensitive patients with the aqueous extract before and after the precipitation of Antigen E, revealed a decrease in specific activity of the extract varying from 10 to 1,000-fold, depending upon the patient tested. The authors concluded that Antigen E was responsible for more than 90% of the activity in the aqueous extract.

In contrast to the purified rye pollen allergens, Antigen E was not digested by trypsin, chymotrypsin, and papain (119); however, partial degradation to a molecular weight of

30,000 was achieved with pepsin, with little loss of activity (173). Antigen E was extensively digested with the bacterial enzyme nagarse, a treatment which completely destroyed the allergenic activity (174). Reduced and alkylated Antigen E possessed only one one-hundredth of the activity of the unmodified molecule (119). Attempts to obtain an inhibiting fragment by trypsin digestion of reduced and carboxymethylated Antigen E were unsuccessful (178).

Recently, King et al (175) have isolated another highly purified allergen, Antigen K, which cross reacted with Antigen E when tested with rabbit antisera and human allergic sera. The allergenic activity of Antigen K was slightly less than that of Antigen E. Of 37 patients examined by direct skin test, one gave a higher cutaneous reaction to Antigen K than to Antigen E, while 6 showed equally reactivity to both allergens. Antigen K had a molecular weight of 38,000 as determined by equilibrium ultracentrifugation and had a similar, though distinct, amino acid composition. No zone electrophoresis data were given as evidence of purity. As found for Antigen E, Antigen K was resistant to digestion with trypsin, chymotrypsin, and papain, but was susceptible to digestion with nagarse with a concommitant loss in activity.

King et al (174) have suggested that the ability of rabbit antiserum to precipitate over 90% of the activity from the whole aqueous ragweed extract may be due to the fact that

Antigen E shares common antigenic determinants with other proteins in the extract. If this were the case, the contribution of Antigen E to the total allergenic activity might be less than 90% should such proteins have different allergenic determinants.

Using the technique of in vitro anaphylaxis of leukocytes obtained from ragweed allergic individuals, Lichtenstein et al (176) confirmed the direct skin testing results of King et al (119), which indicated that Antigen E was the most active allergen in the whole pollen extract.

The antigenic purity of Pool Cc, Antigen E (IV-C), and fraction A.la_2, was investigated by Pruzansky and Patterson (177) by a technique developed to determine the homogeneity of radioactively labelled antigens. Experiments with the three fractions labelled with I^{131} indicated that Antigen E was the most homogeneous (84%), followed by Pool Cc (67%), and fraction A.la_2 (64%).

Robbins and co-workers have recently reported (178) that Antigen E was the major antigen in their fraction A.la_2. These workers developed a modified isolation procedure which yielded an Antigen E preparation with essentially identical properties to those reported by King et al (119). It is very likely that Antigen E also forms the major antigen in Pool Cc.

In view of the reported purity of Antigen E and

its importance as the major allergen in aqueous ragweed extract, a number of investigators carried out studies to determine the efficacy of Antigen E for hyposensitization of ragweed allergic patients. Lichtenstein et al (179) observed a decrease in sensitivity of the leukocytes and an increase in serum blocking antibody levels of ragweed sensitive patients treated with Antigen E. Norman et al (180) and Reisman et al (181) reported that patients could tolerate higher doses of Antigen E than of whole aqueous extract. However, the latter workers noted that treatment with Antigen E did not prove as beneficial to some patients as did treatment with the whole aqueous extract and suggested that these patients were sensitive to other ragweed allergens as well as to Antigen E.

3. The Existence of Multiple Allergens in Short Ragweed Pollen

One of the first indications that ragweed pollen contains more than one "excitant" of hay fever came from studies by Milford (132) on the lipid fraction of the pollen. He showed that reagin sites, injected with the lipid fraction until they gave no further cutaneous reaction, still retained the capacity to react with the aqueous extract. This technique showed that the lipid fraction could not "cross neutralize" the allergenic activity of the aqueous extract.

Similar cross-neutralization studies with the aqueous extract yielded variable results. Unger et al(157) prepared a polysaccharide fraction according to Black (153) and a protein fraction according to Stull et al (156). While the protein fraction neutralized the reaginic activity to both fractions, the polysaccharide fraction neutralized only the reaginic activity to itself. Caulfeild (182) tested patients with five crude fractions of aqueous ragweed and observed that none of the fractions neutralized the whole extract completely and that each fraction varied in its ability to do so. Caulfeild concluded from these results that ragweed contained multiple allergens. Stull et al (183) and later Cohen et al (184), using ammonium sulphate fractionation of the aqueous extract, found that the various preparations they obtained could not neutralize each other. Cohen concluded that there was a minimum of three ragweed allergens. Richter et al (185) provided evidence from cross neutralization studies with partially purified allergens that aqueous extracts of short ragweed pollen contained a minimum of two allergens.

Cece et al (186) performed cross neutralization tests with the three purified fractions: Pool Cc, fraction IV, and fraction A.1, the latter two fractions being penultimate fractions to Antigen E (IV-C) and $A.1a_2$, respectively. The results demonstrated that the three preparations contained similar allergenic determinants. However, the authors concluded that

other allergens exist, since sensitized sites neutralized to any one of the fractions still reacted to the whole extract.

In contrast to the experiments of Cece et al, Reisman et al (169) reported that Antigen E neutralized all the reaginic activity to the whole extract. On a weight basis, however, relatively larger amounts of Antigen E were needed for neutralization and the authors concluded that the whole extract contained allergens other than Antigen E. The fact that the Antigen E preparation neutralized the reaginic activity to the whole extract might have been due to contamination of the preparation by other allergens. Alternatively, the neutralization might have been due to cross-reacting allergenic determinants present on Antigen E and other allergens.

Further evidence for the existence of multiple ragweed pollen allergens was obtained by McKaba and Norman (187) who separated an aqueous extract by starch gel electrophoresis. The relative allergenic activity of the starch gel fractions varied from patient to patient, indicating that the fractions contained allergens of different specificity.

Lichtenstein et al (176) suggested that allergens other than Antigen E were present in the whole extract on the basis of their study of the reactivity of ragweed allergic leukocytes toward Antigen E and a more heterogeneous ragweed fraction A (113). Thus, the weight ratio of fraction A to

Antigen E which caused 50% histamine release from leukocytes varied from one allergic patient to another.

Ishizaka et al (84) have recently reported that a reagin-rich fraction, prepared by ion exchange chromatography and gel filtration, reacted to aqueous ragweed extract but not to Antigen E. Furthermore, the P-K titers of allergic sera to the whole extract did not uniformly correspond with the P-K titers to Antigen E.

4. Aims of the Present Study

As noted above, evidence has been obtained for the presence of components in aqueous ragweed extract with allergenic specificity different from Antigen E (and K). However, no such allergen had been isolated in pure form, and the present studies were undertaken with this objective. The isolation of additional highly purified ragweed allergens was considered to be of interest for several reasons.

a. The observation that Antigen E was not as effective for hyposensitization therapy as the whole extract in some cases, suggests that other allergens are required. Ideally, a battery of purified ragweed allergens should be available for testing and patients treated with only those allergens to which they are sensitive.

b. Studies on the structural nature of pollen allergens have been limited thus far to the few highly purified preparation referred to above. The availability of additional allergens would facilitate comparative structure-function studies. The biological role of peptide and carbohydrate moieties, of primary, secondary and tertiary structures requires elucidation for a number of purified allergens. It is as yet unclear whether atopic allergens have structural features which cause them to induce reaginic-type antibodies in the atopic individual. In this connection the similarity in charge and size of Antigen E and the purified rye allergens I-B and I-C suggest that such properties, among others, might be common features of atopic allergens in general. However, an insufficient number of purified allergens of distinct and common cellular sources have been studied to allow any generalizations to be made concerning this problem.

c. In connection with structural studies, chemical and enzymatic degradation of ragweed allergens might result in the production of haptenic fragments able to effect in vivo neutralization of the reaginic antibodies of allergic patients with no attendant release of pharmacological mediators. Studies on enzymatic proteolysis of Antigen E have not, as yet, resulted in the production of allergenically inhibitory fragments. It is possible, however, that ragweed allergens other than Antigen E might prove more amenable to the preparation of such fragments.

PART B: PRESENT INVESTIGATIONS

CHAPTER III

ISOLATION OF A PURIFIED ALLERGEN, ANTIGEN Ra.3

1. Introduction

In the course of preliminary chromatographic fractionation of aqueous extracts of short ragweed pollen, an allergenically active fraction devoid of Antigen E was found to contain a single antigen (Ra.3) when tested with rabbit antiserum to the whole extract. The final procedure for the isolation of Antigen Ra.3, to be described in this chapter, was based on the method of King et al (113, 119) for the isolation of Antigen E.

2. Materials and Methods

a. Materials

Short ragweed pollen was purchased from Sharp and Sharp, Inc. (Everett, Washington). Sephadex G-25 (medium) and G-100 (40-120 μ) were purchased from Pharmacia (Montreal). Diethylaminoethyl-(DEAE-) and triethylaminoethyl-(TEAE-)

celluloses were of standard capacity and were purchased from Calbiochem. (Los Angeles). All other chemicals were reagent grade and were purchased from Fisher Scientific Co. (Montreal).

b. Column Chromatography

Sephadex gels were routinely suspended in water for three days before use; on the third day, the slurry was consecutively stirred, allowed to settle, and decanted to remove the fines. The slurry was suspended, stirred, and allowed to settle three times in the starting buffer. Columns were poured as a slurry containing an equal mixture of packed gel and buffer. Sephadex G-100 columns were packed under a pressure head of about 20 cm of buffer; Sephadex G-25 columns were packed under a head of 50 to 75 cm of buffer.

DEAE- and TEAE-celluloses were twice washed alternately in 0.2 M NaOH and 0.2 M HCl, and finally with 0.2 M NaOH. The slurries were washed with distilled water until the pH reached 8.0, after which they were suspended in starting buffer three times, prior to being poured into columns.

All gel filtration and anion exchange columns were equilibrated by passage of starting buffer for 24 hours prior to application of the sample. Chromatography was performed at 24 - 26°C and column effluents were collected in a Buchler or Spinco fraction collector, refrigerated at 5°C.

The effluents were monitored by their absorption of ultraviolet light at 280 mμ as measured manually with a Beckman DU spectrophotometer or automatically with an LKB Uvicord spectrophotometer (LKB-Produkter) equipped with a 2 ml flow cell. Total hexose and pentose determinations were done on the effluent fractions using the Tryptophane method (see Chapter VI, 2. Methods).

Pooled fractions were concentrated in a Diaflo ultrafiltration cell at 6°C equipped with a UM-2 membrane (Amicon Corp., Cambridge, Mass.). The yield of Antigen Ra.3 was determined from optical density (O.D.) measurements at 280 mμ, using the experimentally determined coefficient of $E_{1cm}^{1mg/ml} = 1.09$ at pH 7.3 (see Chapter VI, 2. Methods). For storage, solutions of Antigen Ra.3 were millipore filtered (0.22 μ) and kept at 5°C.

3. Experiments and Results

a. Preparation of Aqueous Extract

To defat short ragweed pollen, 2 liters of anhydrous ethyl ether were added to 1000 gms of the pollen, the suspension was stirred for 0.5 hrs., allowed to settle for 1 hr. and the ethereal layer decanted. This procedure was repeated six times until the ethereal layer was free of pigmented substances. The defatted pollen was dried in air for 2 days.

The dried, defatted pollen was extracted by stirring for 4 hours at room temperature in 5000 mls of 0.005 M sodium

phosphate buffer, pH 7.4. A few drops of toluene were added to prevent bacterial growth. The suspension was filtered by suction through 2 layers of Whatman No. 3 filter paper on a Buchner funnel. The residue of insoluble material was washed with an additional 500 mls of phosphate buffer and discarded. The combined filtrates (5000 mls) were brought to pH 7.0 by the addition of 3 N NH_4OH.

The filtrate was cooled to 5°C and brought to 0.9 saturation in ammonium sulphate by slowly adding, with continuous stirring, 620 gms of the salt per liter of extract. The resulting suspension was stirred overnight at 5°C. The precipitate was isolated by centrifugation at 10,000 rpm for 40 mins. in an International B-20 centrifuge using rotor no. 874, which had been precooled to 5°C. The precipitate was dissolved in 500 mls of 0.1 M TRIS-HCl, pH 7.9, to form a viscous, dark brown solution (aqueous extract).

b. Sephadex G-25 Gel Filtration

The aqueous extract was separated into high and low molecular size fractions (A and B, respectively) on a Sephadex G-25 column (10.0 x 95 cms) equilibrated with the eluting buffer of 0.025 M TRIS-HCl, pH 7.9 (Fig. 1). The flow rate was 400 mls/hr and 20 ml fractions were collected. A considerable amount of pigmented material was adsorbed to the column: this could be partially removed by extensive washing of the column

with 1 M acetic acid. The G-25 columns were used only twice and then repoured with fresh gel.

c. DEAE-Cellulose Chromatography

The high molecular size fraction A (Fig. 1) was concentrated by ammonium sulphate precipitation at 5°C (0.9 saturation). The precipitate was dissolved in 0.025 M TRIS-HCl, pH 7.9, dialysed exhaustively and applied to a DEAE-cellulose column (5.0 x 50 cms) previously equilibrated with the same (eluting) buffer. The flow rate was 250 mls/hr and 20 ml fractions were collected. Fraction C, containing Antigen Ra.3, was obtained by this step (Fig. 2). Fraction D, containing Antigen E, was eluted with 0.05 M TRIS-HCl + 0.2 M NaCl, pH 7.9.

d. Sephadex G-100 Gel Filtration

DEAE-cellulose fraction C was concentrated to 150 mls by ultrafiltration at 5°C. Aliquots of 75 mls were dialysed against 0.1 M TRIS-HCl + 0.2 M $(NH_4)_2SO_4$ and separately applied to a bed of Sephadex G-100 (2 Pharmacia columns, each 5.0 x 70 cms, in series), equilibrated with the same (eluting) buffer. The flow rate was 50 mls/hr and 20 ml fractions were collected. As shown in Fig. 3, six fractions (C_I - C_{VI}) were obtained, of which fraction C_{IV} was further purified by TEAE-cellulose chromatography. The bulk of the carbohydrate (total hexose and pentose) was found in the higher molecular size fractions.

e. TEAE-Cellulose Chromatography

Sephadex G-100 fraction C_{IV} was concentrated to 20 mls by ultrafiltration at 5°C, dialysed exhaustively against 0.002 M TRIS-HCl, pH 7.6, and applied to a TEAE-cellulose column (2.0 x 50 cm) equilibrated with the same (eluting) buffer. The flow rate was 35 mls/hr and 5 ml fractions were collected. As shown in Fig. 4 (top), three fractions were obtained (C_{IV-1}, C_{IV-2} and C_{IV-3}).

Fraction C_{IV-2} was rechromatographed on TEAE-cellulose to remove contamination by adjacent fractions. As shown in Fig. 4 (bottom), 2 peak fractions were eluted, both of which contained carbohydrate. The major fraction contained Antigen Ra.3 in a yield of 100 mgs per kg of dry pollen.

4. Discussion

With minor modifications, the method employed by King et al (113, 119) to prepare Antigen E was adopted for the isolation of Antigen Ra.3. In this way a degree of standardization was established between the isolation procedures for the two antigens.

A relatively large scale isolation was carried out, since the yield of Antigen Ra.3 was only 100 mgs/kg of pollen. The processing of a kilogram of pollen instead of the 200 gm

quantities employed for the isolation of Antigen E by King et al did not entail any additional problems except in the initial stages. For example, the isolation by centrifugation of the ammonium sulfate precipitate of the aqueous extract required approximately 12 hours for completion and was done continuously.

The Sephadex G-25 gel filtration and the DEAE-cellulose chromatography procedures were essentially similar to those of King et al. As Sephadex G-100 was reported to be more effective than G-75 for the gel filtration of DEAE-cellulose fraction D (119), the same gel was employed to further resolve DEAE-cellulose fraction C. It was assumed that fraction C would be as heterogeneous as fraction D, so that the ratio of sample volume: column volume was identical to that used by King et al: i.e., 0.01.

Further purification of fraction C_{IV} was effected by ion-exchange chromatography on TEAE-cellulose. Preliminary experiments indicated that DEAE-Sephadex was not suitable for purification of this fraction since extremely low flow rates were encountered with the low ionic strength buffers required for adsorption of the proteins to the resin.

Concentration of the chromatographic fractions was effected by ultrafiltration rather than lyophilization. It was felt that the latter method could lead to relatively greater losses of allergenic activity due to protein denaturation and aggregation, as found for Antigen E (119).

CHAPTER IV

HOMOGENEITY OF ANTIGEN Ra.3

1. Introduction

As described in the previous chapter, chromatography of fraction C_{IV-2} on TEAE-cellulose yielded a major fraction containing Antigen Ra.3. To obtain evidence for its homogeneity, the Antigen Ra.3 preparation was examined by various physico-chemical and immunological techniques. These consisted of: rechromatography on TEAE-cellulose; disc electrophoresis at acid, near-neutral and alkaline pH; analytical ultracentrifugation; gel filtration on Sephadex G-100; and immunodiffusion with rabbit antisera.

2. Materials and Methods

a. Materials

All chemicals were of standard reagent grade, and most were purchased from the Fisher Scientific Co. (Montreal). Chemicals used in the disc electrophoresis experiments were purchased from the Canalco Co. (Rockville, Md.) except for Tris (Hydroxymethyl)aminomethane (TRIS), hydrochloric acid and acetic acid.

Agar gel, employed in immunodiffusion experiments, was prepared by heating in a boiling water bath, a suspension containing 0.85% Oxoid Ionagar no. 2 (Consolidated Laboratories) in 0.14 M NaCl buffered with 0.01 M sodium phosphate, pH 7.4, and 10^{-4} gm/ml of merthiolate. After a clear solution was obtained (approximately 0.5 hrs.), 5 ml aliquots were transferred to petri dishes (5 cm in diameter) and allowed to cool and harden for approximately 20 min. The petri dishes were covered and stored at 5°C.

Antigen E (IV-C) was prepared according to King et al (119) with minor modifications. DEAE-cellulose fraction D (Chapter III) was filtered on Sephadex G-100 to obtain fraction D_{IV}: the column dimensions and elution buffers were identical to those employed for the isolation of Antigen Ra.3. Fraction D_{IV} was chromatographed on TEAE-cellulose to obtain fraction IV-C (Antigen E) using a gradient elution system identical to that used by King et al (119). The Antigen E preparation showed a single precipitin arc on immunodiffusion in agar when tested with a rabbit anti-serum specific to Antigen E.

Two preparations of Antigen E (IV-C) were supplied by the National Institutes of Health (NIH), Bethesda, in sterile vials as a solution in 0.1 M TRIS-HCl, pH 7.9. On immunodiffusion with a rabbit antiserum specific to Antigen E, the NIH preparations showed a line of identity with the Antigen E prepared in the present study.

Antisera to the aqueous extract of short ragweed pollen (Chapter III) were prepared by immunizing rabbits with a mixture containing 1 ml of aqueous extract (35 mg/ml) and 1 ml of complete Freund's adjuvant (Consolidated Laboratories). The rabbits were injected subcutaneously at weekly intervals for four weeks. Ten days after the last injection, the animals were bled via the marginal ear vein. The antisera were stored in small aliquots at -20°C. The rabbits were hyperimmunized over a period of six months and bled at periodic intervals.

Specific antisera to Antigens Ra.3 and E were prepared by injecting rabbits subcutaneously with a mixture of 1 ml of antigen in saline (0.5 mg/ml) and 1 ml of complete Freund's adjuvant. The rabbits received two injections a week apart and were bled ten days after the last injection. The sera were stored in small aliquots at -20°C.

A rabbit antiserum specific to Antigen E was supplied by the NIH.

b. TEAE-Cellulose Chromatography

A TEAE-cellulose column was prepared as described previously (Chapter III, 2.). The column effluent was monitored at 280 mμ with an LKB Uvicord spectrophotometer. The carbohydrate (total hexose and pentose) content of Antigen Ra.3 was determined before and after chromatography as described in Chapter V.

c. Disc Electrophoresis

The disc electrophoresis apparatus consisted of 500 ml capacity upper and lower buffer vessels constructed of plexiglass and fitted with removable carbon electrodes (Speer Carbon, Montreal). The upper buffer vessel contained 8 apertures for housing the electrophoresis tubes and was suspended over the lower vessel by means of a plexiglass stand. A 400 v D.C. power supply (Heathkit) was employed in these experiments.

The method used was essentially that of Davis (188) with some modification. A single 6% running gel was used in conjunction with a discontinuous buffer system. Electrophoresis was carried out in soft glass tubes (0.7 cm i.d. x 12 cm).

Electrophoresis at pH 9.5 made use of the anodic buffer system of Ornstein and Davis (189). The electrode buffer consisted of:

TRIS	0.6 gms
Glycine	2.9 gms
H_2O to	1000 mls, pH 8.3

The running gel was formed from solutions (a), (b) and (c);

(a)	1 N NCl	48 mls
	TRIS	36.6 gms
	TEMED*	0.46 mls
	H_2O to	200 mls, pH 8.9

* TEMED = N,N,N',N' - Tetramethylenediamine

(b)	Acrylamide	48 gms
	BIS*	1.6 gms
	H_2O to	200 mls

(c) 0.07% Ammonium Persulphate in water.

To form the gel, the solutions were combined in the ratio (a):(b):(c) = 1:1:2 (v:v:v).

Electrophoresis at pH 6.6 utilized a cathodic system obtained from the technical literature issued by the Canalco Co. (dated 10/14/63). The electrode buffer consisted of:

2,6-Lutidine	3.82 ml
Glycine	1.37 gm
H_2O to	1000 mls, pH 8.3

The running gel was formed from solutions (a), (b) and (c):

(a)	1 N KOH	48 ml
	Glycine	114 gm
	TEMED	0.4 ml
	H_2O to	600 ml, pH 7.3
(b)	Acrylamide	48 gms
	BIS	1.6 gm
	H_2O to	100 mls

(c) 0.56% Ammonium Persulphate.

