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Short Title:

ISOLATION, STRUCTURE AND ACTIVITY OF Ra3, Ra4 AND Ra5

ISOLATION, STRUCTURE, AND ACTIVITY OF RAGWEED POLLEN ANTIGENS Ra3, Ra4, AND Ra5

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ABSTRACT

A column chromatographic method was developed for sequential isolation of antigens Ra3, Ra4, and Ra5 from short ragweed pollen.

Two major forms of antigen Ra3 (I and II) were separated and found to have the same mol wt (12,000 daltons) and amino acid composition. Their variable carbohydrate content (7%-20%) could be reduced to 3% by preparative acrylamide gel electrophoresis.

Antigen Ra3 was shown to be allergenically independent of antigen E by direct skin test or by histamine release from leucocytes of ragweed sensitive patients.

Antigen Ra4 had a mol wt of 23,000 and an isoelectric point of pH 8.0. Its amino acid composition showed the presence of all common amino acids. Its carbohydrate content (30-100%) could be reduced to the 6% level by ammonium sulfate precipitation.

On immunodiffusion test, Ra4 cross-reacted with the major allergen, antigen E, but the cutaneous sensitivities to these 2 allergens were not associated.

As previously shown for Ra5, the Ra3 and Ra4 proteins were found to have a single polypeptide chain structure. In a collaborative study, N-terminal amino acid sequences were determined for Ra3 (25 residues) and Ra4 (22 residues), the features of which were compared with Ra5 and other protein allergens.

RESUME

Une méthode de chromatographie sur colonne a été développée pour isoler successivement les antigènes Ra3, Ra4 et Ra5 à partir du pollen de Jacobée.

Les deux formes principales de l'antigène Ra3 (I et II) ont été separées; la même composition en acides aminés ainsi que le même poids moléculaire ont été démontrés pour ces deux formes. Leur contenu variable en hydrate de carbone (7-20%) a pu être réduit à 3% par électrophorèse sur gel acrilamide préparatif.

L'antigène Ra3 a une activité allergénique indépendante de celle de l'antigène E. Les épreuves par application directe sur la peau de patients sensibles au Jacobée et par libération d'histamine de leurs leucocytes supportent cette conclusion.

L'antigène Ra4 possède un poids moléculaire de 23,000 et un point isoélectrique au pH 8.0. Tous les acides aminés communs entrent dans sa composition. Son contenu en hydrate de carbone (30-100%) a pu être réduit à 6% par précipitation avec du sulfate d'ammonium.

Ra4 a révélé une réaction croissée, durant l'épreuve d'immunodiffusion, avec l'allergène principal (antigène E) mais la sensibilité cutanée au 2 allergènes n'a pas été associée.

Comme il a été démontré avec Ra5, les parties protéiniques de Ra3 et Ra4 ont révélées une structure d'une seule chaine polypeptidique. Par étude collaborative, la séquence N-terminale d'acides aminés a été déterminée pour Ra3 (25 résidus) et Ra4 (22 résidus), ces caractéristiques sont alors comparées avec Ra5 et d'autres allergènes protéiniques.

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PART A

INTRODUCTION

This study forms part of the effort by numerous investigators to isolate and characterize the components of plant pollens that are allergenic in man. Much of this effort, including the present work on antigens Ra3, Ra4, and Ra5, has centered on the pollen of short ragweed (Ambrosia eliator), the most frequently implicated incitant of acute allergic reactions on the North American continent. An account of the allergens of short ragweed pollen is given in Section I; in the section following we outline the immediate and long-range objectives of the present study.

I. The Allergens of Short Ragweed Pollen

1. Antigen E

The report by T. P. King and associates (1962, 1964) on ragweed pollen antigen E marked a turning point in the history of allergen research. This was the first macromolecular allergen to be isolated in homogeneous form, an outstanding achievement made possible by application of the newly developing techniques of gel filtration and ion exchange chromatography.

The critical element in King's methodology was the division of WSR (water soluble ragweed formed from ether extracted pollen) into a relatively cationic fraction C and relatively anionic D fraction by DEAE-cellulose chromatography. Antigen E and, later, antigen K were obtained by column chromatographic processing of the D fraction. (In contrast, the allergens examined in the present study were initially

isolated from fraction C.) Molecular sieving of fraction D through Sephadex G-100 yielded peaks D-I to D-VII, of which D-IV contained antigen E. The D-IV fraction could be further subdivided into 4 differently charged forms of antigen E, IV-A through -D, according to the sequence of their elution on TEAE-cellulose chromatography.

As indicated by their behaviour on ion exchange chromatography, the 4 forms of antigen E were negatively charged at neutral pH. In a recent study (King, 1972) employing isoelectric focussing in polyacrylamide gel, their isoelectric points were shown to be approx. pH 5.

The 2 major electrophoretic forms of antigen E, IV-B and IV-C, were found to have identical molecular weights (37,800 daltons) and amino acid compositions. The compositions showed no unusual features which might distinguish these allergens as a distinct sub-class of protein antigens, except perhaps for a relatively high cystine content (7 half cystines per molecule). (Analysis gave 0.3 - 0.6 moles free sulfhydryl per mole protein; the presence of a single cysteine residue might explain, at least in part, the tendency of antigen E in solution form to aggregate with time.)

Analysis showed a carbohydrate content of less than 0.5% (measured as arabinose) which could only be considered as contaminant. As will be discussed in Section II, this finding was of fundamental importance to the development of the concept of allergenicity.

In attempts to prepare allergenically active fragments of antigen E, King et al. (1964) studied the effect of proteolytic enzymes on the

allergen. Native antigen E was not digested by trypsin, chymotrypsin and papain. While digestion was observed with pepsin, the pH (2.0) of incubation employed rapidly led to denaturation and inactivation of the protein. Treatment at near physiological pH with the bacterial enzyme, Nagarse, led to extensive degradation but was accompanied by a 1000-fold decrease in its skin activity in patients and the loss of ability to precipitate specific rabbit antiserum (King et al., 1967b).

The allergenic activity of antigen E was dependent on maintenance of the structural integrity of the protein. Thus, conditions which generally induced conformational change, such as exposure to extreme pH, protein denaturants (urea, sodium dodecylsulfate, guanidine); reduction and alkylation; extensive modification of α-amino groups or β- and γ-carboxyl groups, invariably resulted in marked reduction or virtual elimination of the biological activity of antigen E (King and Norman, 1962; King et al., 1964; King et al., 1973).

Antigen E was recently shown to consist of 2 polypeptide chains, a light (α) and heavy (β) chain, of 13,000 and 25,000 daltons, respectively. Griffiths (1972) separated the 2 chains by reduction and carboxymethylation of antigen E followed by chromatography (on Biogel A-5m) in the presence of guanidine -HCl. On the other hand, King et al. reported (1973) that dissociation of the 2 chains could take place without prior reduction of the disulfide bonds, by heating at 50°C in SDS buffer, or by modification of its ϵ-amino groups. Allergenic as well as antigenic activity were lost

as a consequence of chain separation.

The content of antigen E in ragweed pollen was estimated to be 6% of the pollen protein (King et al., 1967b). The contribution of the protein to the allergenic activity of the whole extract was measured by the decrease in activity of the extract after precipitation of antigen E with specific antiserum (King et al., 1964). From the decrease in cutaneous activity of the supernatants tested in 8 ragweed sensitive patients, the authors concluded that antigen E accounted for at least 90% of the activity of WSR. Supporting evidence was provided by Lichtenstein et al. (1966) who showed by leucocyte sensitization that, of the fractions D, D-III, D-IV and D-V, antigen E (D-IV) was most potent in releasing histamine from human leucocytes.

Although there seemed little doubt that, with the isolation of antigen E, the most active of the causative agents of ragweed sensitivity had been found, there was increasing evidence for the existence of additional ragweed pollen allergens.

Lichtenstein et al. (1966) observed that "a unique pattern of sensitivity to different ragweed pollen antigens characterizes the leucocytes of each individual". Ishizaka et al. (1966) reported 2 allergic sera that did not give P-K reactions when skin sites were challenged with antigen E but did with crude ragweed fraction A (King et al., 1964). Whereas Norman and Winkenwerder (1967) reported that patients undergoing hyposensitization treatment tolerated higher doses of antigen E than WSR, Reisman et al. (1968)

observed that clinical treatment with antigen E was not in all cases as beneficial as WSR.

The existence of ragweed pollen allergens other than E was demonstrated in the following years in the isolation of allergens which either shared partial immunological identity with antigen E (antigen K, King et al., 1967a; BPA-R, Griffiths and Brunet, 1971) or were antigenically and allergenically independent (Ra3, Underdown and Goodfriend, 1969; Ra5, Lapkoff and Goodfriend, 1974).

2. Antigen K

A second ragweed pollen allergen, antigen K, was isolated by King et al. (1967a) employing essentially the same methodology used for antigen E. As the charge and molecular weight (38,200 daltons) of antigen K were quite similar to that of antigen E, the preparation of both allergens in good quantity proved to be difficult. An alternative and apparently superior method for the separation of both allergens was recently developed by King (1972). The method was based on ammonium sulphate gradient elution from a column packed with WSR precipitate-celite mixture. The application of this technique for isolation of other pollen allergens has not yet been reported.