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* BIS = N,N' Methylenebisacrylamide

Electrophoresis at pH 3.8 was done with the cathodic system of Reisfeld et al (190). The electrode buffer consisted of:

Beta-Alanine	3.12 gm
Glacial Acetic Acid	0.80 ml
H_2O to	1000 ml, pH 4.8.

The running gel was formed from solutions (a), (b) and (c):

(a)	1 N KOH	48 ml
	Glacial Acetic Acid	17.2 ml
	TEMED	4.0 ml
	H_2O to	200 mls, pH 4.3
(b)	Acrylamide	48 mls
	BIS	1.6 gm
	H_2O to	100 mls

(c) 0.28% Ammonium Persulphate in water.

Except where indicated, sample loads of 0.05 mg in 0.15 ml of 0.005 M NH_4HCO_3 made 3% in sucrose, were applied to the top of the gels and electrophoresis was carried out for 2-3 hours using a current of 4 ma/tube. In the pH 9.5 system, bromphenol blue was used as a marker dye, each gel being run until the blue marker band had travelled a fixed distance. In the cathodic systems, no suitable dye was found and therefore gels were run for a constant time.

After electrophoresis, the gels were removed from the tubes and stained for 0.5 hr. with 0.5% amido black in 7%

acetic acid. After staining, the gels were rinsed in 7% acetic acid for 2 hours and destained electrically.

d. Analytical Ultracentrifugation

Sedimentation velocity experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. A single sector (2°) cell with a KEL-F centerpiece was employed, in conjunction with an AnD rotor. Experiments were performed with a rotor speed of 60,000 rpm and an average rotor temperature of 20°C. An average sedimentation constant was calculated for each run by plotting the logarithim of the distance of the sedimenting boundry from the axis of rotation against time. The sedimentation coefficient was calculated according to the formula:

$$S_{20,b} = 2.303(\log x_2 - \log x_1)/(t_2 - t_1)\ w^2$$

where

$S_{20,b}$ = sedimentation coefficient at 20°C in buffer

x_1 = distance in cm of the sedimenting boundry from the axis of rotation at time t_1

t = time in seconds

w = angular velocity in radians/sec.

The values of x_1 and x_2 were chosen from points which fell on the straight line plot of log x vs. t.

e. Gel Filtration on Sephadex G-100

Sephadex G-100 prepared as described earlier (Chapter III, 2.) was packed into a column (2.5 x 95 cm) and allowed to equilibrate overnight by passage of buffer (0.1 M TRIS-HCl + 0.2 M $(NH_4)_2SO_4$, pH 7.9). The column effluent was monitored with an LKB Uvicord.

f. Immunodiffusion

Double diffusion in agar was carried out by the Ouchterlony technique (191). Appropriate wells were cut out of the agar and filled with reactants. The petri dishes were incubated at room temperature in a humid atmosphere for 24 to 48 hours during which time the appearance of precipitin lines was observed.

3. Experiments and Results

a. TEAE - Cellulose Chromatography of Antigen Ra.3

80 mg of Antigen Ra.3 in 5 mls of 0.002 M TRIS-HCl buffer, pH 7.6, was applied to a TEAE-cellulose column (2.0 x 50.0 cm), previously equilibrated with the same buffer. A single peak emerged from the column after elution of 82 mls (Fig. 5). The carbohydrate content (hexose and pentose) of Antigen Ra.3 was identical before and after chromatography,

i.e., 12.4 ± 2%. The recovery of Antigen Ra.3 was determined by optical density measurements to be 97%.

b. Disc Electrophoresis of Antigens Ra.3 and E

On disc electrophoresis (Fig. 6) at pH 9.5, Antigen E migrated toward the anode as one major, and one minor band, while Antigen Ra.3 migrated toward the cathode and was therefore not observed in the gel. At pH 6.6, Antigen E still migrated toward the anode and therefore did not penetrate the gel; Antigen Ra.3 showed only one component in this system. At pH 3.8, both antigens migrated toward the cathode: Antigen E was observed as a major component, with some trailing; Antigen Ra.3 showed essentially one component with the possibility of a trace contaminant on the cathodal side of the band. Heterogeneity at pH 9.5 was also observed with the two NIH preparations of Antigen E examined at a sample load of 0.15 mg (Fig. 7). The Antigen E prepared in the present study was considered to be less heterogeneous.

c. Analytical Ultracentrifugation of Antigen Ra.3

Samples of Antigen Ra.3 were exhaustively dialysed against a buffer of 0.1 M NaCl + 0.01 M sodium phosphate, pH 7.3, and run at four different concentrations: 1.0, 0.8, 0.6, and 0.4%. The photographs of the schlieren patterns obtained for Antigen Ra.3 at the three highest concentrations are reproduced in

Fig. 8. Only one peak was observed in all cases: the sedimentation coefficients calculated for the four concentrations are given in Table I.

d. Sephadex G-100 Gel Filtration of Antigen Ra.3

Four mls of Antigen Ra.3 (12.0 mg/ml) in 0.1 M TRIS-HCl + 0.2 M $(NH_4)_2SO_4$, pH 7.9, was applied to a Sephadex G-100 column (2.5 x 95 cm) and eluted with the same buffer at a flow rate of 20 mls/hr. A single peak emerged from the column after passage of 380 mls of buffer (Fig. 9).

e. Immunodiffusion of Antigens Ra.3 and E

Antigen Ra.3, at the various concentrations shown in Fig. 10, was allowed to diffuse against a rabbit anti-Ra.3 serum. As can be seen in Fig. 10, Antigen Ra.3 showed only one component at all concentrations examined. Identical results were obtained with 3 rabbit antisera.

Antigens Ra.3 and E, each at a concentration of 1 mg/ml, were allowed to diffuse against an antiserum to the aqueous extract of short ragweed pollen. The results are shown in Fig. 11: each antigen preparation gave only one precipitin arc with no evidence of cross-reaction. Identical results were obtained with 2 rabbit antisera.

The possible cross-contamination of Antigens Ra.3 and

E was investigated by immunodiffusion with specific antisera. The results are shown in Fig. 12. The Ra.3 - anti Ra.3 reference line (Fig. 12 (a)) between well 1 and well 2 was deflected by 0.02 mg Antigen Ra.3/ml in well 3. However, Antigen E in well 4, caused no such deflection. Thus, Antigen E at a concentration of 4.0 mg/ml would contain less than 0.02 mg Antigen Ra.3/ml, or less than 0.5%. Similarly, any contamination of Antigen Ra.3 by Antigen E would be less than 0.5% (Fig. 12 (b)).

4. Discussion

The experiments and results of this chapter demonstrated the homogeneity of Antigen Ra.3 by several criteria.

Rechromatography of Fraction C_{IV-2} (Chapter III) on TEAE-cellulose gave a single peak which was only slightly asymmetric, indicating removal of contaminating components from adjacent fractions C_{IV-1} and C_{IV-3}. The presence of an identical amount of carbohydrate, before and after chromatography provided evidence that this moiety was an integral part of the molecule.

Antigen Ra.3 contained one major component on disc electrophoresis at pH 6.1 and 3.8. Antigen E on the other hand, contained one major and one minor component at pH 9.5. The Antigen E preparations of the present study and those supplied

by the NIH displayed multiple banding. The disc electrophoretic heterogeneity of Antigen E may have been due to the presence of either genetic polymorphs or unrelated contaminants, but this was not studied further. The disc electropherograms revealed electrophoretic differences between Antigen Ra.3 and Antigen E: the former was shown to be a basic protein, the latter, acidic.

Antigen Ra.3 sedimented in the analytical ultracentrifuge as a single peak with a sedimentation coefficient in the range of 1.68 to 1.76 S. No gross contamination with more rapidly sedimenting contaminants was evident. Gel filtration on Sephadex G-100 provided additional evidence for the molecular size homogeneity of Antigen Ra.3.

The immunodiffusion experiments provided evidence for the antigenic purity of Antigen Ra.3. No contaminating components were found on testing Antigen Ra.3 with rabbit antisera to the whole extract or with specific anti-Ra.3 sera. The results obtained with rabbit antisera to the aqueous extract (Fig. 11) demonstrated the antigenic disimilarity of Antigens Ra.3 and E. On the basis of experiments with specific rabbit antisera, any cross-contamination of the two antigens would be less than 0.5%.

CHAPTER V

PHYSICAL AND CHEMICAL PROPERTIES OF ANTIGEN Ra.3

1. Introduction

Some physico-chemical properties of Antigen Ra.3 have already been presented in Chapters III and IV. Antigen Ra.3 was shown to constitute a single size and electrophoretic species of carbohydrate-containing macromolecule. Studies were done to determine the additional physical and chemical parameters of molecular weight, absorption coefficient and spectrum, nitrogen and carbohydrate content and amino acid composition.

2. Materials and Methods

a. Materials

All standards employed for analysis were of reagent grade (Fisher Scientific Co., Montreal). Solution volumes were delivered with Class A (NBS) pipettes but were not calibrated before use.

Samples to be weighed were heated in a Freas oven (Model 825, Precision Scientific), at 105°C to constant weight, and stored over P_2O_5. A Mettler balance (Model S-6) was used for weighing.

Optical density measurements were made with Beckman DU and Coleman Junior spectrophotometers.

b. Determination of Molecular Weight

The sedimentation coefficients calculated from the sedimentation-velocity centrifugation experiments of Chapter III were extrapolated to infinite dilution. The extrapolated values were corrected to standard conditions using the relation:

$$S^{\circ}_{20,w} = S^{\circ}_{20,b} \, (\eta 20,b/\eta 20,w)$$

where

$S^{\circ}_{20,w}$ = sedimentation coefficient corrected to standard conditions of infinite dilution in water at 20°C

$S^{\circ}_{20,b}$ = sedimentation coefficient at infinite dilution in buffer at 20°C

$\eta 20,b/\eta 20,w$ = ratio of the viscosities of buffer and water at 20°C. Viscosity values were obtained from published data (192).

To obtain a diffusion coefficient for Antigen Ra.3, analytical ultracentrifugal runs in a capillary-type synthetic boundry cell were performed in 0.14 M NaCl + 0.01 M sodium phosphate buffer, pH 7.3, at three protein concentrations: 1.0, 0.8, and 0.6%. The concentrations of Antigen Ra.3 were determined from O.D. readings at 280 mμ. Rotor speeds of 9,000 rpm were

employed and, after equilibration for 1 hour, photographs of the schlieren patterns were taken at 32 minute intervals. A diffusion coefficient for each concentration was calculated by the height-area method (193) using the relation:

$$D_{20,b} = K^2A^2/4\pi tH^2$$

where

$D_{20,b}$ = diffusion coefficient in buffer at 20°C

K = enlargement factor (0.676×10^{-2})

A = area under peak after enlargement (cm^2)

H = height of peak after enlargement (cm)

t = time (sec.).

The ratio $A^2/4\pi H^2$ was plotted against time and the slope multiplied by K^2 to yield $D_{20,b}$ for each concentration. The diffusion coefficients were extrapolated to infinite dilution and corrected to standard conditions in an identical manner to that employed for the sedimentation coefficients.

The partial specific volume ($\bar{v}$) of Antigen Ra.3 was calculated by the method of Cohn and Edsall (194) from the amino acid composition (see below).

The molecular weight was calculated from the formula:

$$M.W. = R\ T\ S^{\circ}_{20,w}/D^{\circ}_{20,w}(1 - \bar{v}\rho)$$

where

R = gas constant (8.31 joules/°K/mole)

T = temperature (°K)

ρ = density of sedimenting medium determined from published data (192).

c. Absorption Coefficient and Spectrum

The absorption coefficient of Antigen Ra.3 at 280 mμ was determined on a sample of known optical density previously dialysed exhaustively against 0.005 M NH_4HCO_3. The sample was dried to constant weight at 105°C; the dialysate was weighed as a control. Subsequent protein concentrations were determined using the calculated absorption coefficient.

An absorption spectrum was obtained from optical density readings in the wavelength range 240 - 340 mμ given by a solution (1.04 mg/ml) of Antigen Ra.3 in 0.005 M sodium phosphate buffer, pH 7.3.

d. Nitrogen Content

Nitrogen content was determined by the method of Jacobs (195). Solutions of standards and samples were prepared in 0.005 M sodium phosphate buffer, pH 7.3. The buffer alone served as a blank. Aliquots (0.2 mls) of the various solutions were separately added to 20 ml test tubes containing 0.1 ml of concentrated H_2SO_4 and 1 mg of a catalyst, consisting of $CuSO_4$:K_2SO_4:HgO:Se, 5:15:5:1. After heating for 2 hours the digests were cooled, oxidized with 0.05 ml of 30% H_2O_2 and heated for 4 hours. After cooling, the sulphuric acid was neutralized with 10 mls of 0.4 M sodium citrate buffer, pH 5.5, and an aliquot (0.3 ml) from each tube added to 1.7 ml of 4 M

sodium acetate buffer, pH 5.5. An equal volume of ninhydrin solution (196) was added and the mixtures heated in a boiling water bath for 40 minutes. After cooling, 6 mls of 50% ethanol was added to each tube and the optical density of the solutions determined at 570 mμ.

e. Carbohydrate Content

Standard solutions were prepared in 0.005 M sodium phosphate buffer pH 7.3. Blanks consisted of buffer to which reagent was added, as well as sample solutions with reagent emitted. All determinations were done in duplicate, except for the arabinose standards in the total hexose and pentose assays.

Total hexose and pentose was determined by the Tryptophane method (197). 7 ml portions of 77% H_2SO_4 were separately added to 1 ml aliquots of sample and standard solutions previously cooled to 15°C. After cooling, 1 ml aliquots of 1% Tryptophane solution were added and the mixtures shaken. After thorough mixing, the solutions were heated for 20 minutes in a boiling water bath, cooled to room temperature, and the optical density determined at 500 mμ.

Pentose was assayed by the Cysteine-sulphuric acid method (198). Aliquots (4 mls) of concentrated H_2SO_4 were separately added to 1 ml aliquots of samples and standards previously cooled to 15°C. The reaction mixtures were kept at room temperature for one hour with intermittant shaking, and 0.1 ml

aliquots of 3% Cysteine-HCl were added. The optical density of the reaction mixtures was determined at 390 and 424 mμ.

Hexose was determined by the anthrone procedure (199). Aliquots (2.0 mls) of sample and standards were layered over 4.0 ml aliquots of 0.2% anthrone solution in concentrated H_2SO_4. The mixtures were vigorously shaken in a cold water bath (15°C), brought to room temperature, and heated at 90°C for 16 minutes. After cooling, the optical density of the reaction mixtures was determined at 625 mμ.

Hexosamine was determined by the Elson-Morgan procedure (200). Samples and standards were hydrolysed in vacuo in 2 N HCl at 108°C for 4 hours. After hydrolysis, the solutions were dried by rotary evaporation and the contents of each tube dissolved in 2.0 mls of water, following which, the solutions were quantatatively transferred to 10 ml volumetric flasks. Aliquots (1.0 ml) of a solution of acetylacetone (0.2 ml in 10 ml of 0.5 N Na_2CO_3) were added, and the mixtures heated in a boiling water bath for 30 minutes. After cooling, 3.0 ml aliquots of absolute ethanol, and 1.0 ml aliquots of Erlichs reagent (201) were added with mixing. The flasks were made up to the mark and incubated in a water bath at 37°C for 30 minutes and allowed to cool. Optical densities were read at 540 mμ.

f. Amino Acid Composition

The amino acid composition of Antigen Ra.3 was determined with a Spinco Model 120B amino acid analyser according to the method of Spackman, Stein and Moore (202). The columns were standardized with a mixture containing 0.5 µmoles of each amino acid. Duplicate samples were hydrolysed in 6 N HCl for 24, 48 and 72 hours at 108°C, dried by rotary evaporation (x2), and dissolved in sodium citrate buffer, pH 2.06.

Cysteine and cystine were determined as total cysteic acid on a separate sample which had been oxidized with performic acid by the method of Hirs (203) prior to acid hydrolysis.

Tryptophane was determined by a spectrophotometric method (204) on samples of the intact protein dissolved in 0.1 N NaOH. Optical densities were read at 280 and 294 mu and the tryptophane content was calculated from the formula:

$$M\ tyr/M\ tryp = \frac{0.592 \times O.D._{294} - .263 \times O.D._{280}}{0.263 \times O.D._{280} - 0.170 \times O.D._{294}}$$

where

M = gram moles in 1 gram of protein.

3. Experiments and Results

a. Molecular Weight

The sedimentation coefficients of Antigen Ra.3 were determined for various concentrations (Table I). Extrapolation of the coefficients to infinite dilution (Fig. 13) gave a value of 1.76×10^{-13} sec^{-1}, or 1.80×10^{-13} sec^{-1} when corrected to standard conditions.

The diffusion coefficients were calculated for three concentrations and are given in Table II. Extrapolation of the diffusion coefficients to infinite dilution gave a value of 9.98×10^{-7} cm^2 sec^{-1}, or 10.1×10^{-7} cm^2 sec^{-1} when corrected to standard conditions.

A value of 0.715 was calculated for the partial specific volume of Antigen Ra.3, assuming a value of 0.61 for the specific volume of the carbohydrate (205).

From the aforementioned data, the molecular weight of Antigen Ra.3 was calculated to be 15,172.

b. Absorption Coefficient and Spectrum

A 4.0 ml sample of Antigen Ra.3 in 0.005 M NH_4HCO_3 had an optical density of 1.36 at 280 mμ. The dry weight of the sample was 4.99 mg; the dialysate control had no weight. The absorption coefficient was calculated to be

$$E_{1cm}^{1mg/ml} = 1.09 \pm 0.02.$$

The absorption spectrum of a solution (1.04 mg/ml) of Antigen Ra.3 in 0.005 M sodium phosphate buffer, pH 7.3, was determined over a wave-length range of 240 - 340 mμ. As shown in Fig. 14, the spectrum showed a peak absorbance at 280 mμ.

c. Nitrogen Content

Standard solutions of $(NH_4)_2SO_4$ were prepared containing 0.37, 0.185, 0.093, and .061 mgN/ml. A solution of Antigen Ra.3 (2 mg/ml) in 0.005 M sodium phosphate buffer, pH 7.3, was used for analysis. The colorimetric values obtained are listed in Table III: from the derivative standard curve (Fig. 15) a nitrogen content of 13.5 ± 4% was calculated for Antigen Ra.3.

d. Carbohydrate Content

For the analyses, a solution of Antigen Ra.3 (0.39 mg/ml) in 0.005 M sodium phosphate buffer, pH 7.3, was employed except for the hexosamine determination, for which a solution of 1.0 mg/ml was used.

To determine total hexose and pentose, standard solutions of arabinose were prepared of concentrations: 112.0, 89.6, 67.2, 44.8 and 22.4 μg/ml. The colorimetric values obtained are given in Table IV: from the derivative standard curve (Fig. 16), a total hexose and pentose content of 12.4 ± .2% (as arabinose) was calculated for Antigen Ra.3.

To determine total hexose, standard solutions of galactose were prepared of concentrations: 51.0, 25.5, and 12.75 µg/ml. The colorimetric values obtained are given in Table V: from the derivative standard curve (Fig. 17), a total hexose content of 4.9 ± .2% (as galactose) was calculated for Antigen Ra.3.

To determine total pentose, standard solutions of arabinose were prepared of concentrations: 45.0, 22.4, 14.0 and 9.0 µg/ml. The colorimetric values obtained are given in Table VI: from the derivative standard curve (Fig. 18), a total pentose content of 8.7 ± .2% (as arabinose) was calculated for Antigen Ra.3.

To determine total hexosamine, standard solutions of galactosamine were prepared of concentrations: 72.5, 50.8, 25.4, and 12.7 µg/ml. The colorimetric values obtained are given in Table VII: from the derivative standard curve (Fig. 19), a total hexosamine content of 0.8 ± .02% (as galactosamine) was calculated for Antigen Ra.3.

e. Amino Acid Composition

Duplicate samples each containing 2.3 mg of Antigen Ra.3 were hydrolysed for 24, 48 and 72 hours and the hydrolysates were taken up in 2.7 mls of citrate buffer. Duplicate 24 hour hydrolysates of a performic acid oxidized sample of Antigen Ra.3 was treated in an identical manner. The results of the

amino acid analysis of these hydrolysates are shown in Table VIII. From the data of this table, the % nitrogen recovered was calculated to be 91.3%. From the average values for each hydrolysis time, a best value was determined as the average value for the 3 hydrolysis times or, the value extrapolated to zero hydrolysis time. The average and best values are listed in Table IX. The number of residues of each amino acid listed in the table was calculated on the basis of a molecular weight of 15,172, of which 13% was carbohydrate.

Discussion

The molecular weight of Antigen Ra.3 determined in the analytical ultracentrifuge was 15,172.

The total carbohydrate determined as total hexose and pentose was 12.4 ± .2%, in close agreement with the sum of the separate hexose and pentose determinations: 4.9 ± .2 + 8.7 ± .2 = 13.6 ± .4%. The hexosamine content was 0.8 ± .02%. This value may be minimal since no attempt was made to determine the optimum hydrolysis time for maximum liberation of hexosamine units.

The nitrogen content of 13.5 ± .4% was in accord with the presence of 13% carbohydrate in the molecule.

The absorption spectrum of Antigen Ra.3 showed a single peak with a maximum at 280 mμ, typical of protein.

Amino acid analysis demonstrated the presence of all the common amino acids; of these, proline was present in relatively high concentration (approximately 10%).

CHAPTER VI

ALLERGENIC ACTIVITY OF ANTIGEN Ra.3 AND ANTIGEN E

1. Introduction

In Chapter IV evidence was presented for the presence of a single component in the Antigen Ra.3 preparation. In particular, it was demonstrated that this preparation contained no detectable Antigen E, the major allergen in ragweed pollen (113, 119). In view of the established purity of Antigen Ra.3, an assessment was made of its allergenic activity by direct skin tests of ragweed allergic patients, and by Prausnitz-Kustner (P-K) tests with the sera of ragweed sensitive patients. Simultaneous testing of Antigen E and of the whole aqueous ragweed extract was done to provide data on the relative activity of the three allergen preparations. In addition, the reaginic activity directed to Antigens Ra.3 and E, in the sera of ragweed allergic patients, was assayed by the technique of in vitro anaphylaxis of monkey skin suspensions. Finally, cross neutralization tests were performed to investigate the allergenic cross reactivity of Antigen Ra.3 and Antigen E.

2. Materials and Methods

a. Materials

Antigen Ra.3 and the aqueous extract of short ragweed pollen (water soluble ragweed: WSR) were prepared as described in Chapter III. Antigen E was prepared as described in Chapter IV.

b. Measurement of Total Solid Concentration of Allergen Solutions

The total solid concentration of solutions of WSR was determined by heating aliquots to constant weight at 105°C. The concentration of Antigen Ra.3 solutions was determined spectrophotometrically at 280 mμ using the absorption coefficient determined in Chapter V, and that of Antigen E solutions using the published absorption coefficient (113), $E^{1\ mg/ml}_{1\ cm} = 1.13$ at pH 7.15.

c. Direct Skin and P-K Tests

Prior to testing, each allergen solution was sterilized by filtration through a millipore membrane filter (0.22 μ pore size) at a concentration of 1 mg/ml in 0.14 M NaCl + 0.01 M sodium phosphate, pH 7.4 (phosphate buffered saline: PBS). For testing, the sterile solutions were diluted to an appropriate concentration with sterile PBS.

Direct skin testing of allergen solutions was done by intradermal injection of 0.05 ml aliquots into the forearm of a ragweed sensitive individual. Reactions were read at 15 minutes post-injection and the area of the wheals determined from the dimensions of the major and minor axes.

For P-K tests, normal volunteers (negative atopic history and negative skin test to ragweed extract) were injected intradermally with 0.05 ml aliquots of appropriately diluted ragweed allergic serum at skin sites on the back, 4 to 6 cm apart. After 24 hours, the sensitized sites were challenged with the appropriate allergen solution and the reactions were measured in the same manner as employed in direct skin testing. The relative activity of the various allergen preparations were assayed simultaneously in individual volunteers.

d. In Vitro Anaphylaxis of Monkey Skin Suspensions

Allergenic activity was assayed by an in vitro anaphylactic technique (206). Weighed portions (200 mg each) of rhesus monkey skin fragments were sensitized by incubation with ragweed reaginic serum (2.0 ml of serum made 1:2.5 with Tyrode (207)) for 1.5 hours at 37°C. The sensitized fragment aliquots were separately washed three times with Tyrode and incubated for 15 minutes at 37°C with solutions of allergen prepared in Tyrode. After centrifugation, the supernatants were assayed for the presence of smooth muscle stimulators using the

isolated guinea pig ileum preparation, standardized with histamine. Reactions were quantitated by comparison with those of histamine standards and recorded as histamine equivalents per gram of tissue.

e. Cross-Neutralization Tests

Cross-neutralization tests were performed by diluting reaginic serum with allergen solutions in vitro and incubating at room temperature for 24 hours prior to sensitization of normal skin, as in the P-K test. The injected sites were challenged with appropriate allergen and the reactions measured as in the P-K test. The concentration of allergen solution used to dilute the reaginic serum was such as to just cause complete inhibition of the P-K reaction, and was determined in preliminary experiments.

3. Experiments and Results

a. Comparison of the Allergenic Activity of Antigens Ra.3 and E by Direct Skin Test

Antigen Ra.3 and Antigen E were used in direct skin testing of 46 ragweed sensitive patients. Of this total patient group, 26 had undergone at least one year of hyposensitization therapy with ragweed extract, while the remaining 20 patients

were untreated. For testing, allergen solutions were freshly diluted to a concentration of 10^{-9} gm/ml in sterile PBS. Each patient received a single dose of each preparation.

The results of the direct skin tests are recorded in Table X and Fig. 20. The reactions were of 3 types: 28 patients (Group I) reacted with greater intensity to Antigen E than to Antigen Ra.3; 10 patients (Group 2) reacted with approximately the same intensity to both allergens; 8 patients (Group 3) showed no reaction to either allergen at the concentration used for testing.

b. Assay of the Allergenic Activity of Antigen Ra.3 and Antigen E by P-K Test

P-K tests were performed with the sera of patients representing 2 groups: those reacting predominantly to Antigen E by direct skin test, and those reacting to both allergens with approximately the same intensity. The tests were carried out by sensitizing with a constant dilution of a given allergic serum and challenging with varying concentrations of allergen solution. Alternatively, skin sites were sensitized with varying dilutions of a given allergic serum and challenged with a constant concentration of allergen solution.

Table XI shows the results of experiments using constant serum and varying allergen dilutions. The dilution of a

given allergic serum was adjusted so that the cutaneous reactions obtained for all of the sera would fall within a similar range. Antigen E showed considerably greater activity than Antigen Ra.3 when tested with sera P.H. and A.C., in agreement with the results of direct skin testing of these patients (Table X). Both allergens had approximately the same degree of reactivity when tested with the remaining sera (M.M., K.B., M.C., B.U., M.B.), again in accord with the direct skin test results (Table X).

Table XII shows the results obtained when varying dilutions of allergic serum were used for sensitization and the sites challenged with a constant concentration of allergen solution. The sera of patients P.H. and A.C., who reacted more strongly to Antigen E than to Antigen Ra.3 on direct skin test (Table X), showed greater reaginic activity to Antigen E than to Antigen Ra.3. The sera of patients M.M., and K.B., who gave similar reactions on direct skin test with Antigens Ra.3 and E, showed approximately equal reaginic activity to both allergens.

c. Comparison of the Allergenic Activity of Antigens Ra.3 and E by In Vitro Passive Anaphylaxis of Monkey Skin Suspensions

In vitro passive anaphylaxis was employed to corroborate the relative activity of Antigens Ra.3 and E observed by direct skin and P-K tests. Three sera from patients who, on direct skin test, reacted predominantly to Antigen E, and seven

sera from patients who showed equal reactivity to both allergens, were assayed by this method. Monkey skin suspensions sensitized with allergic serum were challenged with 50 µg of allergen/ml. The results are shown in Table XIII and are represented diagramatically by the histograms in the lower half of Fig. 21. The reactions plotted are the averages of duplicate determinations given in Table XIII. For comparison, the upper half of Fig. 21 shows the direct skin tests results obtained for the same patients. In all cases, the in vitro anaphylactic reactions agreed with the direct skin test data, except for patient F.V. Patient F.V. reacted to both allergens on direct skin test but reacted only to Antigen E by in vitro anaphylaxis.

The activities of Antigens Ra.3 and E were compared by the in vitro anaphylactic technique using sera M.M. and A.C. for sensitization and, for challenge, 10-fold dilutions of approximately equimolar concentrations of the two allergens. The results obtained are shown in Table XIV and Figs. 22 and 23. Serum M.M., (Fig. 22) showed equal reactivity to both allergens while serum A.C. (Fig. 23) reacted only to Antigen E. These results agreed with the in vitro anaphylactic reactions previously obtained employing a single allergen concentration (Fig. 21) and with the direct skin test results (Table X).

d. Comparison of the Allergenic Activity of Antigens Ra.3 and E With WSR by P-K Test

Two allergic sera, M.M. and K.B., showing approximately equal reaginic activity to Antigens Ra.3 and E (Table XI) were employed in these experiments. Normal skin sites sensitized with these sera were separately challenged with ten-fold serial dilutions of the allergen solutions. The results are shown in Table XV. The cutaneous activity of the two purified allergens was approximately 100 to 1000 times greater than the activity of WSR.

e. Allergenic Cross-Reactivity of Antigens Ra.3 and E

To determine the allergenic cross-reactivity of Antigens Ra.3 and E, the two allergens were compared for their capacity to cross-neutralize the reagins in three ragweed allergic sera.

The sera obtained from patients M.M., M.C., and M.B. showed approximately equal reaginic activity to both allergens (Table XI).

Approximately equimolar concentrations of Antigens Ra.3 and E (0.5 µg/ml and 1.0 µg/ml, respectively) were employed to challenge the sensitized sites.

In a preliminary experiment, each allergic serum was separately incubated with serial dilutions of Antigens Ra.3 and

E and the serum-allergen mixtures were used to prepare sensitized skin sites. The sites were separately challenged with the same allergen employed for incubation and the cutaneous reactions obtained are shown in Table XVI and were identical for all sera. As can be seen from the table, a concentration of 5 µg/ml of Antigen Ra.3 and 10 µg/ml Antigen E were required for complete inhibition of the P-K reaction to the respective allergen. These concentrations were employed in the cross-neutralization tests.

To perform the tests, skin sites on three normal volunteers were injected with the allergic serum-allergen mixtures shown in Table XVII. Allergic serum-saline mixtures served as non-neutralized controls. 24 hours following injection, the sites were separately challenged with Antigens Ra.3 and E and the cutaneous reactions obtained are shown in Table XVII. In all cases, Antigen Ra.3 completely neutralized Ra.3-specific antibodies but failed to neutralize the E-specific antibodies. On the other hand, Antigen E neutralized the serum reagins directed to both allergens.

4. Discussion

The allergenic activity of Antigen Ra.3 was established by direct skin testing of ragweed allergic patients (Table X and Fig. 20). Of the patients giving positive cutaneous reactions, 75% reacted predominantly to Antigen E, while 25% reacted

with approximately equal intensity to both allergens. In this respect, no difference was observed between treated and untreated patients.

The relative activities of Antigens Ra.3 and E seen on direct skin test of individual patients was also observed in the P-K and in vitro anaphylactic assays. Thus, patients who reacted predominantly to Antigen E on direct skin test, possessed greater reaginic activity to this allergen than to Antigen Ra.3. On the other hand, patients who reacted with approximately equal intensity to both allergens on direct skin test showed similar reaginic activity to both allergens.

The variation in the relative activities shown by Antigens Ra.3 and E on direct skin testing (Fig. 20) demonstrated that the two allergens had distinct specificities. This was confirmed by assays of allergenic activity in P-K tests with seven allergic sera (Table XI).

Thus, the same preparation of Antigen Ra.3 showed positive reactions for some sera and little or no activity for others, while Antigen E elicited positive reactions with all of the sera. Similar results were obtained in the in vitro anaphylactic assays (Figs. 21, 22 and 23).

The ability of Antigen E to completely neutralize Ra.3-specific reagins could not have been due to contamination of the Antigen E preparation by Antigen Ra.3. The amount of Antigen Ra.3 needed to completely neutralize the Ra.3-specific reagins (Table

XVI) was greater than the amount which could have been present in the Antigen E preparation as contaminant, (0.5%, see Chapter IV, 3.e.).

Since Antigen Ra.3 could neutralize reagins to itself but not those directed to Antigen E, while Antigen E neutralized reagins of both specificities, it would appear that Antigen E possesses both its own unique allergenic determinants as well as allergenic determinants in common with Antigen Ra.3.

CHAPTER VII

DIFFERENCES IN CHROMATOGRAPHIC DISTRIBUTION OF REAGINS TO ANTIGEN Ra.3 AND ANTIGEN E

1. Introduction

It has been reported (81, 82) that reaginic antibodies contained in the sera of ragweed allergic individuals show considerable heterogeneity in elution properties when chromatographed on the anion exchanger, DEAE-Sephadex. This reported heterogeneity may in part reflect the presence in allergic serum of reaginic antibodies directed to diverse allergens in the whole aqueous ragweed extract employed in the studies cited. The experiments of this chapter were designed to provide evidence for this, in view of the availability of two purified ragweed allergens, Antigens Ra.3 and E. For this purpose, two ragweed allergic sera were separately chromatographed on DEAE Sephadex and the derivative fractions were assayed by P-K test for reaginic activity to each allergen.

2. Materials and Methods

a. Materials

DEAE-Sephadex A-50 (Pharmacia) was suspended in water overnight and washed alternately with 0.2 N NaOH and 0.2 N HCl, the last wash being 0.2 N NaOH. The slurry was brought to pH 8.0 by successive washings with distilled water and suspended in starting buffer three times prior to pouring. Solutions of Antigen Ra.3 and E were prepared from sterile stock solutions by dilution with sterile PBS to final concentrations of 0.5 and 1.0 µg/ml, respectively.

Two sera, M.M. and K.B., which contained approximately equal reaginic activity to both Antigens Ra.3 and E (Table XII), were employed in these experiments.

b. Column Chromatography

DEAE-Sephadex chromatography was performed at 5°C, using step-wise elution with TRIS-HCl buffer, pH 8.0, of different molarities. The column effluent was monitored by its absorption at 280 mµ in an LKB Uvicord spectrophotometer equipped with a 2.0 ml flow cell and collected in a Spinco fraction collector.

Effluent fractions were concentrated by positive pressure ultrafiltration (see Chapter III, 2.). After dialysis

against PBS, the fractions were millipore filtered and assayed for reaginic activity by the P-K test (Chapter III, 2.) done in duplicate and at two dilutions.

3. Experiments and Results

A sample (10 mls) of each allergic serum was dialysed overnight against two 2 liter changes of 0.15 M TRIS-HCl, pH 8.0. The samples were separately run on a column (1.8 x 25 cm) which had been equilibrated by passage of the same buffer for 24 hours. After elution of 135 mls, a second buffer of 0.23 M TRIS-HCl pH, 8.0, was applied to the column. After elution of 115 mls, a final buffer of 0.5 M TRIS-HCl, pH 8.0, was applied to remove the remaining protein.

The elution pattern obtained was essentially similar for both sera and is shown in Fig. 24 for serum M.M. Three fractions (I, II and III) were pooled as shown, and concentrated for testing.

The results of the P-K tests are shown in Tables XVIII and XIX and in Figs. 25 and 26, for serum M.M. and K.B., respectively. It can be seen from the results, that the reaginic activity directed toward Antigen E had a different distribution among the DEAE-Sephadex fractions than the activity toward Antigen Ra.3. For both sera, the maximum anti-E activity

appeared in fraction I, whereas the maximum reaginic activity specific for Antigen Ra.3 occurred in fractions II and III.

4. Discussion

The choice of buffer elution conditions employed in these experiments were suggested by the data of Goodfriend and his associates (81, 82). These workers, using a concentration gradient elution technique, found a spread of reaginic activity, with the bulk of the reagins being eluted between 0.10 M and 0.35 M TRIS-HCl, pH 8.0. Accordingly, in the present experiments, buffer molarities of 0.15, 0.23 and 0.5 M were employed for step-wise elution.

To improve the accuracy of the P-K assay, the reagin fractions were tested at dilutions such that the wheal areas fell into the range of 60 - 160 mm^2. Previous P-K testing had indicated that the wheal dimensions in this range were amenable to accurate measurement with a minimum standard deviation. As can be seen from Figs. 25 and 26, the average deviation was 10% from the mean.

The results shown in Tables XVIII and XIX and Figs. 25 and 26, demonstrated that the reaginic antibodies to Antigen E were eluted earlier in the chromatogram than the antibodies to Antigen Ra.3. In view of the relatively small deviation from

the mean (±10%) found in the P-K tests, it was concluded that the differences in elution properties of reaginic activity to Antigens Ra.3 and E were significant.

CHAPTER VIII

GENERAL DISCUSSION

The combination of gel filtration and anion-exchange chromatography proved efficacious for the purification of an allergenically active protein, Antigen Ra.3, from the complex mixture of substances contained in the aqueous extract of short ragweed pollen (Chapter III). The isolation procedure was patterned after the method of King et al (113, 119) for the preparation of Antigen E. This allowed for the simultaneous isolation of Antigens Ra.3 and E and consequently, for a comparative study of the properties of the two allergens. A more efficient isolation procedure might have been developed for Antigen Ra.3, had the latter's physical and chemical properties been known. For example, the polyacrylamide gel, Biogel P-30, might have proven superior to Sephadex G-100 for molecular size fractionation of DEAE-cellulose fraction C. In addition, the complexity of DEAE-cellulose fraction C might have been reduced by eluting with a buffer of lower ionic strength.

The procedures used to purify Antigen Ra.3 resulted in the isolation of a homogeneous preparation when judged by several criteria (Chapter IV). Evidence for charge homogeneity was obtained by TEAE-cellulose chromatography and polyacrylamide gel disc electrophoresis. Analytical ultracentrifugation and

gel filtration on Sephadex G-100 provided evidence for the size homogeneity of the Antigen Ra.3 preparation. Immunodiffusion experiments with two rabbit anti-sera prepared against the whole aqueous extract (WSR) and three rabbit anti-sera against the purified antigen preparation, demonstrated a single precipitin arc, underlining the antigenic purity of the Ra.3 preparation. Immunodiffusion analysis of Antigens Ra.3 and E with rabbit antiserum to WSR demonstrated that cross-contamination of the two allergens, if present at all, would be less than 0.5%.

Physical and chemical analysis (Chapter V) demonstrated that Antigen Ra.3 is a basic protein, containing an appreciable amount of carbohydrate. Evidence was obtained that the carbohydrate forms an integral part of the molecule. Thus, on rechromatography of the Antigen Ra.3-containing fraction, C_{IV-2} (Chapter III), the carbohydrate distribution followed the optical density distribution at 280 mμ (attributed to protein). Furthermore, the carbohydrate content of Antigen Ra.3 showed no change on rechromatography (Chapter IV) or extensive dialysis. Carbohydrate analysis of Antigen Ra.3 revealed the presence of hexose and pentose units, but the nature of these units and of their arrangement remains to be determined.

It is noteworthy, that the absorption spectrum of Antigen Ra.3 showed a single peak at 280 mμ, characteristic of proteins. No peak was evident at 305 mμ suggesting the absence of the 1-amino-1-deoxy-2-ketose linkages postulated by Berrens

to be a common feature of atopic allergens (Chapter I).

A comparison of the properties of Antigens Ra.3 and E is given in Table XX. As can be seen from the table, the electrical charge on the two allergens differ (Chapter IV), Ra.3 being a basic protein, Antigen E, acidic. The lower sedimentation coefficient and higher diffusion constant of Antigen Ra.3 reflects the differences in molecular weight of the allergens. While Antigen E contains only 0.7% pentose, Antigen Ra.3 contains a total hexose and pentose content of 12.4 $\pm$ .2%. The nitrogen content of Antigen Ra.3, 13.5 $\pm$ .4%, is lower than that of Antigen E, 17.1% (119), reflecting the higher carbohydrate content of Antigen Ra.3.

Allergenic testing of Antigens Ra.3 and E established the differences in specificity of these two preparations (Chapter VI). From the observation that both Antigens Ra.3 and E provoked essentially equal cutaneous reactions in some patients, while only Antigen E provoked reactions in others, it seems reasonable to conclude that the two preparations differ in their allergenic specificity. P-K tests and in-vitro anaphylactic experiments with appropriate ragweed allergic sera confirmed the existence of two distinct allergen-reagin systems. Thus, some sera passively sensitized human and monkey skin for cutaneous reactions to both allergens while others sensitized for reactions only to Antigen E.

Antigen Ra.3 had the same level of specific activity as Antigen E in 25% of skin test positive patients. In 75% of such patients, the relative specific activities of the two allergens varied: some patients showing no reactivity to Antigen Ra.3, while others reacted to Antigen Ra.3, but less markedly than to Antigen E. No patients were observed reacting to Antigen Ra.3 but not to Antigen E.

The results of direct skin testing of allergic patients confirmed the importance of Antigen E as the major allergen of ragweed pollen. The higher incidence of reactivity to this antigen might be due to its relative abundance in the pollen. In this connection, the yield of Antigen E in its two major electrophoretic forms, IV-B and IV-C, was reported by King et al (119) to be 1 gm/kg of pollen, which is in marked contrast to the yield of 0.1 gm/kg of pollen obtained for Antigen Ra.3.

The comparative activities of Antigens Ra.3 and E with WSR, were examined by P-K tests, using sera from patients who reacted with similar intensity to Antigens Ra.3 and E by direct skin test. The activities of the two purified allergens were essentially identical, and were 100 to 1000 times higher than that of the WSR preparation.

The results obtained in cross neutralization studies with Antigens Ra.3 and E suggest that the two antigens carry common allergenic determinants. This is in accord with the

suggestion of King et al (174) that Antigen E shares common determinants with other proteins in ragweed pollen extracts. It would appear that Antigen E possesses both "E" and "Ra.3" allergenic determinants, while Antigen Ra.3 possesses Ra.3 determinants only.

Since neutralization of anti-Ra.3 reagins in ragweed allergic sera caused no reduction in the P-K reactions of these sera to Antigen E, the contribution of the Ra.3 determinants to the total cutaneous activity of Antigen E must be relatively minor, at best. The apparent inability of Antigen E-anti-Ra.3 reagin complexes to elicit cutaneous reactions may be due to the presence on the Antigen E molecule of a single Ra.3 allergenic determinant, sufficient to effect neutralization of anti-Ra.3 reagins but ineffectual for eliciting cutaneous reactions with these antibodies. The presence of additional Ra.3 determinants on the Antigen E molecule is not precluded, but such determinants would likely be sterically hindered to render the molecule functionally haptenic with respect to Ra.3 determinants.

The structural similarities of Antigens Ra.3 and E reflected in their allergenic cross reactivity are not apparent from the physicochemical properties of the two proteins. Furthermore, immunodiffusion tests with rabbit antisera failed to show any antigenic cross reaction between the two allergens. However, the physical, chemical, and antigenic dissimilarities

of Antigens Ra.3 and E do not exclude the presence of an Ra.3 region on the Antigen E molecule. Such a region could be relatively small in size, of the order of a penta- or hexapeptide, as has been found for antigenic determinants (208, 209).

Evidence was obtained in the present study (Chapter VII) that the reaginic antibodies in ragweed allergic serum directed to Antigen Ra.3 chromatographed differently than the antibodies to Antigen E. Reagins to Antigen Ra.3 were eluted from DEAE-Sephadex at relatively higher buffer molarities than reagins to Antigen E, suggesting that the antibody types differed in electrical charge. The basis for this difference may reside in the chemical nature of the two allergens. Sela and Mozes (210) have shown that specific rabbit antibodies vary in their overall charge depending on the net charge of the immunogen. Thus, negatively charged immunogens elicited relatively positively charged antibodies, while positively charged immunogens induced relatively negatively charged antibodies. The differences in chromatographic distribution of reaginic antibodies to Antigens Ra.3 and E suggest that the charge on the two allergens influence the physicochemical nature of their respective reaginic antibodies in a similar way to that observed by Sela and Mozes for rabbit antibodies. This could account, in part, for the heterogeneous nature of reaginic antibodies formed to the complex mixture of allergens in ragweed pollen.