The amino acid composition of antigen K was distinct from that of the major antigen. As with antigen E, the small amount of carbohydrate detected ($<1\%$) was considered to be most likely a contaminant.

Immunological cross-reactivity between antigens K and E was deduced from the following: A reaction of partial identity was observed on immunodiffusion of antigens E and K with rabbit anti-WSR antiserum and with some (though not all) antisera separately raised to each antigen; sensitivity of human leucocytes to antigens E and K was found (King et al., 1967a) to be not a random occurrence in the allergic population, but closely linked, with a correlation coefficient of 0.87 ($p < 0.001$).

3. Antigen Ra3

The more cationic DEAE-fraction C provided the source for the preparation of antigen Ra3, the third ragweed pollen allergen to be isolated in what was considered to be a highly homogeneous form (Underdown and Goodfriend, 1969). By analogy with Sephadex G-100 fraction D-IV, a G-100 fraction C-IV was further purified by TEAE-cellulose chromatography. The more retarded TEAE-peak, C-IV-2, contained Ra3.

Homogeneity of the antigen was established by analytical ultracentrifugation, gel filtration through Sephadex G-100, TEAE-cellulose chromatography, polyacrylamide gel disc electrophoresis, and by immunodiffusion analysis with anti-Ra3 and anti-WSR antisera.

The properties of Ra3 differed markedly from E (and K). It had less than half the mol wt (15,000 daltons), a dissimular amino acid composition, and an appreciable carbohydrate content (13 per cent) which apparently remained unchanged through re-chromatography on gel and ion-exchange columns. (The allergen was therefore considered to be a

glycoprotein, although covalent carbohydrate-protein linkage was not established.).

No cross-antigenicity between Ra3 and E was detected on immunodiffusion analysis with corresponding rabbit antisera.

The incidences of human sensitivity to Ra3 and E were found by direct skin test of 46 ragweed sensitive patients to be 44 and 74%, respectively, i.e., some 1 of every 2 patients were sensitive to the newly isolated allergen. Furthermore, half of the Ra3 sensitive patients gave cutaneous reactions (on a weight basis) equal to or greater than antigen E, so that the contribution of Ra3 to total sensitivity of ragweed allergic patients was likely more than minor. The finding that half of the antigen E sensitive patients were unreactive to Ra3 suggested the 2 proteins were allergenically independent; further evidence was obtained from in vitro anaphylactic and cross-neutralization studies (Underdown and Goodfriend, 1969).

Further studies on the isolation and physicochemical properties of antigen Ra3 (Roebber, 1970) showed the presence in column chromatographic eluates of 4 components reacting with anti-Ra3 antiserum but differing in charge and/or size. This suggested the possibility that Ra3 exists in several closely related forms. The major form of the antigen, Ra3-B was shown to be closely similar to the preparation of Underdown and Goodfriend. Small amounts of 3 minor forms (Ra3-A, -C and -D) were isolated in a homogeneous state as judged by gel chromatography and polyacrylamide disc electrophoresis. The 4 forms gave single precipitin arcs on immunodiffusion

test with antiserum to Ra3 in a reaction of antigenic identity while amino acid analysis of the different forms of Ra3 showed general similarities in composition, appreciable divergence was found for proline, arginine, isoleucine and tyrosine; however, these results could be considered as preliminary only, requiring further analyses for confirmation.

4. BPA-R (Antigen Ra4)

After antigen K, BPA-R (basic protein allergen of ragweed) was the second allergen to be reported with antigenic cross-reactivity with antigen E (Griffiths, 1969; Griffiths and Brunet, 1971). Of the several antigens in crude fraction A, it had the most cathodic immunoelectrophoretic mobility - hence Griffiths' nomenclature. It was isolated from DEAE-fraction C by salt gradient elution from CM-Sephadex and gel filtration through Sephadex G-100, and shown to be homogeneous by a (limited) number of physicochemical criteria as well as antigenically by immunodiffusion and immunoelectrophoresis with specific and anti-WSR antisera. An initially reported mol wt of 28,000 daltons (Griffiths and Brunet, 1971) was later (Griffiths, 1972) amended to 23,000.

5. Antigen Ra5

Recently, yet another cationic ragweed pollen allergen was isolated from DEAE-fraction C by gel and ion exchange chromatography in yields of 10-15 mg/kg pollen (Goodfriend and Lapkoff, 1972; Lapkoff and Goodfriend, 1974).

The Ra5 protein was devoid of carbohydrate and homogeneous by a number of physicochemical criteria (Lapkoff and Goodfriend, 1974). Its amino acid composition was distinct from the compositions of E, K, and Ra3, and was characterized by the absence of histidine, threonine, phenylalanine and methionine. The most interesting features of this latest active pollen protein were its small molecular size (5000 ± 200 daltons) and single chain structure, properties which facilitated determination of its complete amino acid sequence (Mole et al., 1975).

Antigen Ra5 was shown to be antigenically and allergenically distinct from antigens E and Ra3 (Lapkoff and Goodfriend, 1974). The incidence of cutaneous activity in man was considerably less than that of antigen E or Ra3. Thus, of 105 antigen E-sensitive patients simultaneously tested with E and Ra5, only 18 showed appreciable Ra5 reactivity while the remainder was insensitive to the allergen (Marsh et al., 1973, 1974). The relatively simple structure of Ra5 and the limited occurrence of sensitivity to it in the allergic population were likely of critical importance to the finding by Marsh et al. (1973, 1974) that IgE-mediated sensitivity to Ra5 was significantly associated with possession of the histocompatibility antigens of the HL-A7 cross-reacting group.

II. Objectives of the Present Study

Studies on the allergens of short ragweed pollen have been in progress for the past decade. As has already been indicated, no special consideration other than the fact of high allergenic activity has motivated the choice of ragweed in place of other pollens or indeed of other sources of allergenically active proteins. The overall aim of these studies has been to assist in the further development of our knowledge of the chemical structure of proteins which subserve their allergenic function of inciting and interacting with reaginic (IgE) antibodies in man. Following the historic studies of T. P. King on antigens E and K, the attention of this laboratory was directed to protein allergens of lower molecular weight potentially present in ragweed pollen which might offer advantages for structure-function studies. As a result, two low molecular weight allergens, Ra3 and Ra5, were isolated and a third, Ra4, of "intermediate" size, has been under study.

In the experimental work for this Thesis, studies were done on problems of a more or less technical nature connected with the isolation and characterization of these allergens, the solutions to which were urgently required. The nature and scope of these experiments are outlined below. Of interest in themselves for defining the chemical and immunological properties of ragweed pollen allergens, these more "immediate" objectives of our study served as a "clearing ground", a necessary preliminary to

more long-range structure-function studies in the development of which some progress was achieved.

1. Multiple forms of antigen Ra3

Previously, evidence was obtained (Roebber, 1970) for the existence of multiple forms of antigen Ra3. Studies were undertaken to isolate 1 or 2 of these forms reproducibly and on a preparative scale (Experiments and Results, I).

2. An improved procedure for isolation of antigen Ra5

Collaborative studies between this laboratory and others have attempted to develop antigen Ra5 as a model for structure-function correlation in the field of atopic hypersensitivity (Mole et al., 1975; Marsh et al., 1975). To further develop this model, it became evident that simplification of the method of isolation and appreciable increase in yield of Ra5 were urgently needed. The low yield (10-15 mg/kg pollen) obtained by the existing procedure (Lapkoff and Goodfriend, 1974) stood in sharp contrast with the Ra5 content of aqueous ragweed pollen extracts (320-350 mg/kg pollen) determined by radio-immunodiffusion assay (Marsh, 1973). Studies were therefore undertaken to develop an improved procedure for isolation of Ra5, one that would allow "simultaneous" isolation of the other, known allergens, in particular antigens Ra3 and Ra4 (Experiments and Results, II).

3. Chemical and immunological properties of antigens Ra3, Ra4, and Ra5 (Experiments and Results, III).

The properties of Ra3 have already been extensively reported (Underdown and Goodfriend, 1969). However, their re-study seemed justified due to the possibility that the initial preparations consisted of 2 forms of the allergen which were undetected due to insufficient sensitivity of the disc electrophoretic technique which had been employed to establish homogeneity of the allergen. Among other parameters, it was of particular interest to determine:

i. the molecular weight by sedimentation equilibrium ultracentrifugation, taking advantage of the more sensitive analytical ultracentrifuge equipped with UV optical system, automatic Scanner and recording apparatus;

ii. the carbohydrate content of Ra3 preparations at various stages of purification;

iii. the amino acid composition of at least 2 forms of Ra3;

iv. the immunological activity of Ra3, in particular, its antigenic relationship to antigen E and the incidence of its allergenic activity in a ragweed sensitive population.

Similar analyses were planned for purified antigen Ra4. Apart from determination of its molecular weight and antigenic cross-reactivity with E, few if any additional chemical and immunological parameters had been reported for this allergen. Indeed, its allergenic activity had been

established (by P-K test) in only 2 ragweed sensitive patients (Griffiths and Brunet, 1971).