From the foregoing discussion, it seems fair to conclude that the principal aim of the present investigations was achieved: namely, the isolation from ragweed pollen of a component distinct in its physicochemical and allergenic properties from the major allergen, Antigen E. The isolation of this component, Antigen Ra.3, has confirmed earlier, suggestive evidence for the presence of multiple allergens in the aqueous extract of the pollen. The availability of Antigen Ra.3, in relatively appreciable quantities, allows for the use of both it and Antigen E in hyposensitization therapy of ragweed-allergic patients. In view of the finding that 25% of ragweed-allergic patients tested were cutaneously sensitive to Antigen Ra.3 as well as to E, the combined use of both allergens may result in greater effectiveness of hyposensitization therapy. The availability of Antigen Ra.3 also makes possible comparative studies with Antigen E, to ascertain those chemical features of the allergens which lie at the basis of their specificities and which may also impart to these proteins their unique immunogenic property of inducing the atopic allergic state.

SUMMARY

1. An allergenically active protein, Antigen Ra.3, was isolated from the aqueous extract of short ragweed pollen by the combined techniques of ammonium sulphate precipitation, gel filtration on Sephadex G-25 and G-100, and ion exchange chromatography on DEAE- and TEAE-cellulose.

2. Antigen Ra.3 was shown to be homogeneous by TEAE-cellulose chromatography, polyacrylamide gel disc electrophoresis at pH 6.6 and 3.8, sedimentation-velocity ultracentrifugation, gel filtration on Sephadex G-100, and immunodiffusion with rabbit antisera prepared to the whole aqueous extract and to the purified allergen.

3. Antigen Ra.3 was shown to be a glycoprotein of molecular weight 15,172 with an $S^0_{20,w} = 1.8 \times 10^{-13}$ sec, $D^0_{20,w} = 10.1 \times 10^{-7}$ cm^2 sec^{-1}, Nitrogen = 13.5 $\pm$ .4%, total total pentose = 8.7 $\pm$ .2%, total hexosamine = 0.8 $\pm$ .02%. Antigen Ra.3 was shown to have all of the common amino acids and an amino acid composition distinct from Antigen E.

4. Antigen Ra.3 was shown by direct skin test to differ in allergenic specificity from the major allergen of ragweed pollen, Antigen E. Antigen Ra.3 had the same level of cutaneous activity as Antigen E in 25% of 38 skin test positive patients.

5. Cross neutralization tests with ragweed allergic sera demonstrated that the two allergens share common allergenic determinants. Antigen Ra.3 neutralized anti-Ra.3 reagins while Antigen E neutralized reaginic activity both to itself and to Antigen Ra.3.

6. Evidence was obtained by DEAE Sephadex chromatography that reaginic antibodies to Ra.3 were relatively negatively charged compared to reagins to Antigen E.

CLAIMS TO ORIGINALITY

1. An allergenically active glycoprotein, Antigen Ra.3, was isolated from short ragweed pollen.

2. Antigen Ra.3 was shown to be homogeneous by physicochemical and immunological analysis.

3. The first physical and chemical parameters including molecular weight, nitrogen and carbohydrate content and amino composition, were determined for this allergen.

4. The allergenic activity of Antigen Ra.3 was analysed by direct skin P-K and in vitro anaphylaxis tests and was shown to differ from the activity of Antigen E, the major allergen in short ragweed pollen.

5. Evidence was obtained that the heterogeneity of ragweed reaginic antibodies was due, in part, to the net charge on the allergenic components of short ragweed pollen.

TABLE I

SEDIMENTATION COEFFICIENT OF ANTIGEN Ra.3 AT VARIOUS SOLUTION CONCENTRATIONS*

Concentration (g %)	Sedimentation Coefficient ($S \times 10^{13}$ sec)
1.0	1.722
0.8	1.682
0.6	1.762
0.4	1.742

*Solutions were prepared in 0.14 M NaCl + 0.01 M Sodium phosphate, pH 7.3.

TABLE II

DIFFUSION COEFFICIENT OF ANTIGEN Ra.3 AT VARIOUS SOLUTION CONCENTRATIONS*

Concentration (g %)	Diffusion Coefficient ($D \times 10^7$ cm^2 sec^{-1})
1.0	8.32
0.8	8.98
0.6	9.02

* Solutions were prepared in 0.14 M NaCl + 0.01 M Sodium phosphate, pH 7.3.

TABLE III

DETERMINATION OF NITROGEN CONTENT OF ANTIGEN Ra.3

Sample	Nitrogen Content (mgN/ml)	Optical Density (570 mμ) 1	2	Average
Standard	0.370	.405	.415	.410
"	0.185	.235	.235	.235
"	0.093	.100	.110	.105
"	0.061	.070	.078	.074
Antigen Ra.3*		.305	.285	.295

*Concentration of Antigen Ra.3 solution: 2.0 mg total solids/ml.

TABLE IV

DETERMINATION OF TOTAL HEXOSE AND PENTOSE CONTENT OF ANTIGEN Ra.3

Sample	Arabinose Content µg/ml	Optical Density at 500 mµ
Standard	112.0	.475
"	89.6	.385
"	67.2	.290
"	44.8	.220
"	22.4	.100
Antigen Ra.3*		.205
"		.210

*Concentration of Antigen Ra.3 solution: 0.39 mg total solids/ml.

TABLE V

DETERMINATION OF TOTAL HEXOSE CONTENT OF ANTIGEN Ra.3

Sample	Galactose Content (µg/ml)	Optical Density at 625 mµ 1	2	Average
Standard	51.0	.610	.575	.593
"	25.5	.301	.292	.297
"	12.8	.148	.162	.155
Antigen Ra.3*		.210	.210	.210

*Concentration of Antigen Ra.3 solution: 0.39 mg total solids/ml.

TABLE VI

DETERMINATION OF TOTAL PENTOSE CONTENT OF ANTIGEN Ra.3

Sample	Arabinose Content (µg/ml)	Δ O.D.* 1	2	Average
Standard	45.0	.662	.648	.650
"	35.0	.495	.495	.495
"	22.4	.305	.280	.292
"	14.0	.218	.202	.200
"	9.0	.152	.150	.151
Antigen Ra.3**		.484	.488	.486

* Difference in optical densities at 390 and 424 mµ.

** Concentration of Antigen Ra.3 solution: 0.39 mg total solids/ml.

TABLE VII

DETERMINATION OF TOTAL HEXOSAMINE OF ANTIGEN Ra.3

Sample	Galactosamine Content (µg/ml)	Optical Density at 540 mµ 1	2	Average
Standard	72.5	.799	.801	.800
"	50.8	.593	.595	.594
"	25.4	.298	.292	.295
"	12.7	.152	.152	.152
Antigen Ra.3*		.102	.096	.099

*Concentration of Antigen Ra.3 solution: 1.0 mg total solids/ml.

TABLE VIII

AMINO ACID COMPOSITION OF ACID HYDROLYSATE OF ANTIGEN Ra.3*

No. of μmoles found (x2)

Amino Acid	A		B		C	
	1	2	1	2	1	2
Lysine	.812	.818	.841	.845	.836	.814
Histidine	.321	.355	.301	.317	.327	.325
Arginine	.402	.466	.491	.455	.471	.451
Aspartic	.833	.857	.888	.916	.831	.791
Threonine	.922	.878	.817	.841	.811	.827
Serine	.556	.548	.453	.467	.426	.416
Glutamic	.950	.942	.958	.946	.933	.949
Proline	1.343	1.043	1.117	1.167	1.324	1.020
Glycine	.948	.970	.931	.911	1.011	.977
Alanine	.770	.754	.731	.737	.755	.745
Cystine/2	.345	.339				
Valine	.761	.707	.741	.753	.748	.762
Methionine	.082	.082	.086	.085	.080	.080
Isoleucine	.497	.517	.513	.491	.521	.503
Leucine	.963	.981	.963	.983	.978	.958
Tyrosine	.290	.286	.241	.253	.307	.333
Phenylalanine	.810	.834	.784	.758	.735	.717

*Acid hydrolysis was carried out in 6 N HCL in vacuo for 24(A), 48 (B) and 72 (C) hours. No. of μmoles based on 0.5 umole amino acid standards.

TABLE IX

AMINO ACID COMPOSITION OF ANTIGEN Ra.3

	Average No. of µmoles found (x2)*					
	24	48	72	Best Value	No. of residues**	Closest Interger
Lysine	.815	.843	.825	.828	7.0	7
Histidine	.338	.309	.327	.325	2.7	3
Arginine	.424	.473	.461	.474	4.0	4
Aspartic	.845	.902	.811	.852	7.2	7
Threonine	.900	.829	.819	.935	7.9	8
Serine	.552	.460	.421	.610	5.2	5
Glutamic	.946	.952	.941	.947	8.0	8
Proline	1.193	1.142	1.172	1.171	9.9	10
Glycine	.959	.921	.994	.958	8.1	8
Alanine	.762	.734	.750	.746	6.3	6
Cystine/2	.342			.342	2.9	3
Valine	.734	.752	.755	.746	6.3	6
Methionine	.082	.086	.080	.083	0.7	1
Isoleucine	.507	.502	.512	.507	4.3	4
Leucine	.972	.973	.968	.971	8.2	8
Tyrosine	.288	.247	.320	.285	2.4	2
Phenylalanine	.822	.771	.726	.875	7.4	7
Tryptophane					3.6	4
Total						101

* See Table VIII and text.

** Based on 1 residue = 0.118 uM.

TABLE X

DIRECT SKIN TEST REACTIONS TO ANTIGENS Ra.3 AND E*

Group I Patients	Wheal Area (mm^2) Antigen E	Ra.3	Group I Patients	Wheal Area (mm^2) Antigen E	Ra.3
A.C.	100	12	A.L.	105	9
M.S.	143	90	H.H.	99	9
B.W.	81	12	M.D.	110	12
W.G.	90	9	M.H.	100	9
P.N.	100	49	H.J.	130	42
A.W.	68	9	R.D.	81	12
M.S.	81	49	T.M.	70	9
P.H.	132	9	W.J.	90	49
M.G.	49	9	W.M.	110	56
J.G.	121	49	I.C.	81	12
K.N.	90	63	L.P.	81	9
H.L.	50	25	W.K.	72	9
M.H.	100	12	J.A.	81	9
C.M.	90	9	T.K.	99	56

(Continued next page)

TABLE X (continued)

Group II Patients	Wheal Area (mm^2) Antigen E	Ra.3	Group III Patients	Wheal Area (mm^2) Antigen E	Ra.3
M.M.	110	143	M.S.	12	12
B.U.	81	99	B.B.	9	9
M.C.	70	90	P.P.	9	9
K.B.	56	64	D.M.	9	9
M.B.	90	126	T.C.	9	12
R.R.	50	35	T.H.	9	15
M.V.	72	99	C.O.	9	20
H.R.	81	80	G.T.	20	9
M.T.	64	72			
A.B.	100	121			

* For the test, 0.05 ml aliquots of solutions (10^{-9} gm/ml) of Antigens Ra.3 and E were separately injected. All patients were injected with 0.05 ml of 0.15 M NaCl as control. The control wheal areas ranged from 9-12 mm^2.

TABLE XI

COMPARISON BY P-K TEST OF THE ALLERGENIC ACTIVITY OF ANTIGENS Ra.3 AND E*

SERA

Allergen Conc. gm/ml	P.H. (1:100)		A.C. (1:100)		M.M. (1:10)		K.B. (1:10)		M.C. (1:10)		B.U. (1:100)		M.B. (1:100)	
	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E
10^{-6}	49	144	42	132	190	210	132	152	89	81	190	210	110	132
10^{-7}	9	110	9	120	168	170	110	130	72	72	100	120	90	100
10^{-8}	9	81	9	100	110	120	72	90	64	56	80	100	81	81
10^{-9}	9	64	9	81	99	100	72	72	9	9	50	90	49	49
10^{-10}	n.d.		n.d.		72	64	25	25	9	9	12	9	9	12

* Reactions expressed as wheal areas (mm^2)

Saline control = 9-12 mm^2

n.d. = not done

TABLE XII

COMPARISON BY P-K TEST OF THE ALLERGENIC ACTIVITY OF ANTIGEN Ra.3 AND E*

Serum P.H.	Challenge Ra.3	E	Serum A.C.	Challenge Ra.3	E	Serum M.M.	Challenge Ra.3	E	Serum K.B.	Challenge Ra.3	E
(1:10)	72	187	(1:10)	90	169	(1:100)	115	100	(1:10)	169	210
(1:100)	12	110	(1:50)	9	144	(1:300)	72	64	(1:30)	81	110
(1:500)	12	81	(1:100)	9	132	(1:900)	25	25	(1:90)	49	63
			(1:200)	9	110				(1:270)	9	12

* Serum sites were challenged with 5×10^{-11} moles of each allergen

Reactions expressed as wheal areas (mm^2)

Saline control = 9-12 mm^2

TABLE XIII

COMPARISON OF THE ALLERGENIC ACTIVITY OF ANTIGENS Ra.3 AND E BY PASSIVE IN VITRO ANAPHYLAXIS OF MONKEY SKIN SUSPENSIONS

	Histamine Equivalents (µg/gm tissue)					
	50.0 µg Antigen E/ml			50.0 µg Antigen Ra.3/ml		
Serum	1	2	Average	1	2	Average
B.U.	0.52	0.48	0.50	0.40	0.40	0.40
M.C.	0.16	0.17	0.17	0.18	0.12	0.16
R.R.	0.11	0.09	0.10	0.12	0.10	0.11
M.B.	1.00	1.00	1.00	0.80	0.60	0.70
M.U.	0.50	0.64	0.57	0.50	0.50	0.50
M.M.	0.18	0.13	0.16	0.50	0.50	0.50
F.V.	0.30	0.25	0.28	0.06	0.06	0.06
P.H.	1.00	0.80	0.90	0.02	0.02	0.02
M.H.	0.35	0.30	0.33	0.04	0.04	0.04
T.W.	0.03	0.03	0.03	0.04	0.04	0.04
D.W.	0.04	0.04	0.04	0.04	0.04	0.04

TABLE XIV

COMPARISON OF THE ALLERGENIC ACTIVITY OF ANTIGENS Ra.3 AND E BY IN VITRO PASSIVE ANAPHYLAXIS OF MONKEY SKIN SUSPENSIONS

		Histamine Equivalent (µg/gm tissue)								
	Concentration	Serum M.M.			Serum A.C.			Serum T.W.		
Allergen	(µg/ml)	1	2	Average	1	2	Average	1	2	Average
Antigen Ra.3	50.0	0.40	0.36	0.38	0.08	0.05	0.07	0.05	0.03	0.04
"	5.0	0.20	0.22	0.21	0.08	0.08	0.08	0.03	0.04	0.04
"	0.5	0.08	0.10	0.09	0.05	0.08	0.07	0.03	0.03	0.03
"	0.05	0.06	0.05	0.06	0.03	0.08	0.06	0.03	0.03	0.03
Antigen E	100.0	0.40	0.40	0.40	0.80	0.60	0.70	0.03	0.03	0.03
"	10.0	0.26	0.26	0.26	0.60	0.70	0.65	0.03	0.04	0.04
"	1.0	0.10	0.10	0.10	0.70	0.70	0.70	0.03	0.02	0.03
"	0.1	0.05	0.05	0.05	0.15	0.25	0.20	0.02	0.03	0.03

TABLE XV

COMPARISON BY P-K TEST OF THE ALLERGENIC ACTIVITY OF ANTIGENS Ra.3 AND E WITH WSR*

Allergen Concentration (gm/ml)	Serum M.M. (1:10)			Serum K.B. (1:10)		
	WSR	Ra.3	E	WSR	Ra.3	E
10^{-6}	99	190	210	72	132	152
10^{-7}	64	168	170	36	110	130
10^{-8}	42	110	120	12	72	90
10^{-9}	9	99	100	12	72	72
10^{-10}	n.d.	72	64	n.d.	25	25

* Reactions expressed as wheal areas in mm^2

Saline control = 9-12 mm^2

TABLE XVI

DETERMINATION OF ALLERGEN CONCENTRATION REQUIRED FOR NEUTRALIZATION OF P-K REACTION

	Wheal Areas						
	Antigen Ra.3 (µg/ml)*			Antigen E (µg/ml)*			
Challenging Dose:	5	0.5	0.05	10	1.0	0.01	Saline*
Antigen Ra.3:							
0.5 ug/ml	9	49	100	-	-	-	115
Antigen E:							
1.0 ug/ml	-	-	-	9	64	105	105

* Skin sites were sensitized with serum M.M. diluted 1:50 with solutions of each allergen at various concentrations and with saline. Skin sites sensitized with saline alone gave wheal areas = 9-12 mm^2.

TABLE XVII

P-K REACTIONS OF RAGWEED ALLERGIC SERA CROSS-NEUTRALIZED WITH ANTIGENS Ra.3 AND E

Serum	Diluted in	Wheal Area (mm^2) on Challenge* Subject 1 E	Subject 1 Ra.3	Subject 2 E	Subject 2 Ra.3	Subject 3 E	Subject 3 Ra.3
M.M. (1:50)	Saline	110	120	100	90	81	88
"	Antigen Ra.3**	115	12	99	9	96	9
"	Antigen E**	9	9	12	9	9	9
M.C. (1:10)	Saline	72	70	-	-	81	90
"	Antigen Ra.3	70	9	-	-	72	9
"	Antigen E	9	9	-	-	12	12
M.B. (1:10)	Saline	110	90	144	110	-	-
"	Antigen Ra.3	115	9	132	9	-	-
"	Antigen E	9	9	9	12	-	-

* Sites challenged with 1 µg Ag.E and 0.5 µg Ag.Ra.3. Saline control = 9-12 mm^2

** Antigens Ra.3 and E: 5 and 10 µg/ml, respectively, in saline.

TABLE XVIII

DISTRIBUTION OF REAGINIC ACTIVITY TO ANTIGENS Ra.3 AND E IN DEAE-SEPHADEX FRACTIONS OF SERUM M.M.

	Serum Fraction Dilution (1:10) Wheal Area (mm^2)						Serum Fraction Dilution (1:20) Wheal Area (mm^2)					
	E*			Ra.3*			E			Ra.3		
Fr. No.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
I	165	182	174	81	90	86	99	108	104	64	42	53
II	100	100	100	99	100	100	80	72	76	81	64	71
III	64	63	64	80	81	81	40	42	41	72	63	68

* Sensitized skin sites were challenged with 1.0 µg Antigen E and 0.5 µg Antigen Ra.3

TABLE XIX

DISTRIBUTION OF REAGINIC ACTIVITY TO ANTIGENS Ra.3 AND E IN DEAE-SEPHADEX FRACTIONS OF SERUM K.B.

	Serum Fraction Dilution (1:5) Wheal Area (mm^2)						Serum Fraction Dilution (1:10) Wheal Area (mm^2)					
	E*			Ra.3*			E			Ra.3		
Fr. No.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
I	165	182	174	99	99	99	120	120	120	63	56	60
II	156	156	156	165	154	160	99	90	95	63	70	67
III	63	56	60	80	99	90	64	56	60	72	72	72

* Sensitized skin sites were challenged with 1.0 µg Antigen E and 0.5 µg Antigen Ra.3

TABLE XX

COMPARISON OF PHYSICAL AND CHEMICAL PROPERTIES OF ANTIGEN Ra.3 AND ANTIGEN E

Property	Antigen Ra.3	Antigen E
Electrophoretic mobility (pH 6.6)	Cathodic	Anodic
$S^{\circ}_{20,w}$	1.80×10^{-13} sec	3.05×10^{-13} sec
$D^{\circ}_{20,w}$	10.1×10^{-7} cm^2 sec^{-1}	7.41×10^{-7} cm^2 sec^{-1}
Molecular weight	15,172	37,000
Nitrogen content	13.5%	17.1%
Total hexose + pentose content	12.4%	0.5%

(continued)

TABLE XX (continued)

No. of Residues	Antigen Ra.3	Antigen E
Lysine	7	18
Histidine	3	6
Arginine	4	16
Aspartic	7	49
Threonine	8	17
Serine	5	26
Glutamic	8	25
Proline	10	15
Glycine	8	37
Alanine	6	31
Cystine/2	3	7
Valine	6	24
Methionine	1	7
Isoleucine	4	20
Leucine	8	21
Tyrosine	2	4
Phenylalanine	7	12
Tryptophane	4	6
TOTAL	101	341

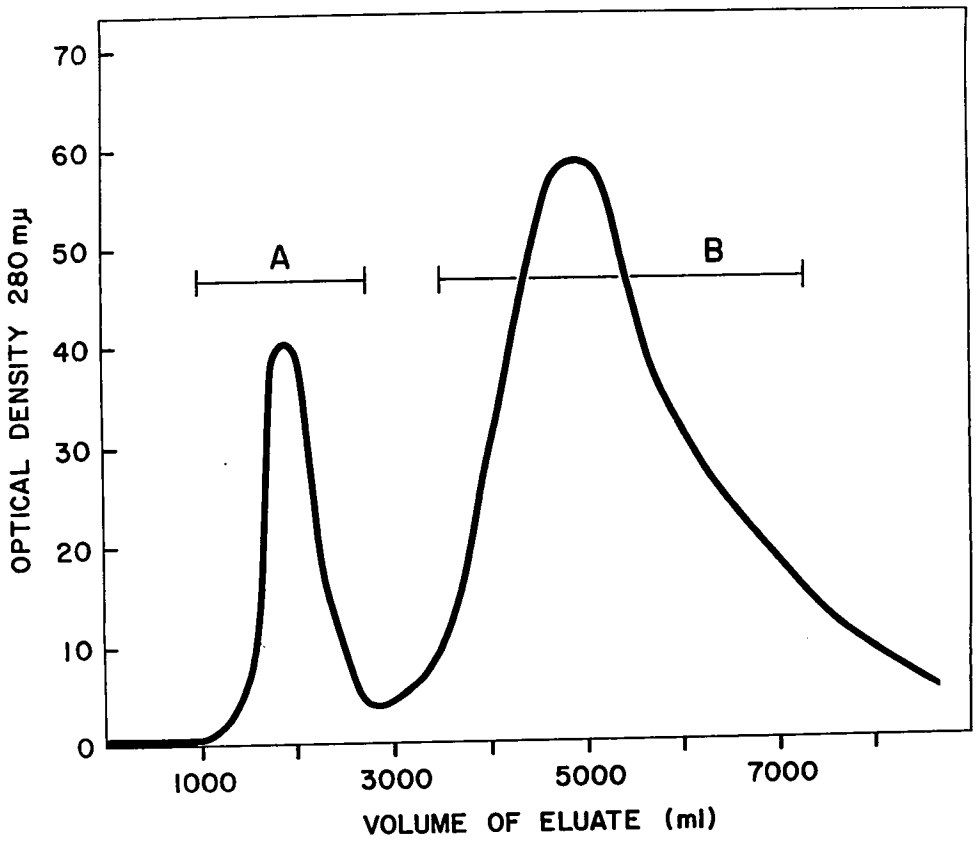


Fig. 1. Gel filtration of the aqueous extract of short ragweed pollen on Sephadex G-25.

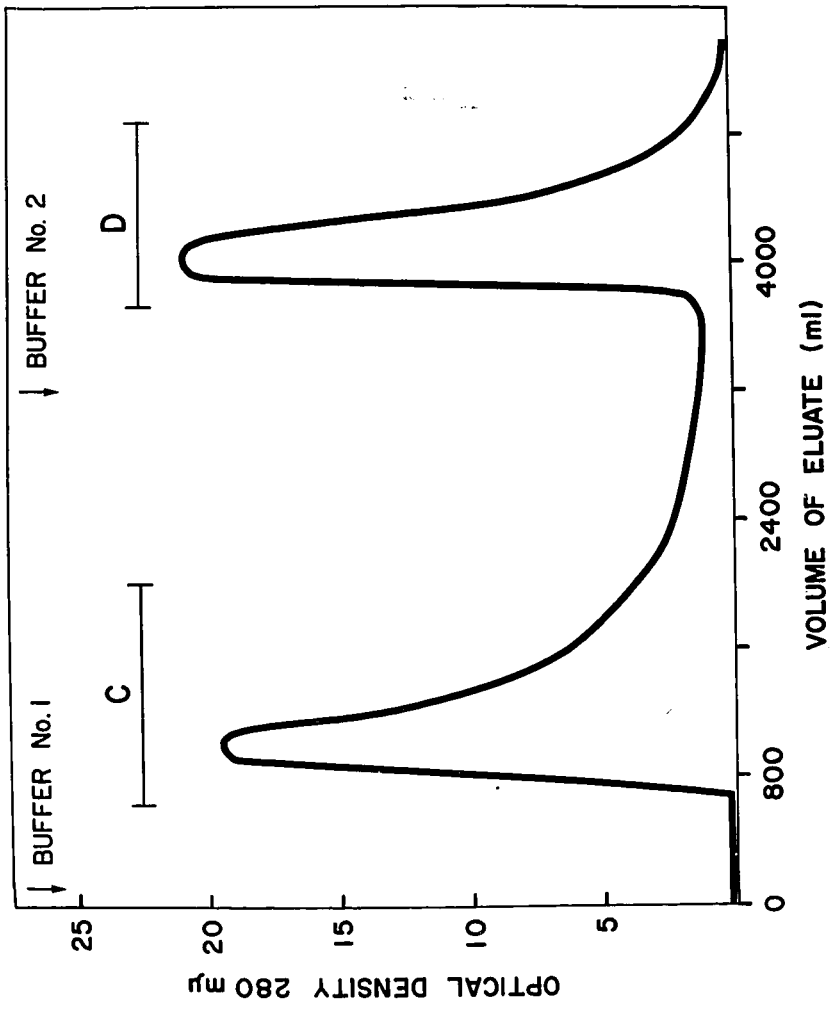


Fig. 2. DEAE-Cellulose chromatography of Sephadex G-25 fraction A.

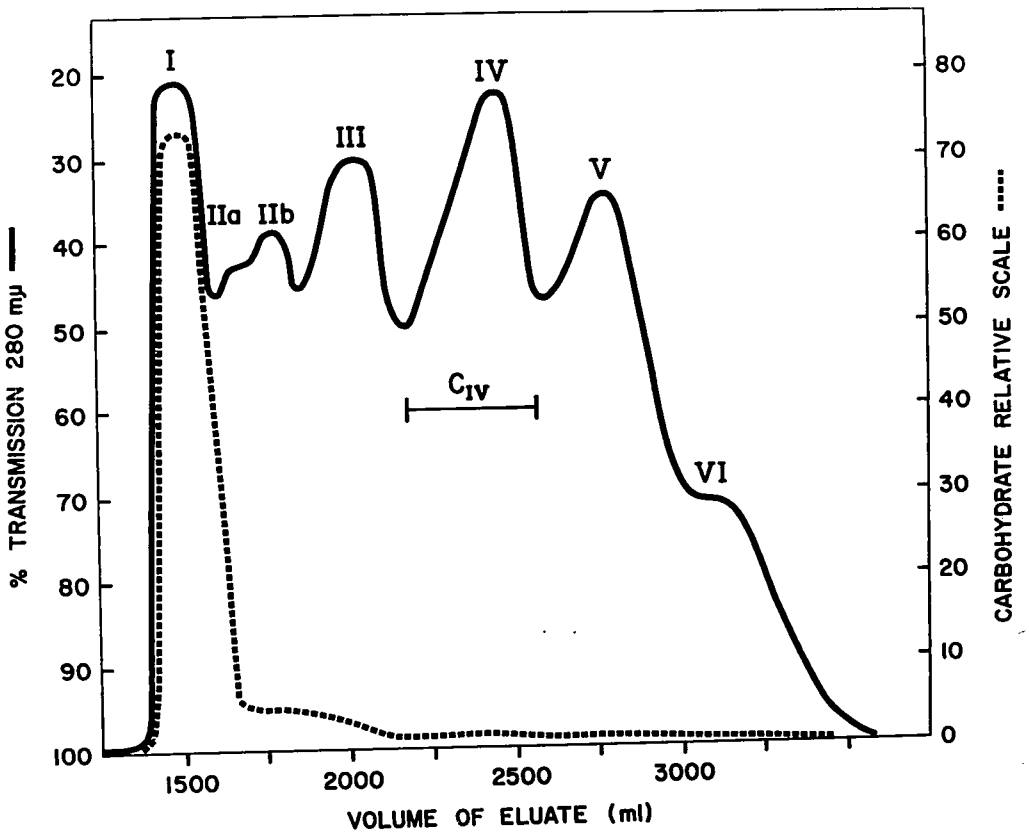


Fig. 3. Gel filtration of DEAE-cellulose fraction C on Sephadex G-100.

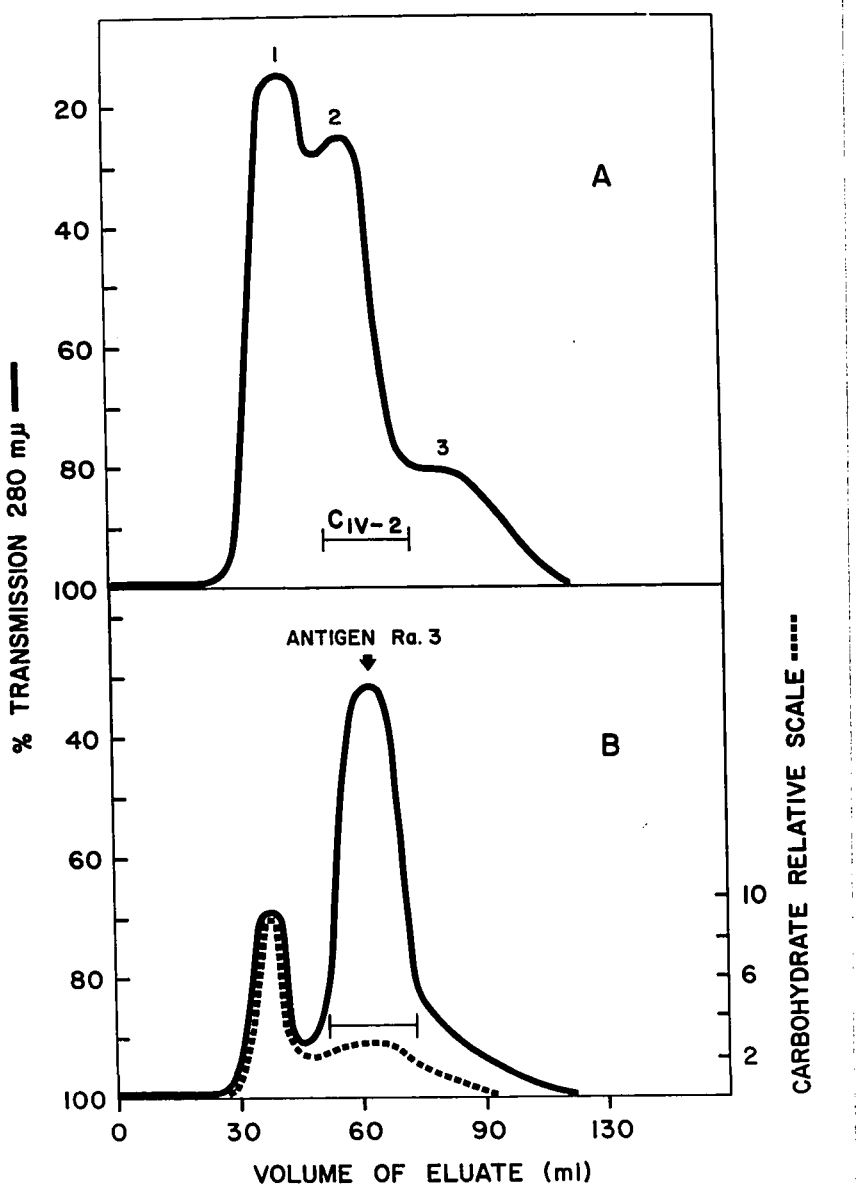


Fig. 4. TEAE-Cellulose chromatography of Sephadex G-100 fraction C_{IV} (top) and of the subfraction C_{IV-2} (bottom).

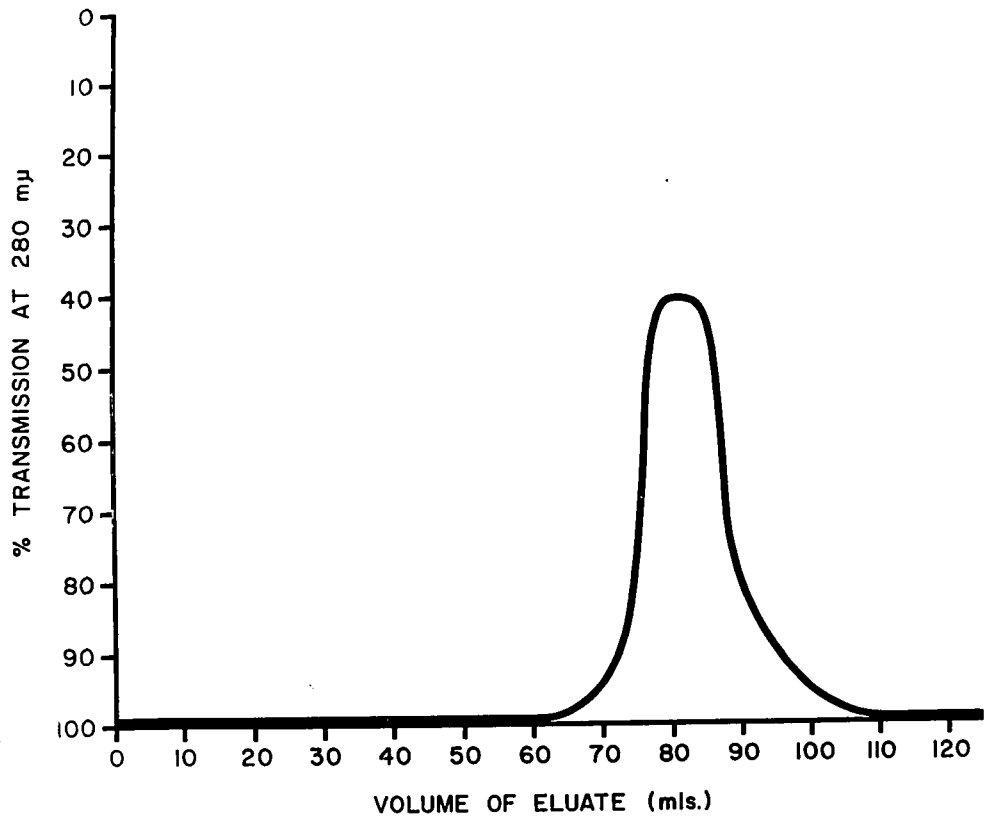


Fig. 5. TEAE-Cellulose chromatography of Antigen Ra.3.

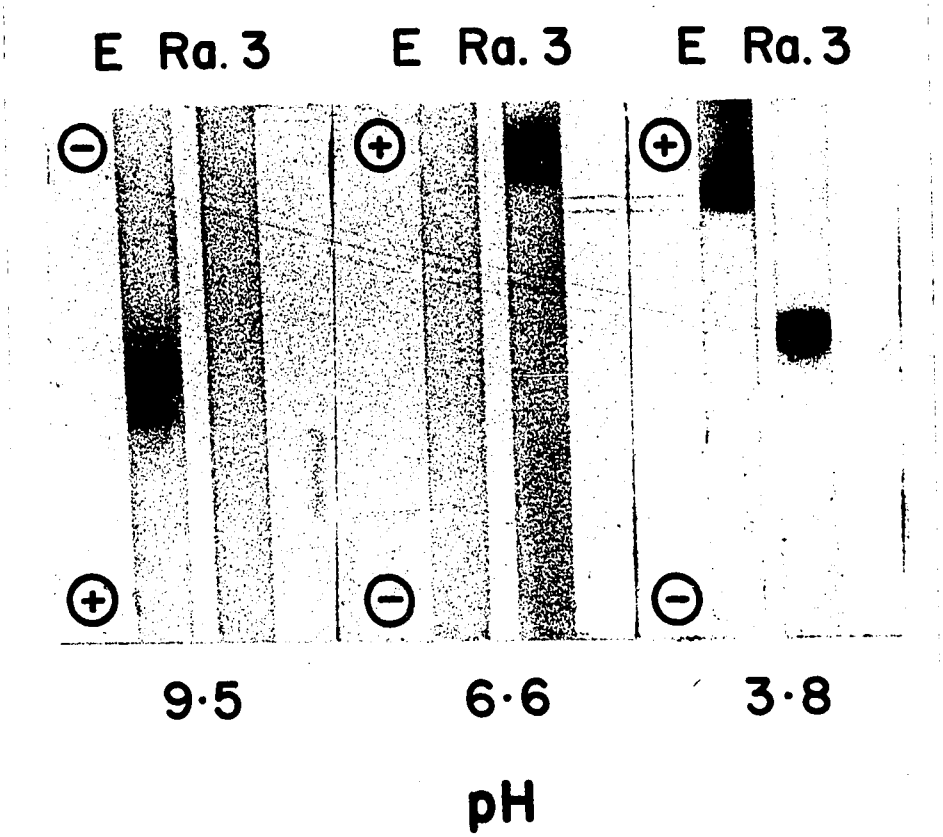


Fig. 6. Disc electrophoretic analysis of Antigens Ra.3 and E.

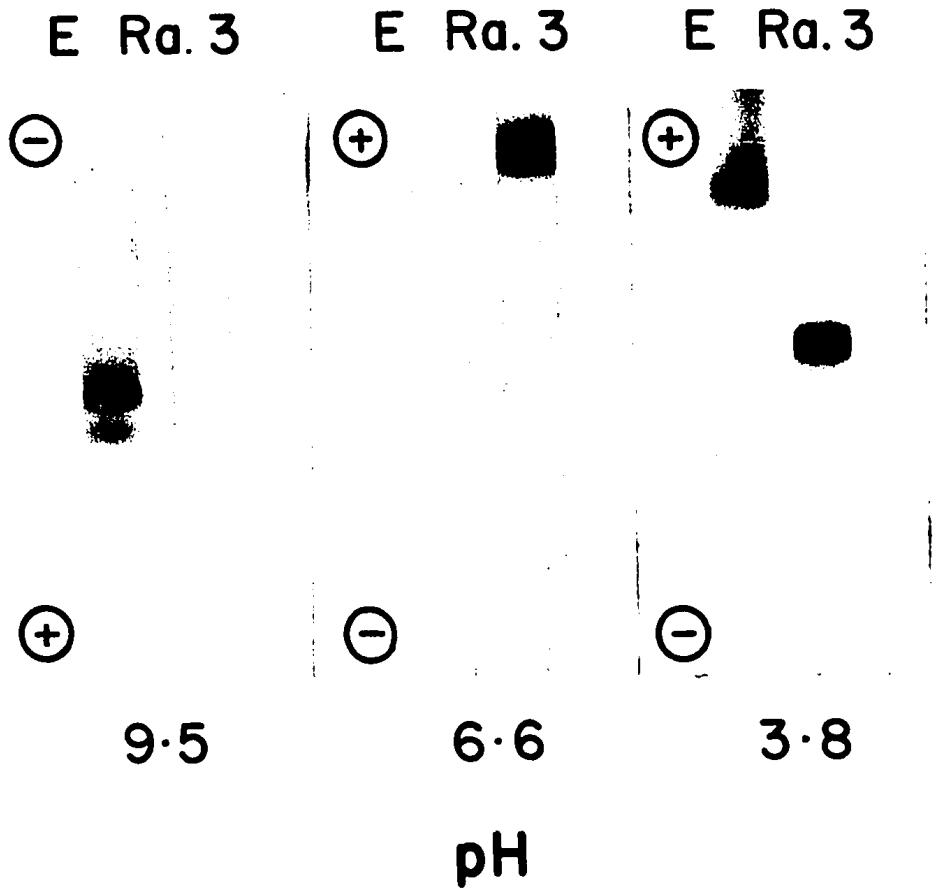


Fig. 6. Disc electrophoretic analysis of Antigens Ra.3 and E.

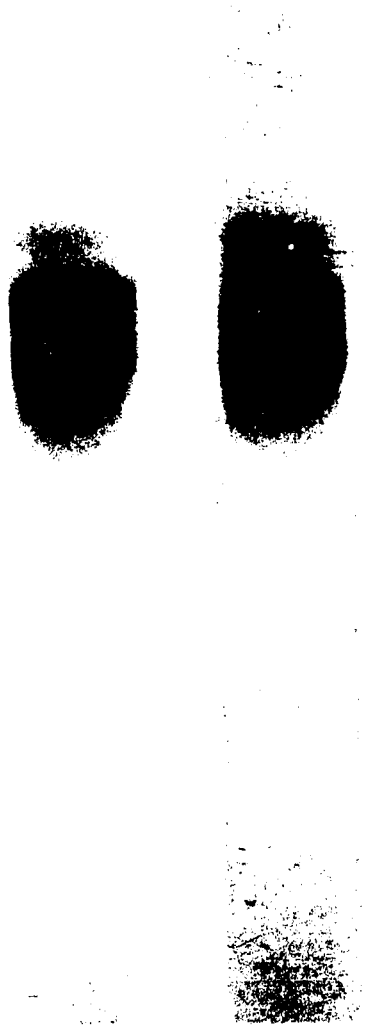


Fig. 7. Disc electrophoretic analysis of two NIH Antigen E preparations.

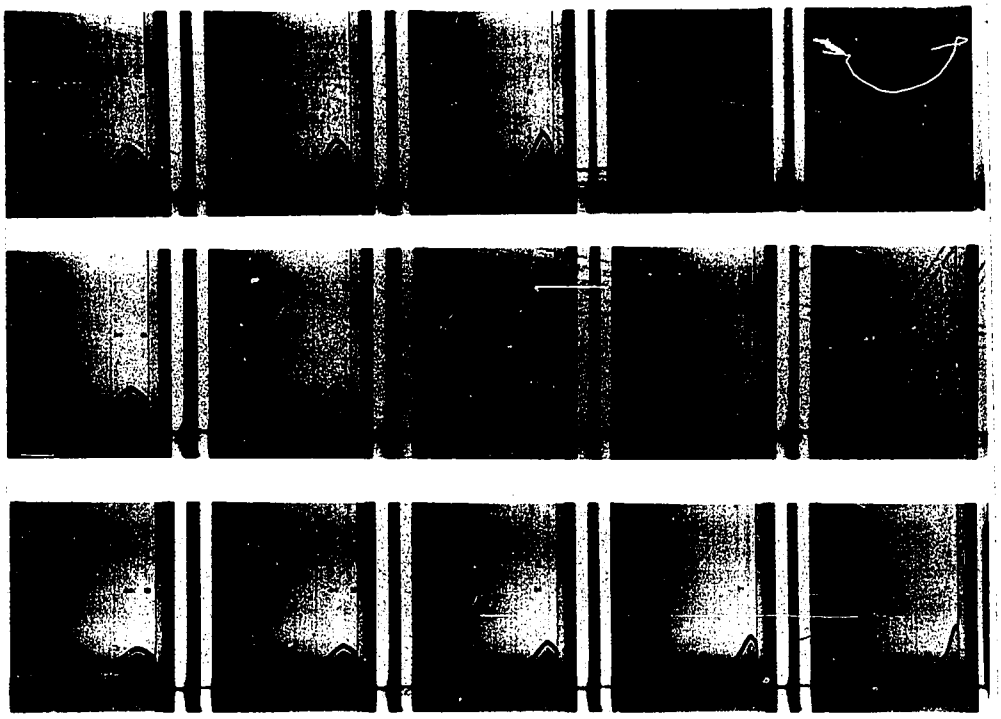


Fig. 8. Sedimentation-velocity ultracentrifugation of Antigen Ra.3. Photographs were taken at 16 min. intervals. Concentrations: 1% (top); 0.8% (middle); 0.6% (bottom). Direction of sedimentation: from right to left.