It was also planned to determine the allergenic (cutaneous) activity of antigen Ra5, when isolated by a modified procedure, in a random population of ragweed sensitive patients, as well as some chemical parameters not previously established.

4. Structure-function studies

At the present stage of knowledge, it appears that the structure of macromolecular allergens must subserve at least 2 functions: interaction of allergen with (mast) cell-bound IgE antibodies for release of pharmacological mediators and interaction with lymphocytes for biosynthesis of these antibodies.

Certain requirements concerning chain or subunit structure of allergens appear to be implied by their role in bridging of adjacent cell-bound IgE antibodies, a reaction mechanism which is believed to trigger the symptom complex of the immediate hypersensitivity state (Ovary and Taranta, 1963). The general view is that a minimum number of 2 antibody binding determinants should be present on the provoking (allergen) molecule. Stanworth (1973) has recently proposed that, in the simplest case, these allergenic determinants should be identical and distributed symmetrically on each of 2 subunits or chains at the critical distance for cross-linking of adjacent cell-bound antibody molecules. The allergenically active proteins β-lactoglobulin (Bull, 1946; Townend and Timasheff, 1957;

Bleumink and Young, 1968) and phospholipase A (Shipolini et al., 1971) do indeed meet the requirement for 2 equal subunits. Recently, an α-β (non-identical) subunit structure was established for antigen E (King et al., 1973) so that "adjacent" IgE antibodies may also bridge non-identical allergen determinants. The concept of Stanworth of obligatory subunit structures for protein allergens appears to be contradicted, however, by the activity of antigen Ra5 and codfish allergen M, both of which consist of single polypeptide chains. For purposes of generalization, it would be of interest to determine the polypeptide chain structure of additional allergens, such as antigens Ra3 and Ra4 of the present study. Antigen Ra4 would be of particular interest as it may constitute the free heavy chain of antigen E.

Regarding the role of allergen in the biosynthesis of reaginic antibodies, the general notion has pervaded the literature that some special structural features are common to all macromolecular allergens, serving to distinguish them as a class from other antigens (cf. Strejan, 1973). Early speculation of the protein versus carbohydrate nature of allergens (for review, see Richter and Sehon, 1960) was followed by the concept that the allergens of atopic hypersensitivity are glycoprotein in nature (Berrens, 1962; Stanworth, 1963). This tenet could not be reconciled with an increasing number of reports on isolation of carbohydrate-free protein allergens; ragweed pollen antigens E, K (King et al., 1963, 1967) and Ra5 (Goodfriend and Lapkoff, 1972); codfish allergen M (Elsayed and

Aas, 1971). Data on the carbohydrate content and the amino acid composition of several ragweed and rye grass pollen allergens and of allergens from cod and honey bee do not point to any consistent compositional features (D. Marsh, 1975) except for a relatively high content of half-cysteine (Mole et al., 1975).

It would seem that only determination of the complete amino acid sequence of a number of protein allergens from diverse sources might reveal structural features of importance to their allergenic function. Studies in this direction have been only fragmentary to date. The sequence of bee venom phospholipase A (Shipolini et al., 1971) was determined prior to implication of this protein in hymenoptera sensitivity (Sobatka et al., 1975). A partial sequence of codfish allergen M has been reported (Elsayed et al., 1973). Recently, collaborative studies between this laboratory and that of Dr. J. Don Capra have established the complete amino acid sequence of Ra5 (Mole et al., 1975). A long-range perspective of the present study was to extend these sequence determinations to include antigens Ra3, Ra4 and other pollen protein allergens for clarification of the role (if any) of common primary structural elements.

Another objective of comparative studies of this type should be to gain some insight into the chemical nature of the regions which combine with IgE antibodies. There has not yet appeared in the literature any report on the successful preparation of a fragment bearing a single (haptenic) allergen determinant or the chemical definition of such a determinant. Such haptenic determinants may be present in aqueous pollen

extracts. Malley et al. (1963, 1964) described an haptenically active preparation obtained from the dialyzable fraction of an aqueous extract of timothy grass pollen. This preparation inhibited the precipitin reaction of total extract with rabbit antibodies raised to the extract. However the isolate was still able to induce cutaneous reactions in grass allergic individuals. Attallah and Sehon (1969) isolated a low molecular weight fraction (A-2RS-I) from the dialysate of the aqueous extract of short ragweed pollen. They reported that the fraction was able to specifically inhibit cutaneous (P-K) activity of the whole aqueous extract, suggesting the presence of monovalent fragments able to combine with reaginic antibodies directed to all the allergenic determinants in the crude extract. If confirmed, these findings would obviously have great potential for clinical treatment of ragweed sensitive patients, and for study of the chemical nature of allergen determinants. However, the yield of fraction A-2RS-I would appear to be very low, a factor which may account for the limited clinical and chemical characterization of it reported to date.

While these reports encourage the search for "naturally occurring" monovalent fragments of allergens, they also point to the desirability at the present stage of allergen research for "planned" preparation of such fragments by enzymatic and/or chemical degradation of homogeneous and well defined protein allergens. Encouraging support for efforts in this direction has come from studies on the antigenic determinants of a number of diverse

proteins of known amino acid sequence (Benjamini et al., 1972). The general finding emerging from these studies, particularly with low molecular weight proteins and polypeptides, such as ACTH, gastrin, and glucagon, is that immunological (determinant) activity resided in defined sequences of amino acids in addition to tertiary configurations. If similar structural elements governed the binding of allergens with reagins, one would expect that the availability of low molecular weight protein allergens (of the size of the antigens of the present study) would facilitate successful preparation of haptenic allergen fragments.

The availability of a number of allergens of known amino acid sequence and chemical modification of such allergens may lead to definition of the chemical nature of the carrier regions of protein allergens, i.e., regions which interact with thymus dependent (T-) lymphocytes in the process of IgE antibody synthesis (Goodfriend, 1974; Marsh et al., 1975). Tada et al., (1971) and Ishizaka et al., (1972) established that the collaboration between carrier-specific helper (T) lymphocytes and hapten-specific precursors of antibody forming (B) lymphocytes was required for maximal anti-hapten IgE antibody response. An allergen molecule would therefore incorporate within its structure carrier regions to interact with Ir gene determined receptors on T cells, and haptenic regions to bind to specific B cells. Differentiation of these 2 regions was strikingly demonstrated recently by Ishizaka et al. (1974). The authors prepared urea denatured and reduced and alkylated antigen E which was no longer

able to bind with human or rabbit IgG antibodies and did not induce wheal and erythema reactions in ragweed sensitive individuals. Denatured antigen E as well as isolated α and β subunits were found to stimulate carrier-specific helper cells for IgE antibody production in the rabbit to the same extent as the native allergen. All of the modified preparations induced DNA synthesis in peripheral lymphocytes of ragweed sensitive patients but not of normal individuals.

From the foregoing results it can be inferred that the carrier regions of antigen E may be largely, if not entirely, sequence dependent, while such is clearly not the case with antibody binding regions of this allergen. These findings if generalized to other allergens, would point to new possibilities in immunotherapy, as injections of patients with peptidyl fragments bearing active carrier sequences might be employed to alter the T cell population in a beneficial manner without the danger of causing allergic reactions.

In line with the objectives outlined above, studies were initiated to determine the polypeptide chain structure of antigens Ra3 and Ra4, and, in collaboration with Dr. J. D. Capra and his associates, the complete amino acid sequence of these proteins. Progress achieved to date will be presented in Section III of "Experiments and Results".

PART B

MATERIALS AND METHODS

I. Chemicals

All chemicals were of reagent grade and were purchased from Fisher Scientific Co., Montreal, unless otherwise indicated.

II. Ragweed Pollen Allergen Preparations

DEAE-Fraction C was prepared from ether defatted short ragweed pollen (Sharp & Sharp, Everett, Wash.) according to the method of Underdown and Goodfriend (1969) with minor modifications (Roebber, 1970). The fraction was Millipore filtered (see below) and stored at 5°C until used.

Antigen E was donated by Dr. T. P. King, Rockefeller University, New York, N. Y..

Antigen BPAR (Ra4) was donated by Mr. B. Griffiths, Biologics Control Laboratory, Canadian Communicable Diseases, Department of National Health and Welfare, Ottawa.

III. Antisera

Rabbits received weekly subcutaneous injections of 0.5 mg antigen Ra3 or Ra4 in 1 ml aliquots of PBS made 40% in Freund's incomplete adjuvant (Canadian Laboratory Supplies, Montreal). Immunization was continued for 4 weeks and, after a 4 weeks' rest, continued for an

additional 4 weeks. Rabbits were bled 8 days after the last injection from the main artery of the ear using a vacutainer needle.

Rabbit antisera to WSR and antigen E were obtained from the NIH (Bethesda, Md.). Rabbit antiserum to antigen Ra5 and goat antiserum to antigen E were prepared in this laboratory according to the above schedule. Goat antiserum to Ra5-ovalbumin conjugate was obtained from Dr. David G. Marsh.