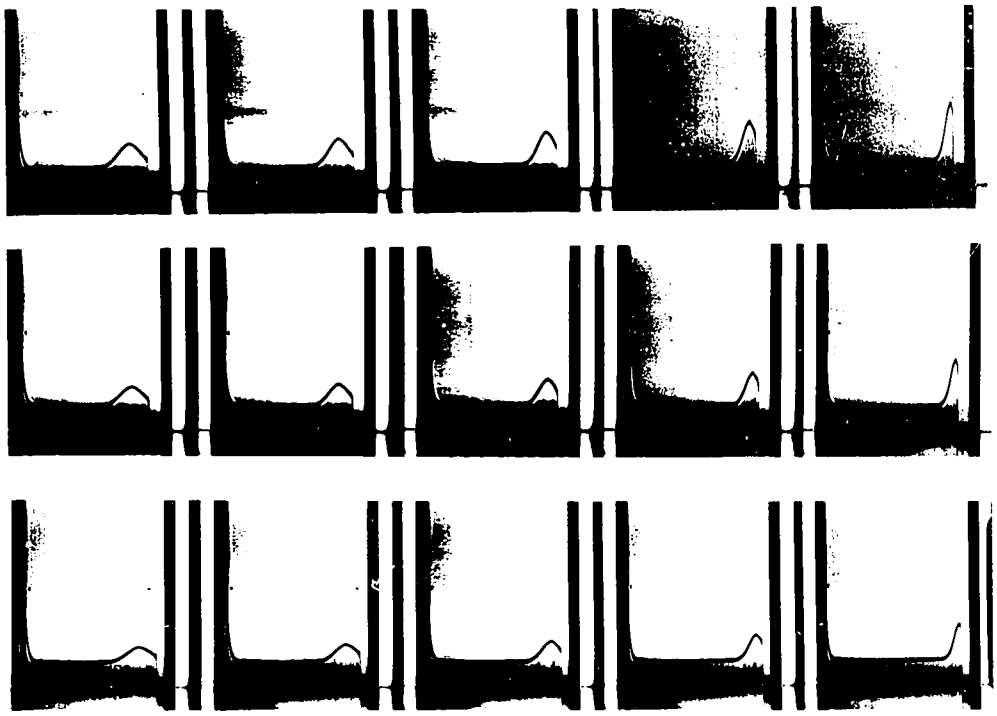


Fig. 8. Sedimentation-velocity ultracentrifugation of Antigen Ra.3. Photographs were taken at 16 min. intervals. Concentrations: 1% (top); 0.8% (middle); 0.6% (bottom). Direction of sedimentation: from right to left.

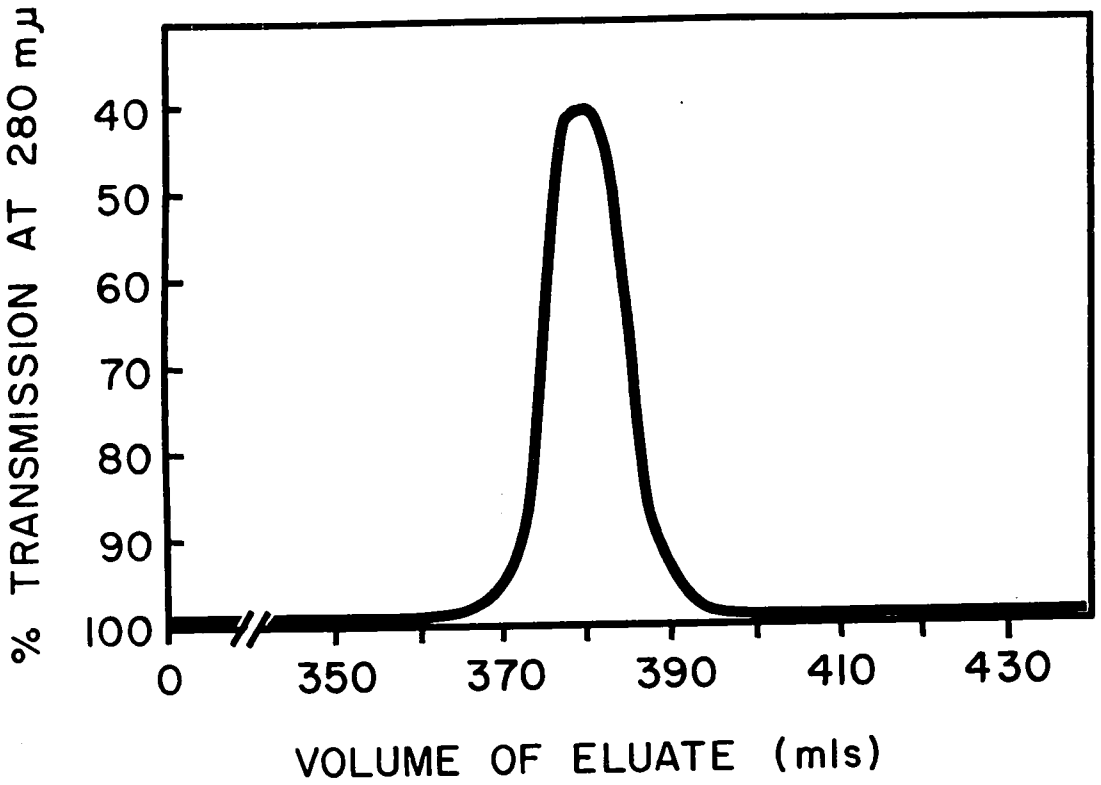


Fig. 9. Gel filtration of Antigen Ra.3 on Sephadex G-100.

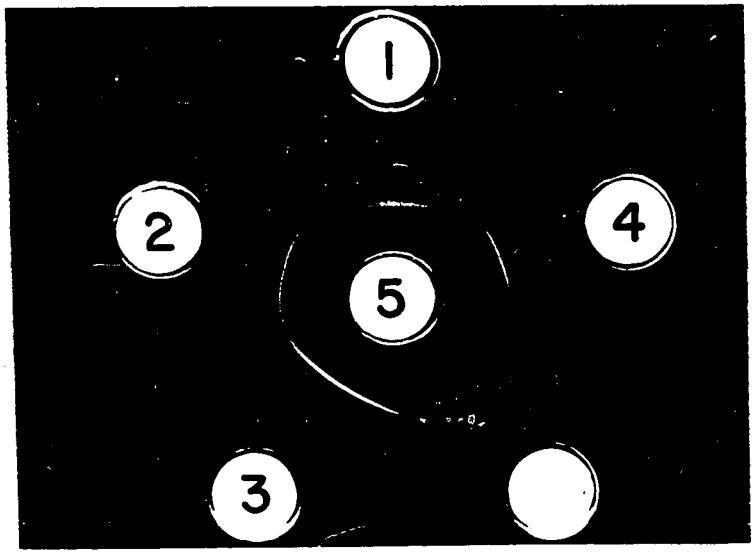


Fig. 10. Immunodiffusion analysis of Antigen Ra.3. Antigen Ra.3 in wells: 1 (0.50 mg/ml); 2 (0.10 mg/ml); 3 (0.20 mg/ml); 4 (0.05 mg/ml). Rabbit antiserum to Antigen Ra.3 in well 5.

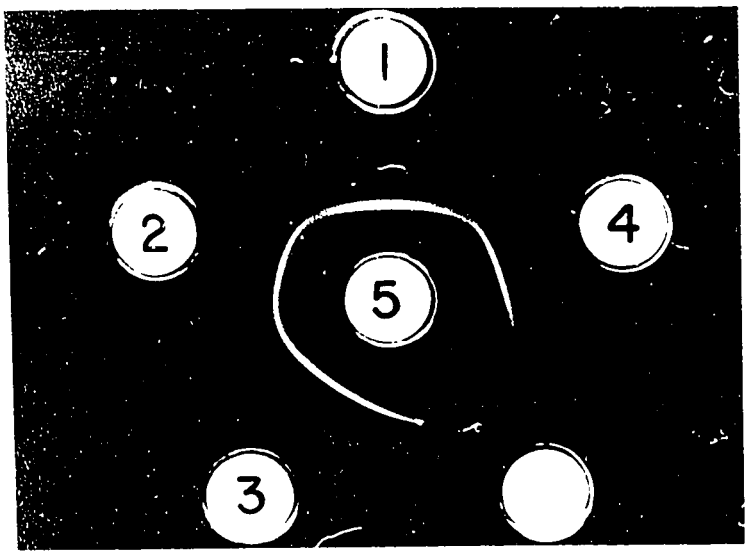


Fig. 10. Immunodiffusion analysis of Antigen Ra.3. Antigen Ra.3 in wells: 1 (0.50 mg/ml); 2 (0.10 mg/ml); 3 (0.20 mg/ml); 4 (0.05 mg/ml). Rabbit antiserum to Antigen Ra.3 in well 5.

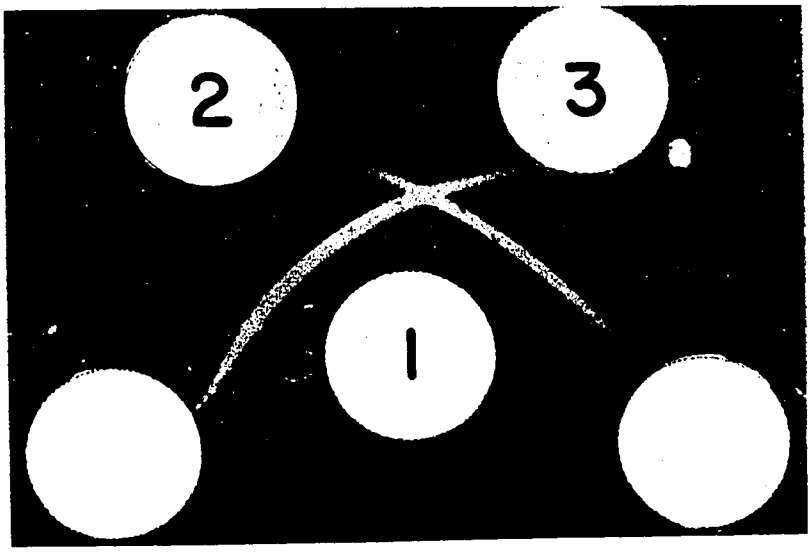


Fig. 11. Immunodiffusion analysis of Antigens Ra.3 and E. Antigen Ra.3 (1.0 mg/ml) in well 2; Antigen E (1.0 mg/ml) in well 3; rabbit antiserum to WSR in well 1.

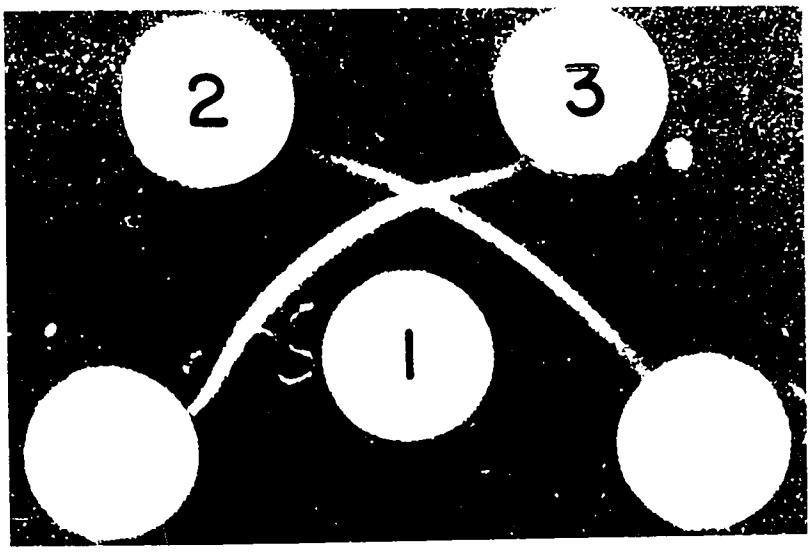
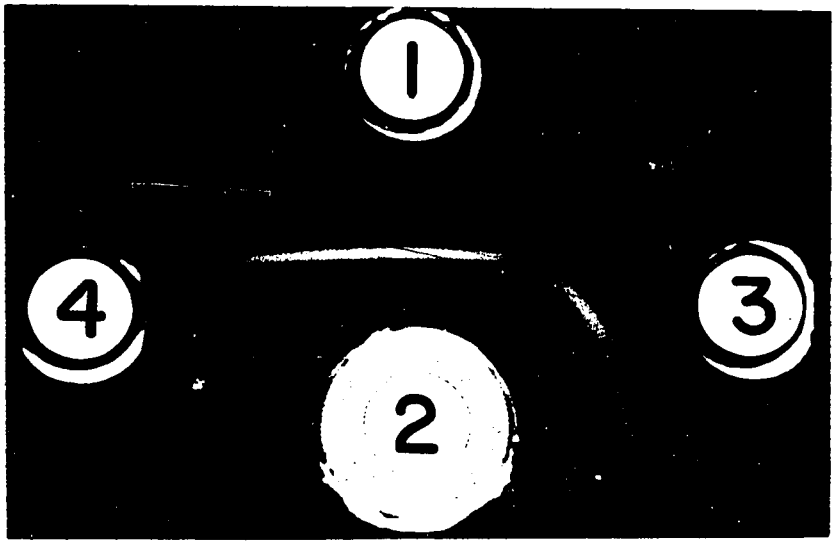


Fig. 11. Immunodiffusion analysis of Antigens Ra.3 and E. Antigen Ra.3 (1.0 mg/ml) in well 2; Antigen E (1.0 mg/ml) in well 3; rabbit antiserum to WSR in well 1.



(a)

Fig. 12. Immunodiffusion analysis of Antigens Ra.3 and E. Antigen Ra.3 in wells 1 (0.5 mg/ml) and 3 (0.02 mg/ml); Antigen E in well 4 (4.0 mg/ml); rabbit antiserum to Antigen Ra.3 in well 2.

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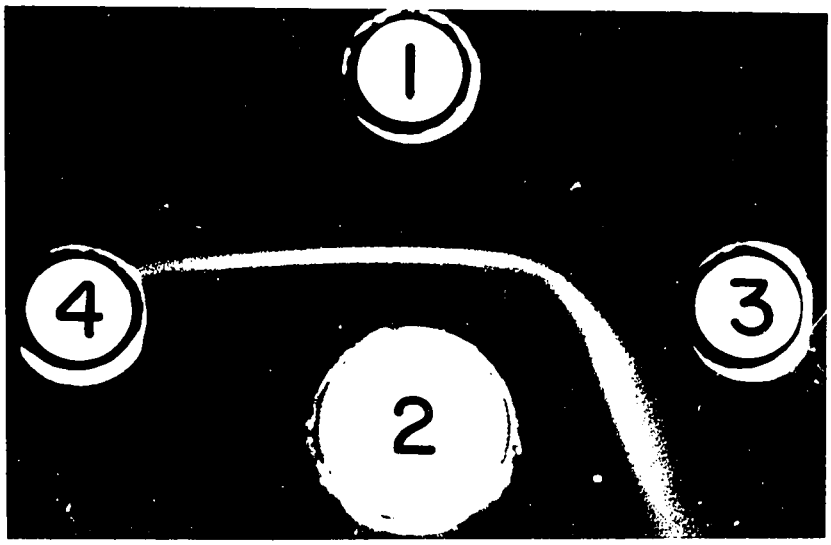

(a)

Fig. 12. Immunodiffusion analysis of Antigens Ra.3 and E. Antigen Ra.3 in wells 1 (0.5 mg/ml) and 3 (0.02 mg/ml); Antigen E in well 4 (4.0 mg/ml); rabbit antiserum to Antigen Ra.3 in well 2.

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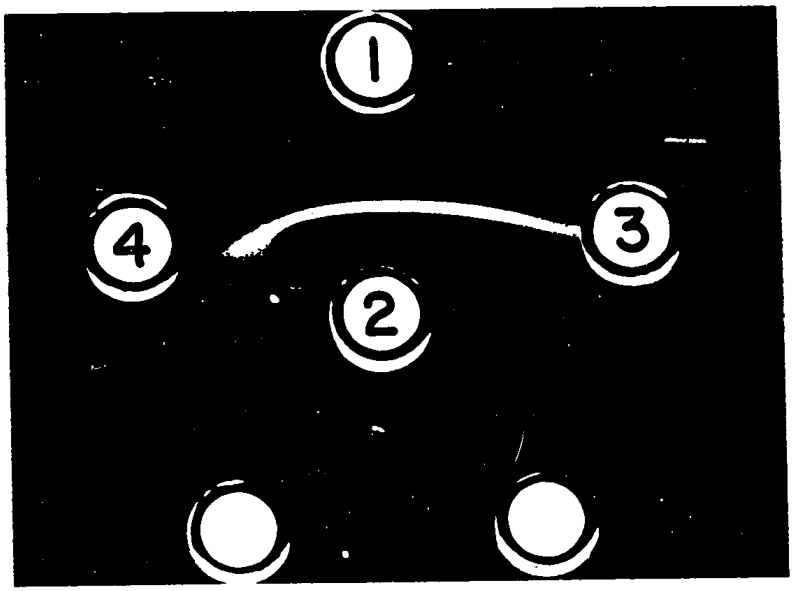
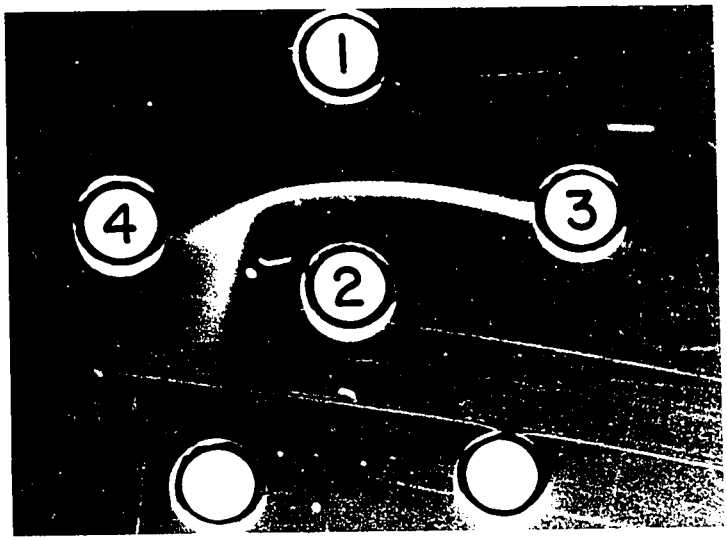


(b)

Fig. 12. (cont'd). Immunodiffusion analysis of Antigens Ra.3 and E. Antigen E in well 1 (0.5 mg/ml) and well 4 (0.01 mg/ml); Antigen Ra.3 in well 3 (2 mg/ml); rabbit antiserum to Antigen E in well 2.



(b)

Fig. 12. (cont'd). Immunodiffusion analysis of Antigens Ra.3 and E. Antigen E in well 1 (0.5 mg/ml) and well 4 (0.01 mg/ml); Antigen Ra.3 in well 3 (2 mg/ml); rabbit antiserum to Antigen E in well 2.

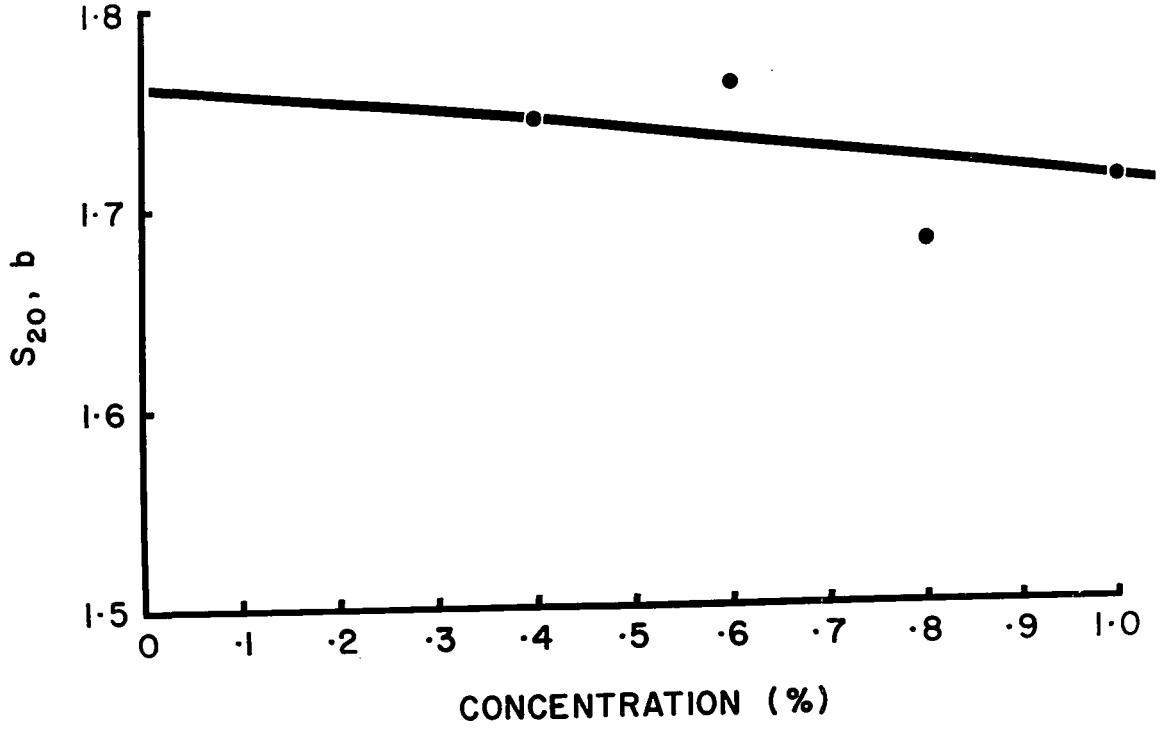


Fig. 13. Determination of sedimentation co-efficients to infinite dilution.