IV. Column Chromatography

Sephadex gels and ion exchangers (Pharmacia, Montreal) and Biogels (Biorad Labs., Mississauga, Ont.) were allowed to swell overnight in starting buffers and fines were removed by repeated decantations. Columns were poured with the aid of reservoirs (Pharmacia) and packed under hydrostatic heads determined by gel and resin type. Equilibration was routinely achieved by passage of starting buffers for 18 hr prior to application of the sample to the column top. All columns were run at 10°C in an LR/FL50 Puffer Hubbard Chromatography Refrigerator (Allied Scientific, Montreal). Column effluents were monitored at 280 nm with a Uvicord II (LKB Productor) equipped with a 2 mm flow cell and by $O.D._{280nm}$ measurements in a Coleman R-4 Perkin Elmer double beam spectrophotometer equipped with a Model CDR Concentration Computer (Fisher Scientific, Montreal). Protein concentrations in column effluents were expressed as

the number of $O.D._{280nm}$ units. Where necessary, especially in the developmental stages of an isolation procedure, several fractions spaced at equal distances around each protein peak of a column chromatogram were tested for the presence of antigen by immunodiffusion against specific antisera.

V. Ultrafiltration and Sterilization

All antigen solutions were concentrated to volumes ranging from 20-40 ml at 5°C in Diaflo ultrafiltration cells fitted with UM-2 membranes (Amicon Corp., Cambridge, Mass.). Solutions were sterilized by passage through 0.22 u filters (Millipore Corp., Mississauga, Ontario) into sterile flasks.

VI. Dialysis

Solutions to be applied to ion exchange columns were dialysed against starting buffer when they were of higher ionic strengths; it was otherwise not found to be necessary. Dialysis was carried out overnight with several buffer changes using Spectrapor membrane tubing (Spectrum Medical Industries, Los Angeles) with mol wt cut-offs appropriate to the allergen under study.

VII. Molecular Weight Determination

The molecular weights were determined at 20°C in a Spinco Model E ultracentrifuge equipped with UV optics scanner and recorder and employing the "overspeed" modification of the conventional sedimentation equilibrium technique (Chervenka, 1970). A solution (0.12 ml) of antigen in 0.05 M Tris-HCl, pH 7.6, or 0.03 M sodium phosphate buffer, pH 6.8, was overlayered on 0.03 ml FC-43 fluorocarbon oil (Spinco, Palo Alto, Calif.) in the sample sector of a double sector cell; 0.15 ml of the appropriate buffer was deposited in the solvent sector. Scans were taken at 12,000 rpm to ensure an $O.D._{280nm}$ in the range of 0.2 to 0.3. The rotor was oversped for 90 min at 44,000 rpm for Ra3 and 30,000 rpm for Ra4. Operating speeds of 28,000 rpm for Ra3 and 20,000 rpm for Ra4 were then maintained overnight. On the next day, 2 consecutive scans were made 1 hr apart to verify equilibrium distribution of $O.D._{280nm}$ throughout the cell. The $O.D._{280nm}$ distributions were corrected for the base-line (Chervenka, 1970). The molecular weight was calculated from equation: $M = \frac{2RT}{(1-\bar{v}\rho)\omega} 2.303 \frac{d \log c}{d (r^2)}$ where: M = molecular weight; R = gas constant = 8.313×10^7 ergs/degree mole; T = absolute temperature = 293°K; ρ = density of solution, taken as 1 gm/ml; $\bar{v}$ = partial specific volume in cc/g, calculated from the amino acid composition by the method of Cohn and Edsall (1943); ω = angular velocity (rad/sec); c = concentration ($O.D._{280nm}$); r = distance from center of rotor (cm). The slope $\frac{d \log c}{d (r^2)}$ was obtained by least square analysis of the experimental values (Dickson, 1968).

VIII. Isoelectric Focussing

Isoelectric points were determined in a 110 ml LKB preparative isoelectric focussing column (LKB Productor) thermostated at 5°C by a Haake cooling bath and connected to a Buchler Model No. 3-1014A power supply set for constant voltage.

The method generally followed the outline supplied in the LKB 8100 Ampholine Instruction Manual. The recommendations for basic proteins were followed, a glycerin rather than sucrose density gradient was employed. Solutions were pumped into the appropriate compartments of the column with a 12,000 Varioperpex peristaltic pump (Fisher Scientific Co.) in the following order: dense electrode solution, dense gradient solution, light gradient solution, light electrode solution. The composition of the solutions were as follows:

Dense electrode solution:

12.0 ml	glycerol
5.0 ml	1 N NaOH
7.7 ml	H_2O

Light electrode solution:

0.2 ml	conc. H_2SO_4
20.4 ml	H_2O

Dense gradient solution:

27.0 ml	glycerol
26.2 ml	H_2O
1.8 ml	Ampholine carrier ampholyte

Light gradient solution:

54.0 ml	H_2O containing the protein
0.9 ml	Ampholine carrier ampholyte

After 4 days at 400-600 volts, the column content was collected at 50 ml/hr by peristaltic pumping in fractions of approx 2 ml and the % transmission of the effluent was monitored at 280 nm. The pH of each fraction was measured immediately and the O.D. read manually.

IX. Polyacrylamide Gel Electrophoresis

1. Disc electrophoresis

The model 1200 Canalco Research Disc Electrophoresis apparatus connected to a 500 volt DC power supply (Gelman) by a safety interlock adaptor was used for analytical electrophoresis. Gels 12-15% in acrylamide (International Scientific, Ottawa) were polymerized in glass tubes (0.5 x 7.5 cm). Sample loads were 9 to 20 µg in protein in buffer volumes of 0.2 to 0.8 ml.

Electrophoresis at pH 4.3 was carried out with the cathodic system of Reisfeld et al. (1962) as modified by Canalco (1968). Generally, the electrophoresis time was 2.5 hr at 2 mA/tube. Gels were fixed and stained as described in part IV of this section.

2. Sodium dodecylsulfate (SDS) gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed initially by the method of Weber and Osborne (1969), utilizing the following components (per 100 ml):

a. Gel Buffer pH 7.0

0.78 g $NaH_2PO_4 \times H_2O$

3.86 g $Na_2HPO_4 \times 7\ H_2O$

0.2 g SDS

b. Acrylamide Solution

22.2 g acrylamide

0.6 g methylene bisacrylamide

c. Catalyst

1.5 g ammonium persulfate

d. Tracking dye

0.05 g bromphenol blue

To form a working solution for 12 gels, 15.0 ml of gel buffer were mixed with 13.5 ml of acrylamide solution, 1.5 ml of ammonium persulfate and 0.045 ml of N,N,N',N'-tetramethylene diamine. To make 1 l of tray buffer, 500 ml of gel buffer were diluted 1:2 with water.

Electrophoresis was carried out for 4 hr at 8 mA/tube.

In more recent analysis by SDS gel electrophoresis, the method of Wachsmeldt et al. (1971) was used with minor modifications. The following solutions were employed, each made up to 100 ml with dist H_2O:

a. Gel Buffer pH 8.9

48 ml 1N HCl

36.6 g Tris

0.46 ml N,N,N',N'-tetra methylene-diamine

b. Acrylamide solution

48 g acrylamide

0.64 g N,N' methylene bisacrylamide

c. Catalyst

140 mg ammonium persulfate

d. SDS solution

0.8 g SDS

e. Tracking dye

0.15 g bromphenol blue

The working solution contained 1 part gel buffer, 1 part SDS solution, 2 parts acrylamide solution, and 4 of catalyst.

The tray buffers contained in 1000 ml:

Upper buffer (pH 8.9)	Lower buffer (pH 8.1)
6.32 g Tris	12.1 g Tris
3.94 g glycine	50 ml 1N HCl
1.0 g SDS	1.0 g SDS

The working solution and tray buffers were made 0.1% in dithiothreitol (Nutritional Biochemicals Corp., Cleveland, Ohio).

Electrophoresis was carried out for approx 1 hr at 2-3 mA/tube, by which time the tracking dye had travelled 4.8 cm.

3. Preparative electrophoresis

Preparative polyacrylamide gel electrophoresis was performed in a Kontes K565000 Preparative Electrophoresis Apparatus (Canadian Scientific Company, Montreal) with a 125 ml capacity gel chamber. The cathodic pH 4.3 system of Reisfeld et al. (1962) was employed as for the analytical runs. All steps were carried out in a 5°C coldroom. To form the gel, the lower end of the gel chamber was sealed off by securing a double layer of parafilm over the end. A solution of a 20% acrylamide (12 ml) was added to the chamber and allowed to polymerize to form a plug. A 10% running gel solution (60 ml) was added to the chamber, allowed to polymerize, and 1 hr later a solution of 9-10 mg protein in 20% sucrose was layered beneath the upper buffer. The column was connected to a 500V power supply (Gelman) and electrophoresis was carried out at 10 mA for 40 hr.

The gel column was removed from the chamber by rimming with a 2", 22 gauge needle under a stream of water. A 0.5 cm longitudinal guide strip was cut with the aid of a gel slicer (Kontes K565050) and incubated with the Coomassie blue stain of Koening et al. (1970) for 5-10 min (see part IV of this section). Gel segments containing protein were macerated, stirred with 10 vol buffer overnight and the supernatant collected by centrifugation.

4. Staining of polyacrylamide gels

i. Protein bands were fixed for 1 hr in 1% sulfosalicylate/5% trichloroacetic acid (TCA) and subsequently washed 3 x with cold tap water and 3 x with dist H_2O. Gels were stained for 2 hr with 0.5% Coomassie blue in 7% acetic acid/10% TCA. Background stain was removed by several washes with 7% acetic acid; gels were stored in the same solution.

ii. Fixing and staining of protein bands in a single solution was carried out using the method of Koening et al. (1970). A 0.025% solution of Coomassie Brilliant Blue (Schwarz-Mann, Orangeburg, N.Y.) was prepared with 10% TCA in acetic acid:methanol:water, 14:40:160 and incubated overnight with acrylamide gels and SDS acrylamide gels. Background stain was washed out with acetic acid:methanol:water, 14:40:160.

iii. Macromolecular carbohydrate was detected in polyacrylamide gels by a modification of the PAS stain described by Kapitany and Zebrowski (1973). The gels were incubated for 1 hr in 12.5% TCA and 2 hr in 1% periodic acid. They were placed in capped but perforated polyethylene tubes, stirred in 4 l of 1% acetic acid for 2 hr to remove the periodic acid, and finally incubated in Schiff's reagent (British Drug Co., Toronto) for 2 hr at 5°C in the dark. Background stain was removed in the dark by washing in 7% acetic acid.

X. Carbohydrate Analysis

Total carbohydrate was determined by the tryptophane method (Kabat and Mayer, 1961). Standard solutions were prepared containing 10 - 50 µg of arabinose (British Drug Co., Toronto). Proteins to be assayed were in solution in Tris or phosphate column buffers given in Part C, Experiments and Results. Reagent blanks were prepared containing the same buffer. Aliquots of 7 ml of 77% H_2SO_4 were added to 1 ml of standard and sample solutions at 15°C. One ml portions of 1% tryptophane (British Drug Co., Toronto) were added followed by vortexing, and the solutions were heated for 20 min in a boiling water bath. The $O.D._{500nm}$ were read after cooling at rm temp for 0.5 hr.

XI. Amino Acid Analysis

The amino acid compositions were determined in a Spinco Model 120B Amino Acid Analyzer, according to the method of Moore, Spackman and Stein(1958). The amino acid mixture (Spinco) employed for standardization was diluted to 0.1 uM of each amino acid.

Antigens were precipitated from their buffer solutions by addition of acetone (1:20,v:v), redissolved in H_2O, and reprecipitated with acetone. The samples were dried overnight in vacuo in dessicator and subsequently dissolved in double distilled 6N HCl (A & C American Chemicals, Montreal). Tubes were sealed in vacuo ($\angle$ 70 µ)

and hydrolyzed for 24 hr and, where feasible, for 48 and 72 hr at 110 ± 1°C in a Freas oven (Precision Scientific). Hydrolysates were filtered through 0.45 μ Millipore filters, dried by rotary evaporation and dissolved in 2.3 ml of 0.2 N sodium citrate buffer, pH 2.19. One ml aliquots of the mixture were applied to the short and long columns of the Analyzer. The number of residues per mole protein was calculated from the micromolar composition, assuming 100% recovery of the amino acids.

Cystine/2 was assayed as cysteic acid and as carboxymethyl cysteine in acid hydrolysates of proteins after performic acid oxidation, and after reduction and carboxymethylation.

Tryptophane was determined by the spectrophotometric method of Beaven and Holiday (1952).

The molar ratio of tyrosine to tryptophane was calculated from the formula:

$$\frac{M_{Tyr}}{M_{Try}} = \frac{0.592\ O.D._{294nm} - 0.263\ O.D._{280nm}}{0.263\ O.D._{280nm} - 0.170\ O.D._{294nm}}$$

XII. Performic Acid Oxidation (Blackburn, 1968)

Prior to oxidation, 2 mg of antigen Ra3 was precipitated from buffer solution by addition of 20 vol acetone (x 2) and the precipitated protein dried in vacuo. The protein was redissolved in 1.9 ml of 99+% formic acid and the solution brought to 5°C. An aliquot of 0.1 ml

performic acid was added, the glass container stoppered and kept in an ice bath for 4 hr. A volume of 0.3 ml of 48% hydrogen bromide was added and the solution rotary evaporated at 40°C. The dried residue was maintained overnight in vacuo over NaOH pellets to ensure complete removal of HBr. The amino acid composition was determined on duplicate portions of the dried sample.

XIII. Sulfhydryl Assay

The sulfhydryl content was determined by:

1. Method of Ellman (1959).

A solution was prepared of 39.6 mg of 5,5'-Dithio-bis-2-nitrobenzoic acid (DTNB)(Pierce Chemical Co., Rockford, Ill.) in 10 ml of phosphate buffer, u = 1, pH 7.0. Prior to assay, proteins were dialyzed against 0.02 M phosphate buffer pH 7.8. An aliquot of 3 ml of dialyzed solution was placed in a 1 cm quartz cuvette, 0.02 ml of DTNB solution added, and the $O.D._{412nm}$ determined after 5 min. Moles of sulfhydryl (C) were calculated by the formula $C = \frac{A}{E} + D$ where

A = absorbance at 412 nm

E = extinction coefficient = 13,600/M/cm

D = dilution factor

2. Method of Rohrbach et al.(1973)

A stock solution was prepared of 6-7 mg of 4,4'-bis-dimethylamino diphenylcarbinol (BDC-OH) (ICN - K & K Laboratories, Cleveland) in 10 ml acetone. Aliquots of 10 ul were added to protein solutions (2-20 uM) prepared with 0.04 M sodium acetate, pH 5.1, containing 4.0 M guanidine hydrochloride. The absorbance at 612 nm was determined 20 min after the addition of BDC-OH.

The protein concentration was plotted against $O.D._{612nm}$ and the number of sulfhydryls calculated from -slope/E_m where E_m = apparent molar absorption coefficient for BDC^+ = 70,800 $M^{-1}cm^{-1}$.

XIV. Reduction and Alkylation

i. One of the procedures employed was a variation of that described by Waxdal et al. (1968).

Six mg of antigen Ra3 were dissolved in a solution of 3 ml of 0.01 M sodium phosphate buffer, pH 7.2, containing 1% SDS and 4 mg of DTT. The solution was maintained at 50°C for 4 hr, cooled to rm temp, and made 0.3 mM in iodoacetamide. After 20 min in the dark, the mixture was applied to a column of Sephadex G-50s (see Part C. Experiments and Results).

ii. For disc electrophoretic analysis, proteins were reduced according to the procedure of Virella and Parkhouse (1973) with minor modifications.

One mg of protein was incubated for 3 hr at 37°C in 0.2 M Tris, pH 8.2, made 1% in SDS and 1% in dithiothreitol (DTT). Aliquots of 0.04 ml of this mixture were subsequently applied to polyacrylamide gels (see Part C. Experiments and Results).

XV. N-Terminal Amino Acid Determination

The N-terminal amino acid of antigen Ra3 was determined by Edman degradation (Edman, 1970).

A sample of 1 mg of the antigen was dissolved in 1 ml pyridine:water, 1:1, made 0.4 M in N-dimethylallylamine. The pH was adjusted to 9 with dilute aqueous trifluoroacetic acid, 50 µl of phenylisothiocyanate (PITC) was added, the tube was flushed with N_2, capped, and allowed to stand 1 hr at 40°C. The solution was extracted x 3 with 2 ml aliquots of benzene and the aqueous phase lyophilized. The dry PTC preparation was dissolved in 100 µl of anhydrous trifluoroacetic acid and incubated at 40°C for 15 min. The degraded protein was precipitated (x 2) with 3 ml aliquots of ethylene chloride and the combined ethylene chloride extracts were evaporated to dryness in a stream of N_2. To the dry sample of thiazolinone, 0.2 ml of 1 N HCl was added, the tube flushed with N_2, and heated at 80°C for 10 min. The PTH amino acid was extracted (x 3) with 1 ml aliquots of ethylacetate and the combined extracts dried in a jet of N_2, and the residue dissolved in 200 ul of ethylene chloride.

The N-terminal amino acid was identified by thin-layer chromatography on preformed Silica gel G plates (Eastman-Kodak, Montreal). Aliquots (50 ul) of PTH-standards (Pierce Chemical Co.) and samples were applied to the bottom of 3 plates which were separately run in D, E, and H solvent systems (Edman, 1970). After 40-50 min, the plates were dried at 100°C for 15 min and the spots located by UV light for Rf determination.

XVI. Amino Acid Sequence Determination

Amino acid sequences were determined by Dr. J. Donald Capra (Southwestern Medical School, Dallas, Texas) and his associates using the automated Edman degradation method in a Beckman Model 890 sequenator. The phenylthiohydantoin derivatives were identified by thin layer and liquid-gas chromatography (Mole and al., 1975).

XVII. Immunological Methods

1. Immunodiffusion

Double diffusion in agar (Ouchterlony technique (Ouchterlony, 1958)) was done in Hyland immunoplates (patterns B and C), using enlarged wells where necessary. Precipitin lines were developed overnight by incubating in a humid atmosphere at rm temp.

2. Human leucocyte histamine release assay

The assays were performed by Dr. L. M. Lichtenstein (Good Samaritan Hospital, Baltimore, Md), using a modification (May, 1970) of the procedure of Lichtenstein and Osler (1964). The allergen specificity of histamine release in the direct assay was tested by inhibition with specific antisera (Lichtenstein and Osler, 1966).