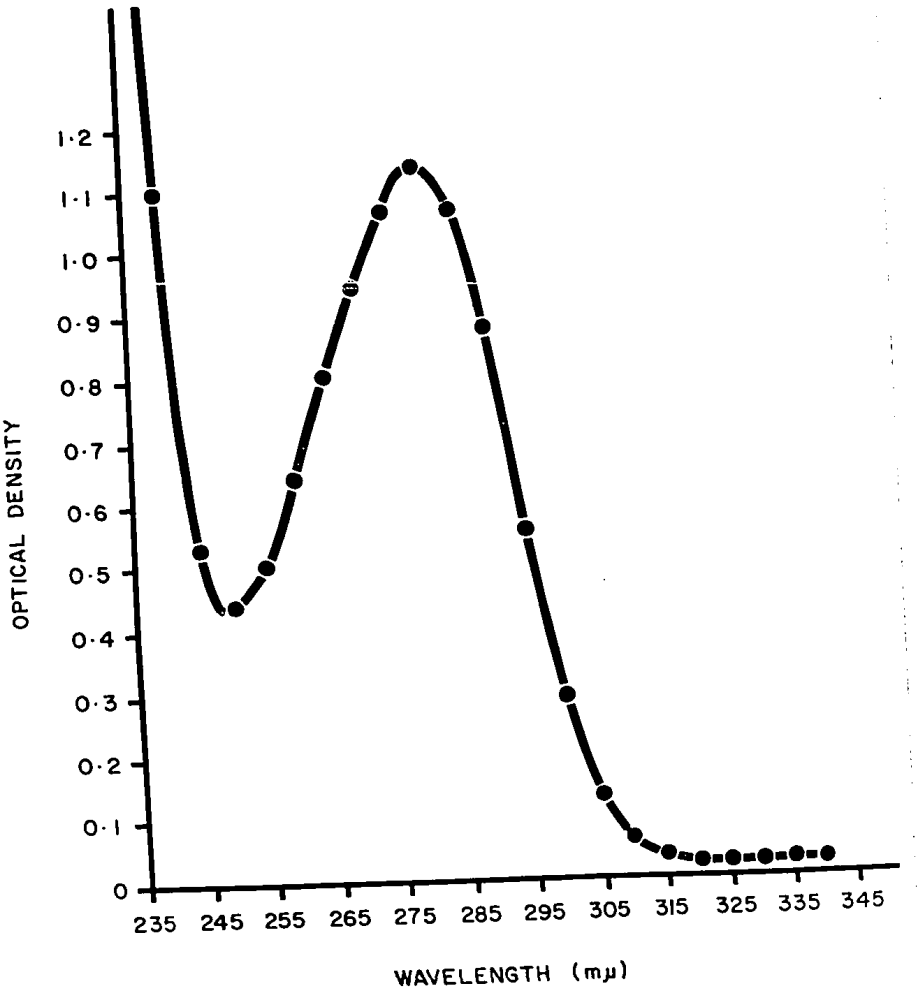


Fig. 14. Absorbance spectrum of Antigen Ra.3.

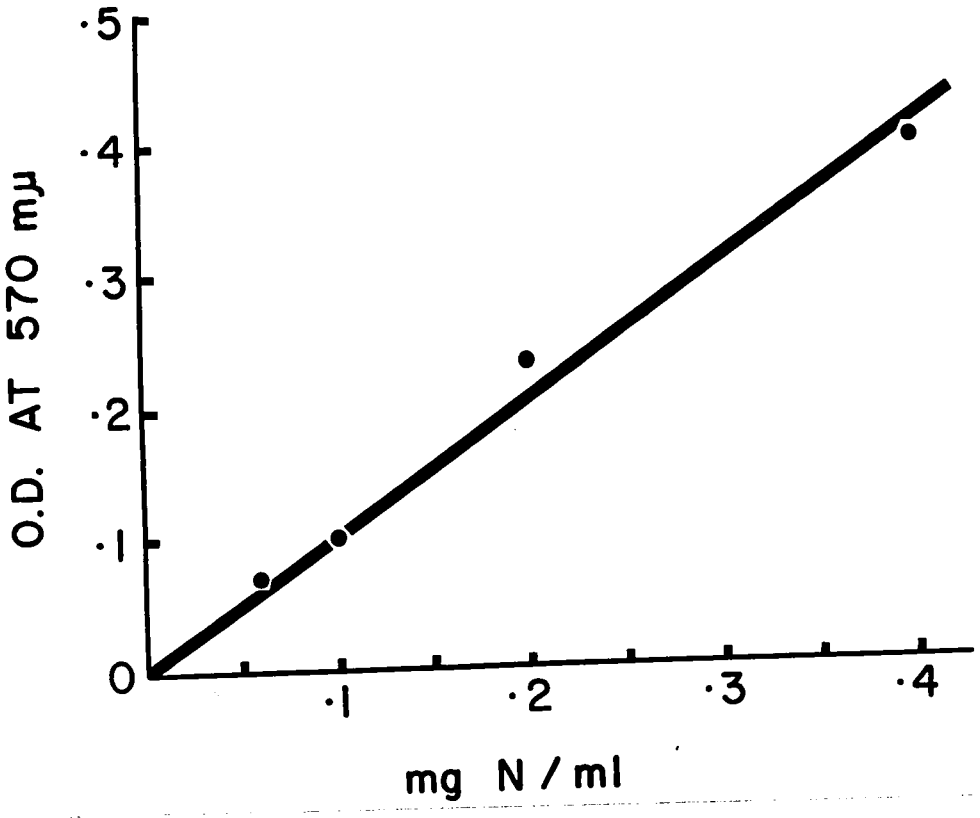


Fig. 15. Standard curve for the determination of nitrogen content of Antigen Ra.3.

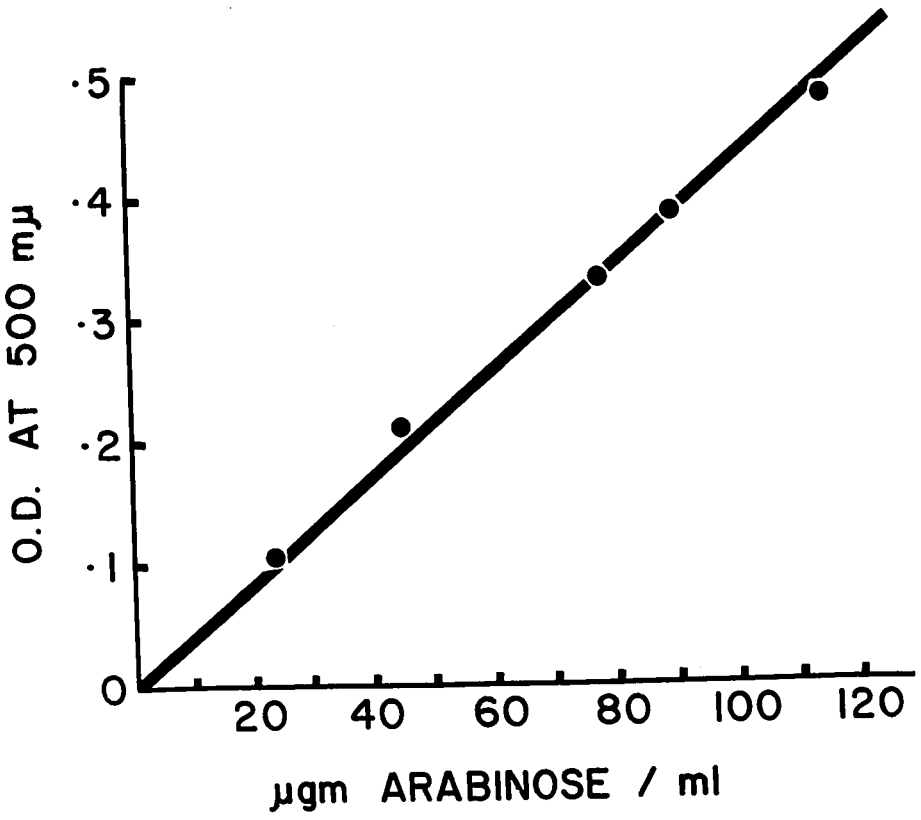


Fig. 16. Standard curve for the determination of total hexose and pentose content of Antigen Ra.3.

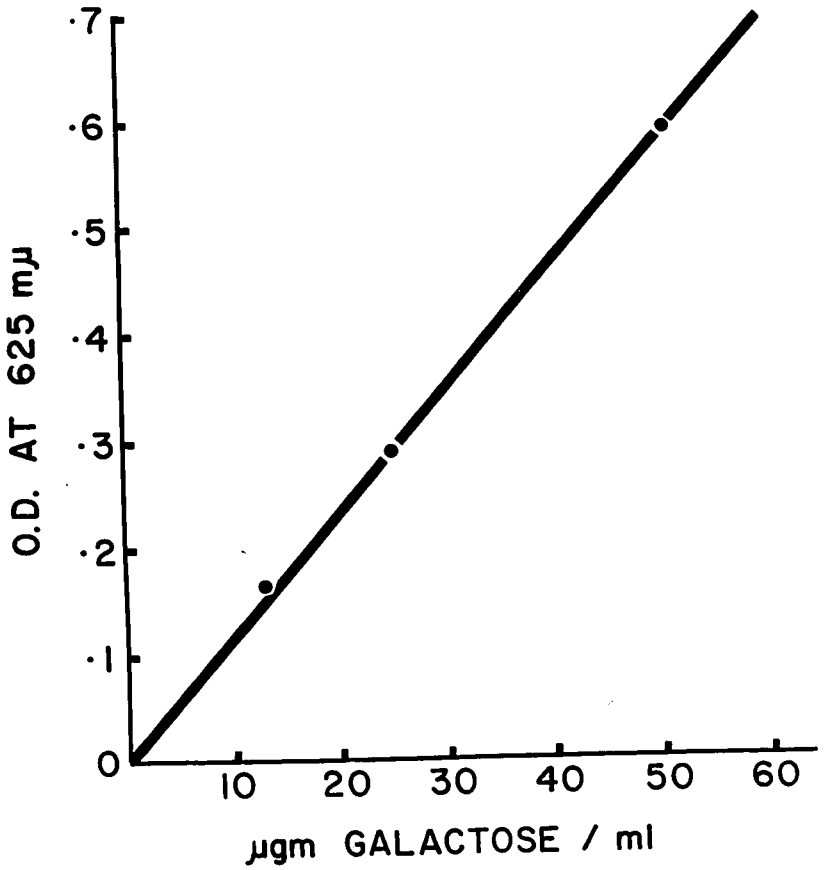


Fig. 17. Standard curve for the determination of total hexose content of Antigen Ra.3.

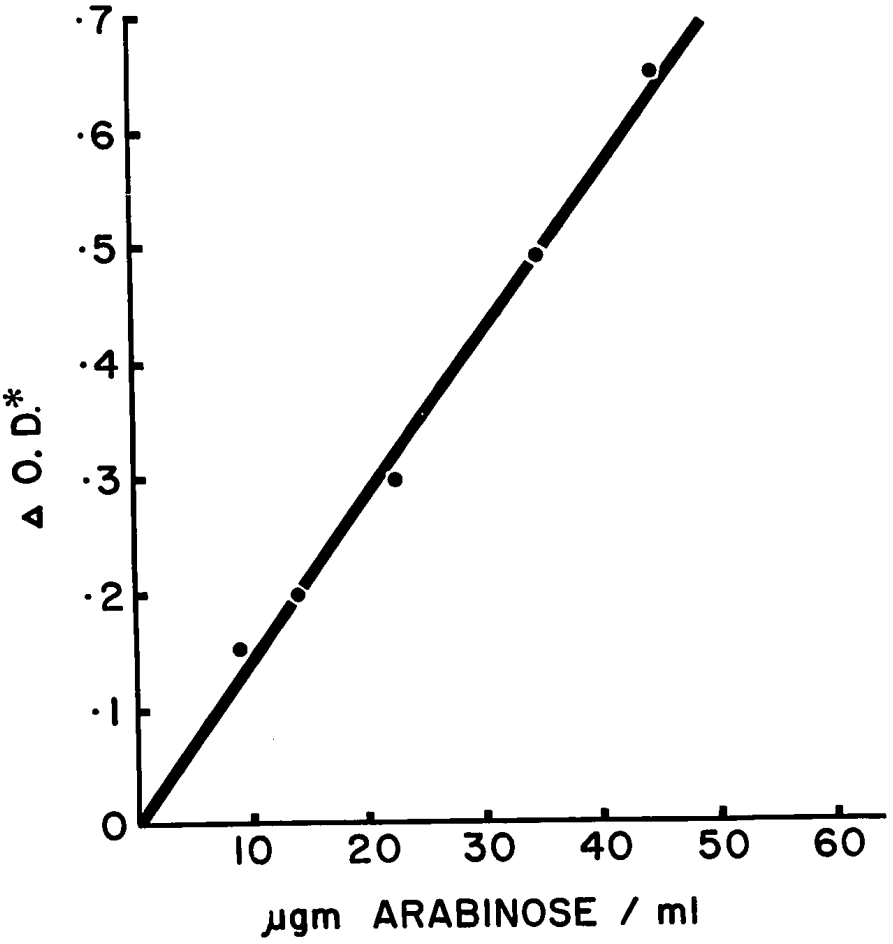


Fig. 18. Standard curve for the determination of total pentose content of Antigen Ra.3.
* O.D. 390 mµ - O.D. 424 mµ.

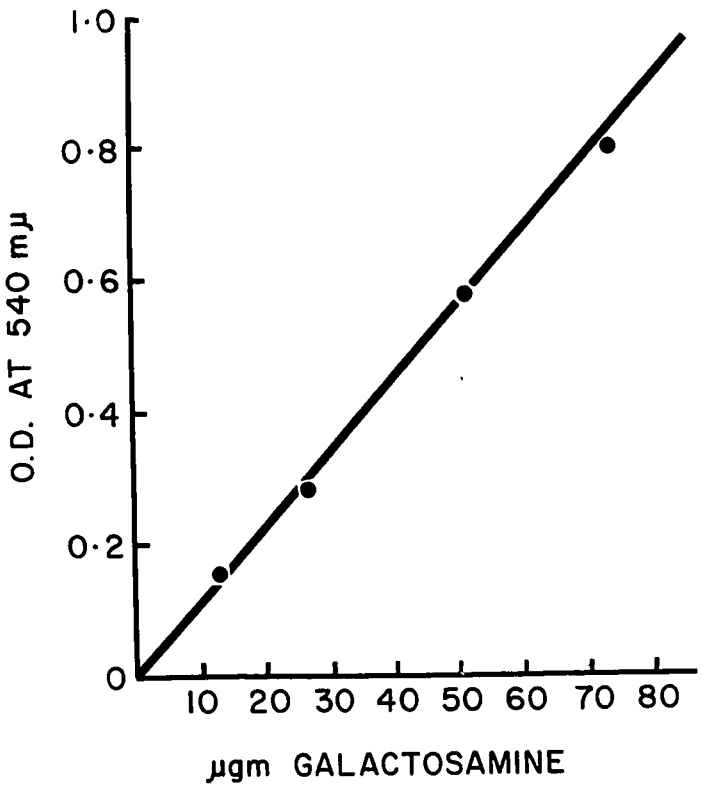


Fig. 19. Standard curve for the determination of total hexosamine for Antigen Ra.3.

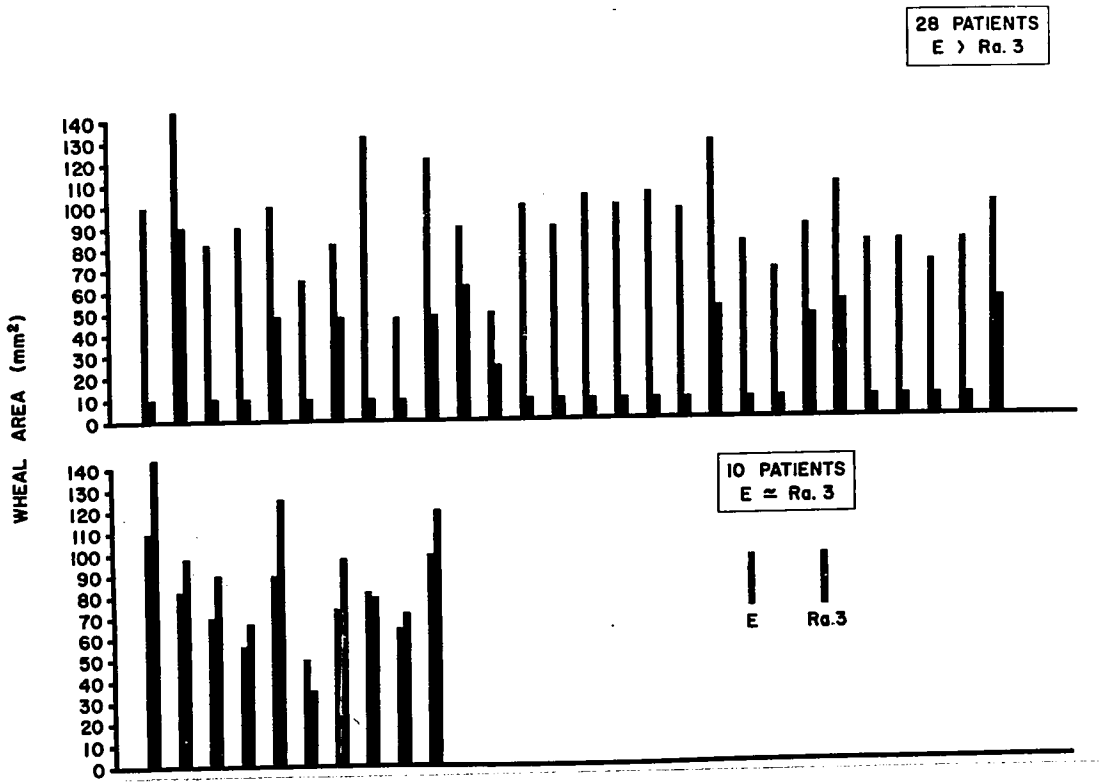


Fig. 20. Direct skin testing of allergic patients with Antigens E and Ra.3. The concentration of each antigen solution was 10^{-9} gm/ml and 0.05 ml aliquots were employed for testing.

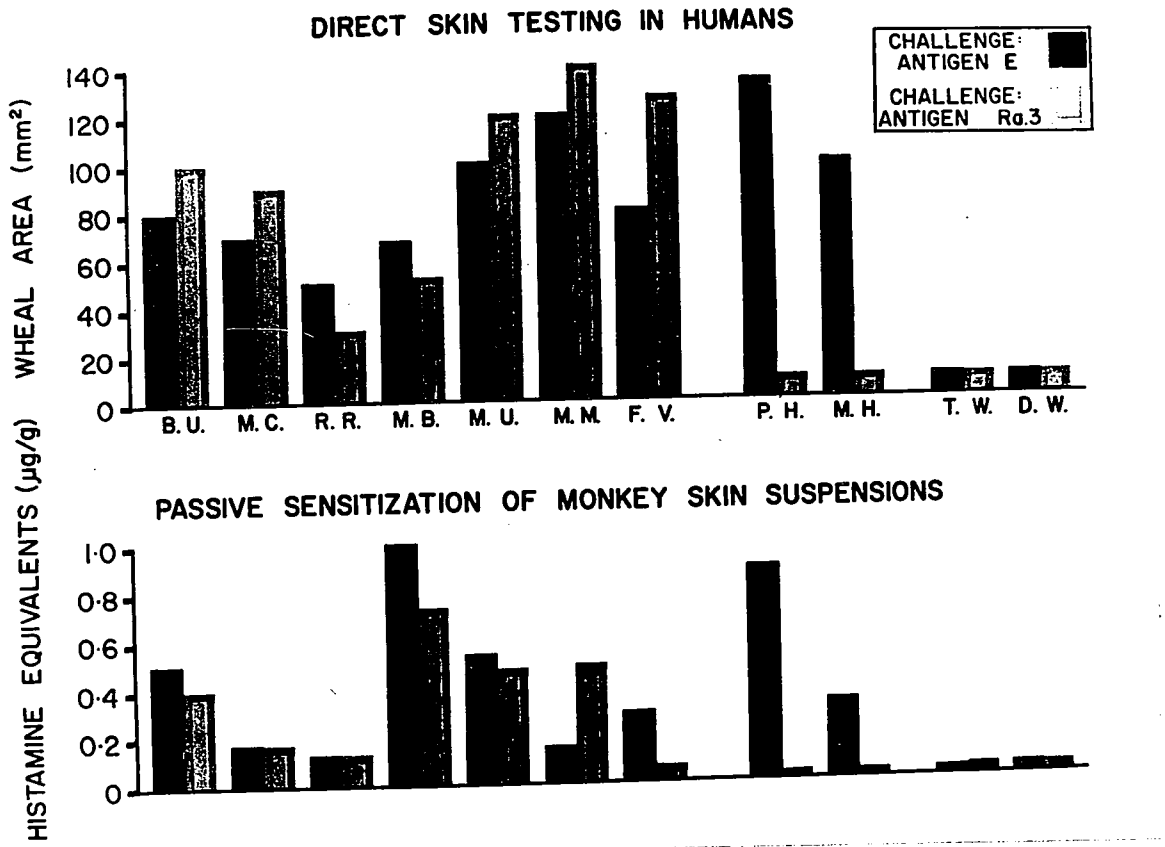


Fig. 21. Comparison of allergenic activity of Antigens Ra.3 and E. Concentration of Antigen solutions: direct skin test, 10^{-3} ug/ml; in vitro anaphylaxis, 50 ug/ml.