3. Direct skin test

The tests were performed in the allergy clinic of the Royal Victoria Hospital, Montreal. Ragweed allergic patients were injected intradermally with 0.025 ml of allergen in 0.01 M sodium phosphate buffered physiological saline (PBS). Reactions were read 15 min after injection and graded according to wheal size compared to PBS controls.

Skin puncture tests were performed by Dr. John Santilli on allergic patients at Scott Airforce Base, Illinois. The skin surface was scratched with a scarifier and 0.02 ml of allergen solution (30 ug/ml) applied simultaneously at different sites. Reactions were graded 1+ to 4+ according to average wheal diameters.

PART C

EXPERIMENTS AND RESULTS

I. Isolation of the 2 Major Forms of Antigen Ra3

In our previous studies (Roebber, 1970) a method of isolation of antigen Ra3 was developed that incorporated CM-cellulose ion exchange as a major purification step. However, in follow-up experiments, the CM-cellulose step proved to be a source of large variation in yield of the antigen and was therefore replaced by the chromatographic procedure employed by Underdown and Goodfriend (1969), except for the use of the G-50 rather than G-100 type of Sephadex, DEAE-Sephadex in place of DEAE-cellulose and altered buffer elution conditions. A flowsheet of the entire isolation procedure is given in Fig. 1. The final step of gel filtration through Sephadex G-50s permitted isolation of the 2 major forms of antigens Ra3 (I and II) on a preparative scale.

1. Fraction C-III

An aliquot of 25 ml of fraction C (King et al., 1964; Underdown and Goodfriend, 1969) equivalent to 125 g of ragweed pollen was applied to a column of Sephadex G-50m (5 x 95 cm). The chromatogram was developed with 0.05 M Tris-HCl, pH 7.6, at a flowrate of 60 ml/hr and the eluate collected in 20 ml fractions.

As shown in Fig. 2, 5 clearly defined peaks (C-I to C-V) were obtained, of which the third (C-III) contained all the Ra3 as determined by immunodiffusion against specific rabbit antiserum.

Fraction C-III (180 ml) was concentrated 10-fold* and equilibrated with 0.001 M Tris-HCl, pH 7.6, by passage through a column of Biogel P-2 (2.5 x 95 cm). The Ra3-containing fraction was collected in an effluent volume of approx. 200 ml.

Three additional aliquots of fraction C were separately processed as above and the derivative C-III fractions combined; the C-III pool (corresponding to 500 g pollen) was concentrated to 35 ml.

2. Fraction C-III-2

Fraction C-III (35 ml) was applied to a column of DEAE-Sephadex A-50 (5 x 25 cm) and the chromatogram developed with 0.001 M Tris-HCl, pH 7.6, at a flow of 120 ml/hr. Fig. 3 shows the eluate O.D. profile generally obtained. Ra3 was localized to the second peak (C-III-2) which contained approx. 60 O.D. units in a 170 ml volume. The fraction was found to contain 2 components on disc electrophoresis (Fig. 4).

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*Here, as throughout this study, protein solutions were concentrated by ultrafiltration at 5°C in Diaflo cells which were equipped with UM-2 membranes (see Part B, Materials and Methods).

3. Fractions C-III-2a and -2b

Fraction C-III-2 (170 ml) was concentrated to 20 ml and applied to a column of Sephadex G-50s (5 x 95 cm) equilibrated with 0.025 M Tris-HCl, pH 7.6. The chromatogram was developed with the same buffer at a flowrate of 20 ml/hr and the eluate collected in 7 ml fractions (Fig. 5A to D). As shown in the figure for this experiment, C-III-2 was resolved into peak and shoulder fractions (C-III-2a and C-III-2b), (Fig. 5A), although in later runs 2 well separated peak fractions could be obtained. Fractions 2a and 2b in the run of Figs. 5A and 5B contained 40 and 12 O.D. units, respectively; the fractions were Millipore filtered and stored at 5°C.

Fractions C-III-2a and -2b derived from a total of 1 kg pollen were separately pooled and recycled (x 2) through Sephadex G-50s as described above.

The final yields of disc electrophoretically pure C-III-2a and C-III-2b (Fig. 5C and D) were approx 40 and 10 O.D. units, respectively. Both fractions gave precipitin arcs in a reaction of identity with rabbit anti-Ra3 antiserum and rabbit antiserum to WSR (Fig. 6 and 7). In addition to this finding, the results to be given subsequently of physicochemical and chemical analyses of the 2 fractions demonstrated that they represented 2 forms of the antigen, Ra3-I and Ra3-II, respectively.

II. Development of a More Integrated Method of Isolation of Antigens Ra3, Ra4 and Ra5

As indicated in the Introductory Part of this Thesis, isolation of the allergenically active proteins Ra3, Ra4 and Ra5 from WSR provided 3 potentially useful models for structure-function studies, each having a special interest. However, the methodology of isolation of each allergen had developed more or less independently of the others, utilizing different chromatographic sequences and start-up chromatographic fractions. It became evident that, if extensive structure-function studies on the allergens were to be developed simultaneously, a more integrated and rationalized method for their isolation would be desirable from the standpoint of speed and economy.

An additional stimulus to the development of a more efficacious method was the finding by Marsh (1973) that aqueous short ragweed pollen extract contained 320-350 mg Ra5 per k pollen, a surprising result in view of the relatively low amounts of Ra5 (10-15 mg/kg pollen) obtained on first isolation of the antigen in this laboratory (Lapkoff and Goodfriend, 1971). As the extract assayed by Marsh was prepared without the usual preliminary ether extraction of the pollen, a method of isolation of Ra5 was developed utilizing non-ether extracted pollen as start-up material (Roebber et al, 1975).

The method was extended to include the sequential isolation of antigens Ra3 and Ra4 and the entire procedure is schematized in Fig. 8. Major elements of the procedure are: the use of non-defatted short ragweed pollen for preparation of the aqueous extract; the use of DEAE-Sephadex for partial depigmentation of the aqueous extract; molecular sieving of the partially depigmented extract through Biogel P-10 to obtain partially purified fractions of antigens Ra4, Ra3 and Ra5 (in order of their elution); work up of the latter 3 fractions to final purification by ion exchange and molecular sieve chromatography.

1. Aqueous pollen extract (WSR)

A batch of 200 g of short ragweed pollen was stirred in 1.6 l of a solution consisting of 1 part borate buffer (6.2 g boric acid, 9.5 g sodium tetraborate, 4.3 g of sodium chloride), pH 8.4, and 39 parts of 0.9% saline for 20 hr at 5°C. The extract was separated from the pollen by centrifugation in an IEC B-20 centrifuge (rotor 874) for 15 min at 10,000 rpm. The water soluble ragweed (WSR) was filtered successively through Millipore filters of 14 μ, 1.2 μ, 0.45 μ, and 0.22 μ and concentrated to 300 ml.

2. Partially depigmented WSR

The WSR was divided into 2- 150 ml portions and the latter

were simultaneously passed through 2 columns (2.5 x 30 cm) of DEAE-Sephadex A-25. The chromatograms were developed with 0.05 M Tris-HCl, pH 8.0, at a flow of 80 ml/hr. An effluent volume of 400 ml collected from each column contained all of antigens Ra3, 4 and 5 (as well as antigen E) detectable by immunodiffusion test. The 2- 400 ml fractions were pooled and concentrated to 100 ml.

3. Fractions II, III and VI

The partially depigmented WSR was applied in 2 equal portions (of 50 ml) to 2 columns (5 x 90 cm) of Biogel P-10 (100-200 mesh). The chromatograms were developed with 0.05 M Tris-HCl, pH 8.0, at a flow of 60 ml/hr and the eluates collected in 20 ml fractions. A complex but reproducible pattern of 8 major eluate peaks was observed as shown for one of the chromatographic runs in Fig. 9. By immunodiffusion tests done on the eluate with specific animal antisera, antigens E, Ra4, Ra3 and Ra5 were eluted (in order of their size) in peaks 1, 2, 3, and 6, respectively. There was some overlap between Ra4 and Ra3; in addition, the distribution of Ra3 coincided with the descending branch and shoulder of peak 3. In the run for Fig. 9, the eluate volumes were: for Ra4, 275 ml; Ra3, 180 ml; Ra5, 150 ml.

The corresponding fractions (see Fig. 9) from the 2

P-10 columns were pooled, concentrated to approx. 50 ml, Millipore filtered, and stored at 5°C. The entire procedure was repeated to accumulate fractions II, III and VI corresponding to 400 g pollen. These fractions served as start-up material for isolation of antigens Ra4, Ra3 and Ra5, respectively.

4. Antigen Ra4

i. Fraction II-2

Fraction II (100 ml) was applied to a column (2.5 x 30 cm) of CM Sephadex C-50 and the chromatogram developed with 0.05 M phosphate buffer, pH 6.8, at a flow of 30 ml/hr. After the first unretarded, strongly pigmented fraction was eluted (fraction II-1, Fig. 10) a linear gradient of 0 - 0.5 M NaCl formed in 2 l of the same buffer eluted several peak fractions. As some 3 to 4 of the latter gave precipitin arcs with rabbit antiserum to Ra4, the entire material eluted by the salt gradient was pooled and the pool (fraction II-2) concentrated to 30 ml for further work-up. In later runs the salt gradient was replaced by a single chromatographic step using 0.35 M NaCl in the column buffer (Fig. 10).

ii. Fraction II-2A

Fraction II-2 (30 ml) was divided into 3- 10 ml aliquots and each aliquot cycled through a column (2.5 x 90 cm) of Sephadex G-75. The chromatogram was developed with 0.05 M Tris-HCl, pH 7.6, containing

0.2 M $(NH_4)_2 SO_4$ at a flow of 15 ml/hr and the eluate collected in 7.5 ml fractions. Three virtually identical chromatographic patterns were obtained, each showing a large (Ra4) peak with the suggestion of a shoulder in the ascending branch and a smaller peak on the descending side (Fig. 11a). Surprisingly, Ra4 was detected mainly in the descending branch of the main peak. Cuts, each containing 15 O.D. units in 25-30 ml, were made as shown in the figure and pooled; the pool was concentrated to 10 ml and re-run on the same column. A single homogeneous peak was eluted and a peak fraction (II-2A) of 32 O.D. units in approximately 70 ml was derived (see Fig. 11b). The fraction, which was almost colorless, contained more than 100% carbohydrate (assuming $E^{1\text{ cm, }1\text{ mg}}_{280\text{ nm}} = 1.0$ for Ra4).

The carbohydrate content of fraction II-2A was drastically reduced by precipitation with ammonium sulfate. An aliquot (14 ml; 13 O.D. units) of fraction II-2A in 0.05 M Tris-HCl, pH 7.6, was brought to 60% saturation at 4-5°C by the addition of sat. $(NH_4)_2SO_4$ prepared in the same buffer. After centrifugation, the precipitate was dissolved in 8 ml of the Tris buffer and dialyzed overnight at 5°C against the same buffer. After clarification by Millipore filtration, 7 O.D. units were recovered. The Ra4 preparation contained 10% carbohydrate, assuming $E^{1\text{ cm, }1\text{ mg}}_{280\text{ nm}} = 1.0$.

A duplicate aliquot (14 ml) of fraction II-2A was brought to 60% saturation as above, the precipitate dissolved in 14 ml of the Tris buffer, reprecipitated with $(NH_4)_2SO_4$, and dialyzed as above. The Ra4 preparation obtained in this way contained 4.2 O.D. units and 5.6% carbohydrate. The yield from the singly and doubly precipitated Ra4 preparations were combined (approx. 11 O.D. units) for cycling through CM-Sephadex.

A solution of ammonium sulfate treated fraction II-2A containing 11 O.D. units in 7 ml of 0.05 M Tris-HCl, pH 7.6, was applied to a column (1.5 x 27 cm) of CM-Sephadex. The chromatogram was developed with 0.05 M phosphate buffer, pH 6.8 (Fig. 12). After a small unretarded peak was eluted with the starting buffer, a linear gradient of 0 - 0.5 M NaCl in 400 ml of the same buffer eluted a single, somewhat spread peak containing 9 O.D. units of Ra4 in 100 ml. The Ra4 peak fraction was concentrated to 20 ml and Millipore filtered; the colorless solution was stored at 5°C.

5. Antigen Ra3

i. Fraction III-2

Biogel P-10 fraction III was concentrated to 50 ml, dialyzed exhaustively against 0.001 M Tris-HCl, pH 7.8, and applied to a column (2.5 x 30 cm) of DEAE-Sephadex. The chromatogram was developed with the same buffer at a flowrate of 60 ml/hr and the eluate collected in

20 ml fractions. A large peak was eluted with a prominent shoulder on the ascending side (Fig. 13a). Ra3 was located mainly in the descending branch of the peak. A cut of 160 ml was made as shown in the figure, concentrated to 50 ml and recycled through DEAE-Sephadex under the same chromatographic conditions. Two well-separated peaks were eluted, of which the second (III-2) contained all the Ra3 (80 O.D. units) in 2 disc electrophoretic forms (Fig. 13b).

ii. Ra3-I and II

Fraction III-2 was concentrated from 200 to 30 ml and sieved through Sephadex G-50s. The chromatogram was developed with 0.025 M Tris-HCl, pH 7.6, at a flowrate of 25 ml/hr. Two peak fractions were eluted (Fig. 14a) corresponding to Ra3-I and II in a total yield of 75 O.D. units. The Ra3-I cut was recycled twice on the same column to obtain a homogeneous peak containing disc electrophoretically pure Ra3-I (26 O.D. units) (Fig. 14b). The solution was concentrated to 10 ml, Millipore filtered, and stored at 5°C.

The Ra3-II peak fraction was not further purified; it was concentrated to 20 ml, Millipore filtered, and stored at 5°C.

6. Antigen Ra5

i. Fraction VI-2

Ra5-containing fraction VI (150 ml) was concentrated to 50 ml

and applied to a column (2.5 x 30 cm) of CM-Sephadex C-25. The chromatogram was developed with 0.04 M sodium phosphate buffer, pH 6.8. Two well separated fractions were eluted (Fig. 15), the second of which contained almost pure Ra5. Fraction VI-2 (240 ml) was concentrated to 50 ml and recycled through CM-Sephadex as previously. A single homogeneous peak was eluted; the colorless peak fraction (100 O.D. units) was concentrated to 25 ml.

Fraction VI-2 (25 ml) was applied to a column (2.5 x 85 cm) of Biogel P-10 (200-400 mesh). The chromatogram was developed with 0.025 M Tris-HCl, pH 7.6, at a flow of 12 ml/hr and the eluate collected in 6 ml fractions. Antigen Ra5 was eluted as a sharp peak (Fig. 16). To remove trace contaminants, 10 ml aliquots of the Biogel P-10 fraction, each containing approx 30 O.D. units, were cycled (x 3) through a column (2.5 x 90 cm) of Biogel P-4. The chromatograms were developed with the 0.025 M Tris at a flow of approx 25 ml/hr (Fig. 17). The final yield of Ra5 was 75 mg/kg pollen (based on $E^{1\text{ cm, }1\text{ mg}}_{280_{nm}} = 2.36$ (Goodfriend and Lapkoff, 1972).

III. Chemical and Immunological Properties of Antigens Ra3, Ra4, and Ra5

The isolation of antigens Ra3, Ra4, and Ra5 from the aqueous extract of short ragweed pollen was described in the previous section. Purification was continuously monitored by acrylamide gel disc electrophoresis until, as a minimal requirement, the antigen preparations displayed single bands in an acidic buffer system (Figs 4, 18, 19). Additional data bearing on homogeneity were concomitantly obtained in the course of chemical and immunological examination of the antigens and will be summarized in part D, III, 1.

The data obtained for antigens Ra3 and Ra4 relate to their molecular size and charge, carbohydrate content, amino acid composition, polypeptide chain structure, and antigenic and allergenic activity. Chemical and immunological properties of antigen Ra5 were described by Lapkoff and Goodfriend (1974). In the present study, a few additional parameters were examined, viz., the isoelectric point, free sulfhydryl content, and chain structure.

In collaborative studies between this laboratory and that of Dr. J. Donald Capra, the complete amino acid sequence of antigen Ra5 (Fig. 20) was recently determined (Mole et al., 1975). In continuation of these studies, partial sequences have to date been obtained of the N-terminal region of antigens Ra3 and Ra4 and are presented in this section.

1. Antigen Ra3

i. Molecular weight

The mol wts of the 2 forms of antigen Ra3 were determined by analytical equilibrium ultracentrifugation.

A partial specific volume of 0.731 was calculated from the amino acid composition (Tables I, II) and adjusted to 0.722 to take into account an 8% carbohydrate content (Gottschalk, 1966). The values for the slope of log c vs r^2 for Ra3-I from duplicate runs was calculated by least square analysis to be 0.251 and 0.260 (Fig. 21). Substitution in the standard equation gave mol wts of 11,900 and 12,300 daltons corresponding to the 2 slopes respectively. The average mol wt was therefore 12,000 daltons with 2.5% experimental uncertainty. A single analytical run gave a slope of 0.260 for Ra3-II (Fig. 22); the corresponding mol wt was 12,300 daltons. Thus, no difference in molecular size between the 2 forms of Ra3 was detected.

ii. Isoelectric point

A solution of 10 mg Ra3-I in dist H_2O was incorporated in a glycerol density gradient together with 2.5 ml of pH 7-9 carrier ampholytes with the aid of a gradient mixer. The solution was pumped into a 110 ml electrofocussing column and 600 volts were applied across the column for 4 days. The column contents were collected in 2 ml fractions, the effluent was continuously monitored for % T at 280 nm but the pH's of

the 2 ml fractions were separately determined. As shown in Fig. 23, Ra3-I eluted as a sharp symmetrical peak with an isoelectric pH of 8.6.

iii. Carbohydrate content

Variable and relatively high carbohydrate contents were found for Ra3 during the course of this study. Evidence was obtained from the following experiments that the carbohydrate was present largely, if not entirely, as a contaminant and not as an integral part of the molecule.