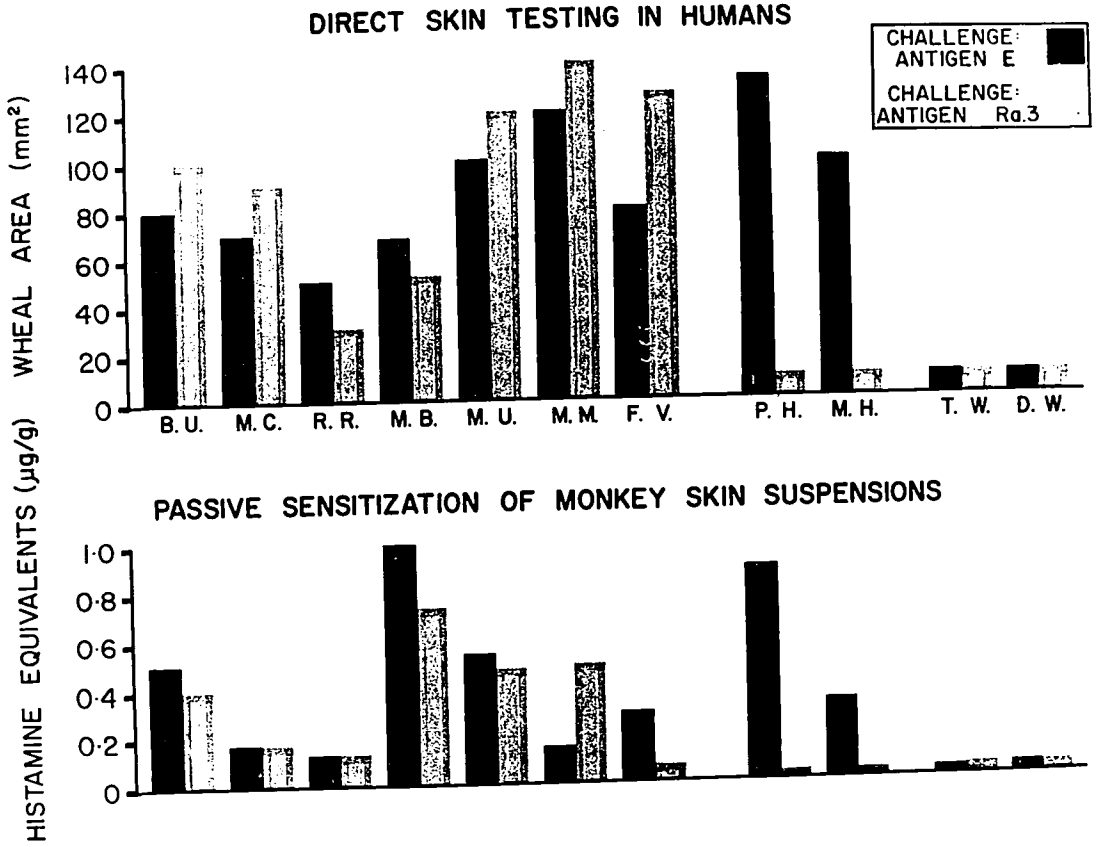


Fig. 21. Comparison of allergenic activity of Antigens Ra.3 and E. Concentration of Antigen solutions: direct skin test, 10^{-3} ug/ml; in vitro anaphylaxis, 50 ug/ml.

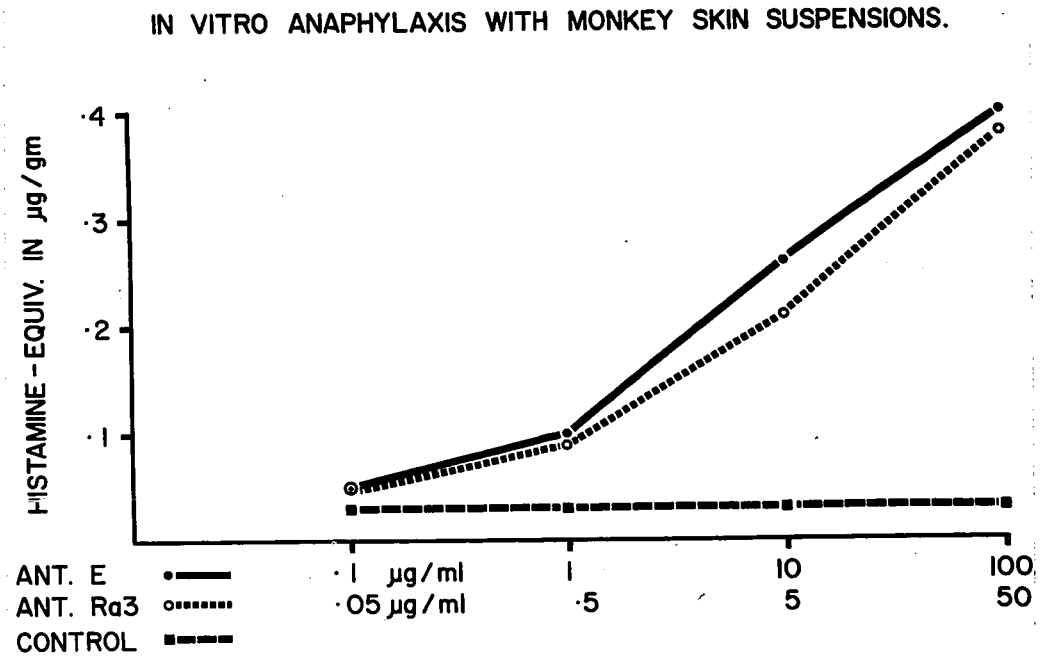


Fig. 22. Assay of allergenic activity of Antigens Ra.3 and E by in vitro anaphylaxis of rhesus monkey skin suspensions passively sensitized with serum of patient M. M.

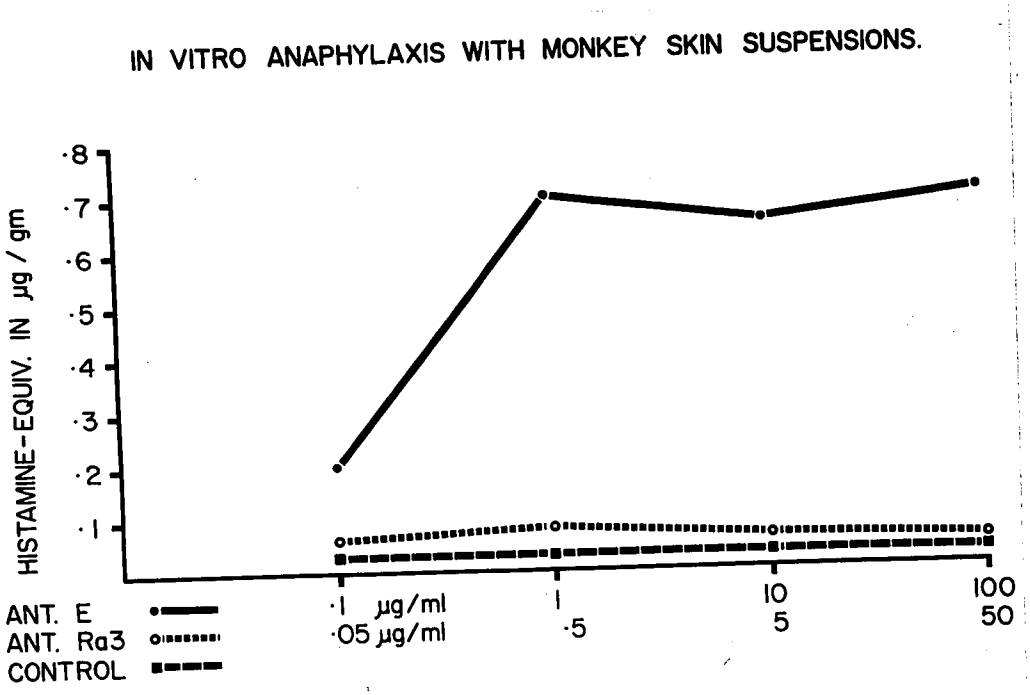


Fig. 23. Assay of allergenic activity of Antigens Ra.3 and E by in vitro anaphylaxis of rhesus monkey skin suspensions passively sensitized with serum of patient A. C.

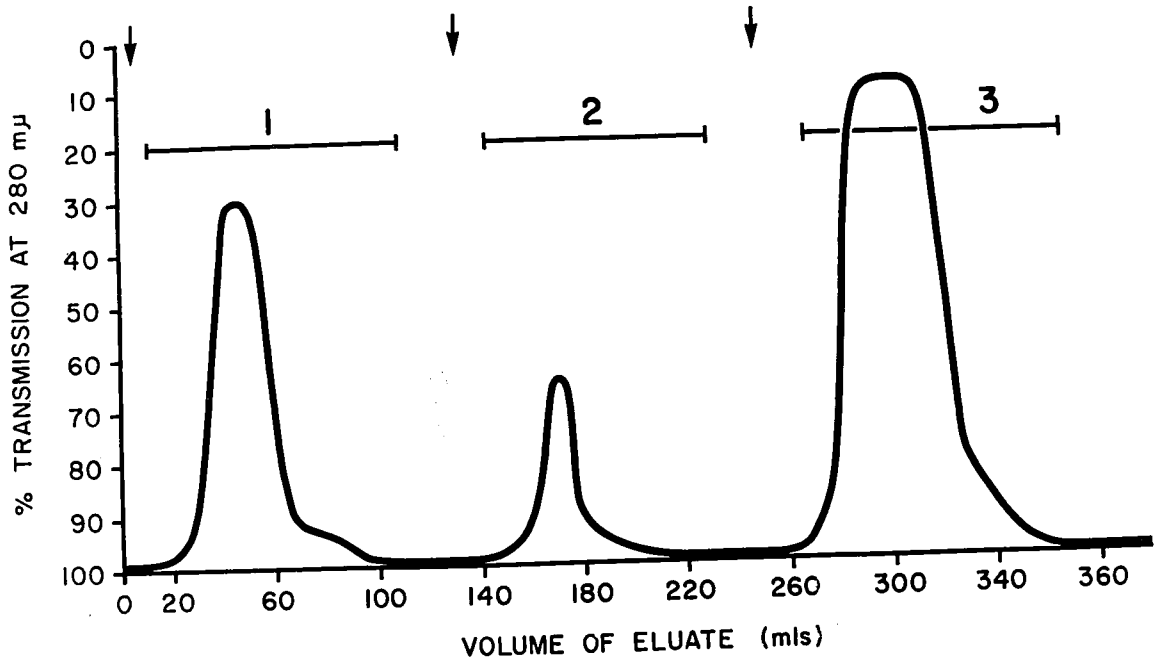


Fig. 24. DEAE-Sephadex chromatography of ragweed allergic serum M.M.
The arrows indicate points of application of buffer (see text).
1, 2 and 3 refer to cuts made to obtain fractions I, II and III, respectively.

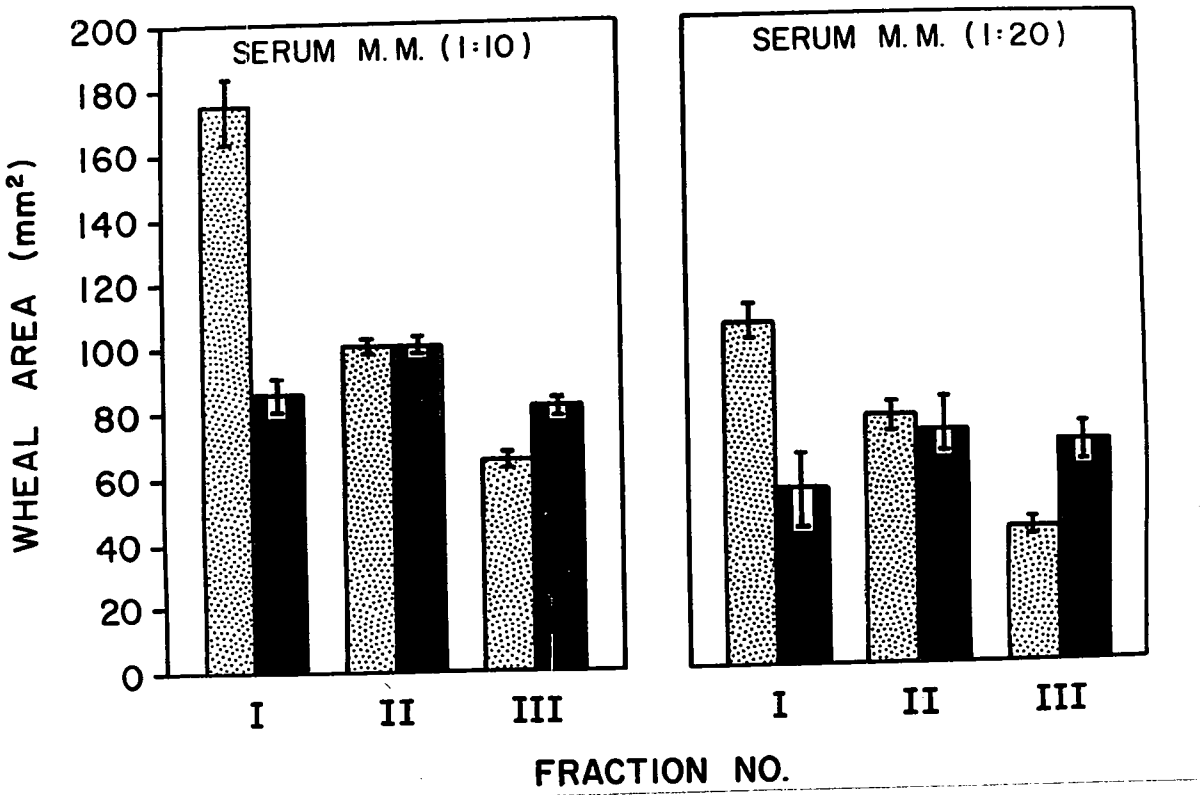


Fig. 25. Reaginic activity of DEAE-Sephadex fractions of serum M.M. separately tested with 0.5 µg Antigen Ra.3/ml and 1.0 µg Antigen E/ml.

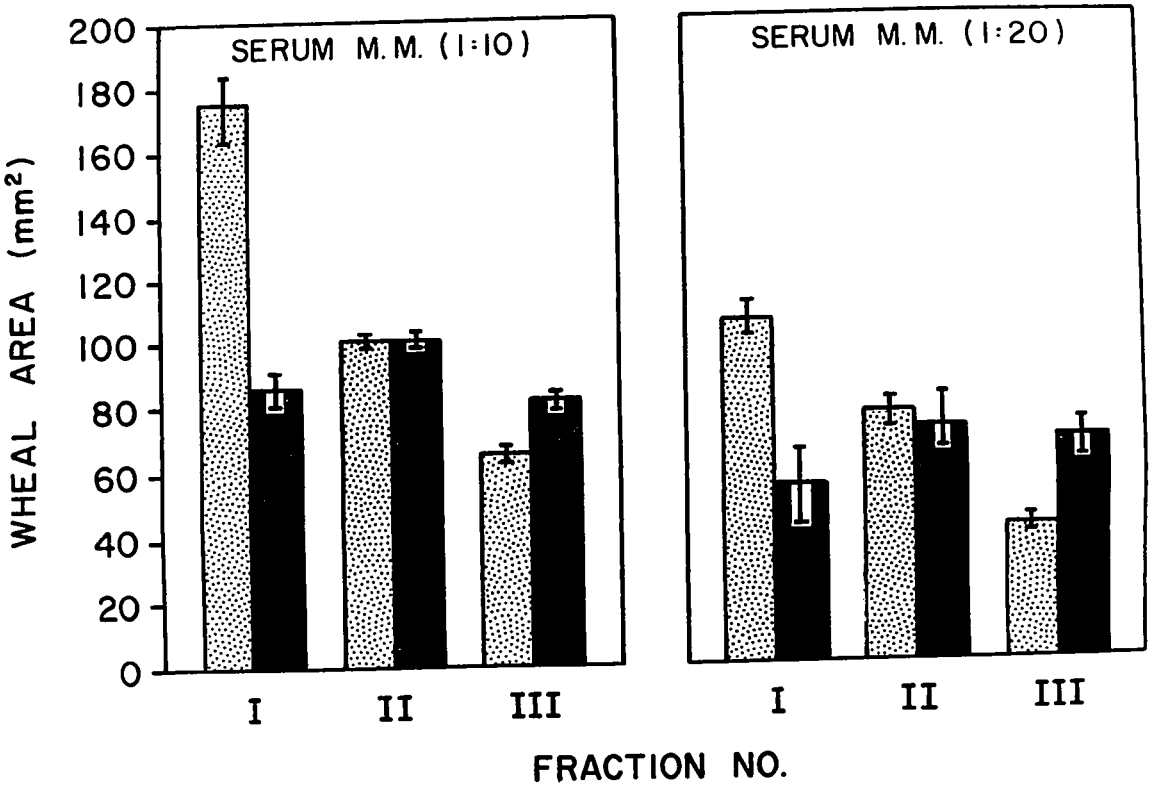


Fig. 25. Reaginic activity of DEAE-Sephadex fractions of serum M.M. separately tested with 0.5 µg Antigen Ra.3/ml and 1.0 µg Antigen E/ml.

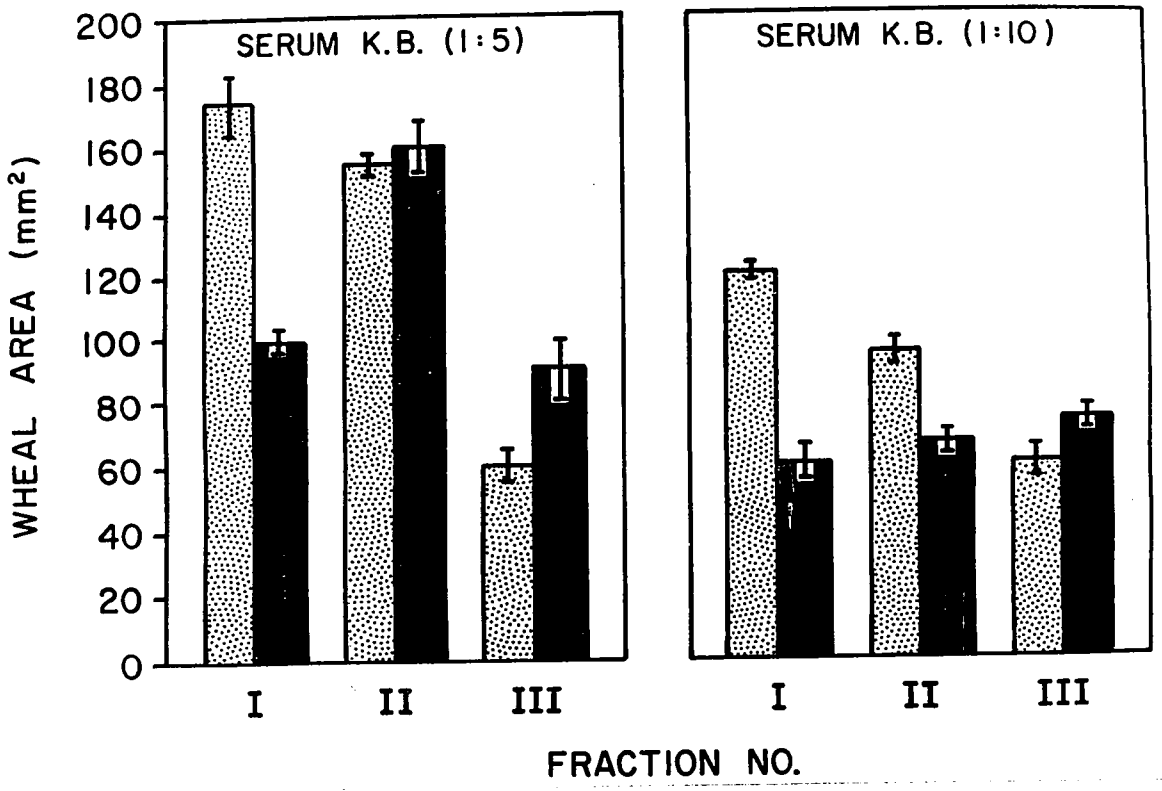


Fig. 26. Reaginic activity of DEAE-Sephadex fractions of serum K.B. separately tested with 0.5 µg Antigen Ra.3/ml and 1.0 µg Antigen E/ml.

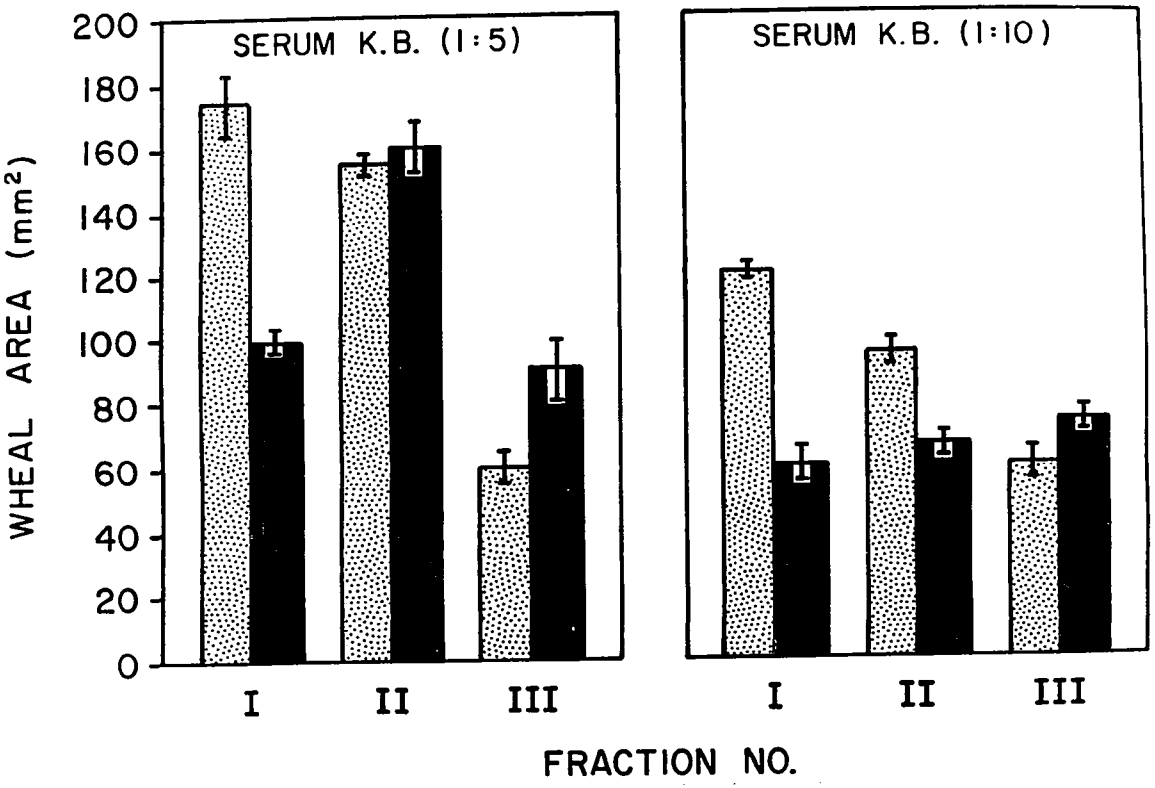


Fig. 26. Reaginic activity of DEAE-Sephadex fractions of serum K.B. separately tested with 0.5 µg Antigen Ra.3/ml and 1.0 µg Antigen E/ml.

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