a. A sample of Ra3-I was cycled through a column (2.5 x 90 cm) of Biogel P-30 using 0.025 M Tris-HCl, pH 7.6, for development. The eluate fractions were assayed for protein by O.D. (280 nm) measurements and for carbohydrate, by the tryptophan method. The protein and carbohydrate peaks did not coincide (Fig. 24a). The cut (c) shown in the figure was cycled through the same column (Fig. 24b) and a similar cut of the eluate peak cycled as previously (Fig. 24c). On the 3rd cycle, protein and carbohydrate peaks almost coincided. The final Ra3-I preparation contained 7% carbohydrate, while a pool of cuts (d) of the descending branches contained 20%. Amino acid analysis of the 2 preparations showed no significant differences in composition (Table III).

b. Antigen Ra3 (30 mg) obtained by DEAE-Sephadex chromatography was sieved through a column (5 x 90 cm) of Sephadex G-50s, using 0.025 M Tris-HCl, pH 7.6, for development. Assays done as above on the eluate fractions showed a marked dissociation between the carbohydrate and protein eluate profiles (Fig. 25).

c. An experiment was done to determine if and to what extent carbohydrate could be removed from Ra3 by preparative polyacrylamide gel electrophoresis. A sample (10 mg) of Ra3 obtained from the DEAE-Sephadex column chromatographic step contained both forms of the allergen and 10% carbohydrate. The sample was run for 40 hr on a polyacrylamide column in the pH 4.3 system. A vertically cut strip of the column was stained for 10 min in Coomassie blue and showed 2 separate but diffuse bands (3.8 and 5.1 cm from the top of a 12 cm column). Using the stained strip as guide, the 2 unstained bands were separately sliced, fragmented and separately stirred overnight in 10 ml volumes of 0.1 M Tris-HCl, pH 7.6. The suspensions were filtered and the clear supernatants concentrated to 2 ml by analytical polyacrylamide disc electrophoresis at pH 4.3, 80-90% of the upper band preparation consisted of Ra3-I, the remainder, Ra3-II, and vice versa for the lower band (Fig. 26).

Protein yield was determined from O.D. (280nm) readings against the Tris buffer blank. (Previous determinations had demonstrated that essentially no UV absorbing materials were leached from protein-free regions of acrylamide gel.). The yield of Ra3-I (with some II) from 10 mg starting material was 0.70 mg, the yield of Ra3-II (with some I), 0.42 mg. Analysis on duplicate aliquots of each allergen solution (Table IV) showed the continued presence of carbohydrate, but at a markedly reduced level of 3% for both Ra3 forms.

iv. Amino acid composition

The amino acid composition of Ra3-I was determined on 3 aliquots of 0.5 mg each separately subjected to 24, 48 and 72 hr acid hydrolysis. The number of amino acid residues was found by calculating the number of moles of each residue per 11,000 g of protein (Table II). All of the usual amino acids were found to be present, except methionine, for a total of 101 residues.

Performic acid oxidized Ra3-I did not show an increase in cystine plus cysteine (determined as cysteic acid) compared to unoxidized Ra3-I (Table V). Thus, 3 cys/2 residues was obtained in both cases. However, analysis of reduced and alkylated Ra3 (see below) showed the presence of 4 carboxymethylcysteine residues.

The amino acid compositions of Ra3-I and Ra3-II were compared using 24 hr acid hydrolysates of the respective proteins. The number of residues were virtually identical except for a few of the amino acids, e.g., proline and glycine. The residue differences appeared to lie within the error of the analytical method (Table VI).

v. Free sulfhydryl

Free sulfhydryl content of Ra3-I was determined by 2 colorimetric methods: that of (a) Ellman (1959), using the reagent DTNB, and (b) Rohrbach et al. (1972), using BDC-OH.

a. From the O.D. reading (Table VII), the -SH content for BSA was calculated to be 0.64 M/M protein, in close agreement with literature values (Putnam, 1965). A value of 0.11 M/M protein was found for Ra3, a result that was probably due to the slight turbidity which developed in the Ra3 solution following reaction with the colorimetric reagent.

b. The free sulfhydryl content of BSA was 0.57 M/M protein (Fig. 27, Table VIII), in reasonable agreement with the previous assay. Free sulfhydryl content of Ra3-I was determined at allergen concentrations ranging from 2-10 nM/ml. There was no decrease in absorbancy at 612 nm with increase in protein concentration (Table VIII), i.e., free sulfhydryl was not detected.

vi. Polypeptide chain structure

The number of constituent polypeptide chains of Ra3-I was determined by SDS-acrylamide gel disc electrophoresis after reduction and alkylation of the protein.

a. Preliminarily, 6 mg of Ra3-I in 5 ml 0.05 M Tris-HCl, pH 7.6, was recycled through a column (2.5 x 90 cm) of Sephadex G-50s (Fig. 28). The chromatogram was developed with 0.01 M sodium phosphate buffer, pH 7.1. The antigen was recovered essentially quantitatively in a homogeneous peak at an elution volume of 266 ml.

The Ra3 solution was concentrated to 3 ml, made 1% in SDS, and incubated with 4 mg of DTT for 4 hr at 50°C. It was subsequently cooled to rm temp, made 0.3 M in iodoacetamide and allowed to stand for 30 min.

The reaction mixture was sieved through Sephadex G-50s as above, except for the inclusion of 0.1% SDS in the equilibrating and eluting buffer. Ra3 was eluted essentially quantitatively as a sharp peak at an elution volume of 190 ml along with 2 subsequent minor peaks (Fig. 28).

Samples of the major (Ra3) and minor peaks were examined at a sample load of 10 and 20 ug in the SDS-polyacrylamide gel system of Weber and Osborn (1969). On staining of the gels with Coomassie blue, the major peak showed a heavy band along with a sharp faint band at the top and bottom of the acrylamide gel column (Fig. 28). The 2 minor peaks did not appear as protein staining bands at the concentration examined.

To determine the extent of reduction and alkylation of the Ra3 preparation, the Sephadex G-50s peak fraction was dialyzed overnight at 5°C against 0.05 M Tris-HCl buffer, pH 7.6, concentrated to 2 ml, precipitated (x 2) with acetone, redissolved in dist H_2O, and an appropriate aliquot analysed for amino acid composition. The results (Table IX) showed the presence of 4 carboxymethylcysteine residues, i.e., 1 residue higher than the number of half-cystines established by amino acid analysis of unoxidized or performic acid oxidized Ra3.

b. Additional evidence concerning the chain structure of Ra3 was obtained as follows. An aliquot (1 ml) of the same (initial) solution of Ra3 employed for the reduction and alkylation experiments described above was reduced with DTT according to Virella and Parkhouse (1973).

Reduced Ra3 was subjected to polyacrylamide gel disc electrophoresis using the basic pH-SDS system of Wachneldt (1971). A sample (1 mg) of antigen Ra5 reduced in the same manner and a reagent blank were run simultaneously. As shown in Fig. 29, a single major band was obtained for Ra3 and Ra5, along with several minor bands which were, however, present in the electropherogram of the reagent control. The migration distances of the major bands were 4.1 and 4.5 cm for Ra3 and Ra5, respectively. As the tracer (bromphenol blue) incorporated in the runs migrated a distance of 4.8 cm, the possibility was precluded that additional band(s) may have migrated out of the gel.

vii. N-Terminal amino acid

The N-terminal amino acid of antigen Ra3-I was found to be glycine. No other amino acid was detected.

viii. N-Terminal amino acid sequence

Studies are in progress in collaboration with Dr. J. Donald Capra and his associates on the complete amino acid sequence of antigen Ra3. Studies to date, utilizing the Backman automated Sequencer have established the sequence of 25 residues in the N-terminal region of Ra3-I (following reduction of the antigen with 2-mercaptoethanol and alkylation with iodoacetamide). The sequence (Capra, J.D. Personal communication) is given in Fig. 30.

ix. Immunological properties

a. Antigenic purity and specificity of Ra3 were established by immunodiffusion tests with a number of antisera. A single precipitin arc was observed for Ra3-I and -II with rabbit antiserum to the whole aqueous extract (Fig. 7). Furthermore, the 2 forms of Ra3 gave a single precipitin arc in a reaction of complete identity with rabbit antiserum raised to Ra3-I (Fig. 6). On the other hand, the antiserum to Ra3 failed to react with antigens E, Ra4, or Ra5. Similarly, no reaction was observed between Ra3 and antisera to the latter antigens.

b. The incidence of allergenic (cutaneous) activity of Ra3 was compared with that of antigen E in patients sensitive to whole aqueous extract (Greer) of short ragweed pollen (assays performed by Dr. John Santilli). Cutaneous reactions were obtained by skin puncture tests (see Part B. Materials and Methods) at a fixed allergen concentration (30 µg/ml). The incidence of patient reactivity to the allergens is given in Table X. Some 80% of the ragweed sensitive patients reacted to antigen E, while 40% reacted to Ra3, a relative incidence similar to that reported by Underdown and Goodfriend (1969). It is noteworthy that Ra3 elicited cutaneous reactions in 4 of the 20 patients who failed to react to antigen E, emphasizing independence in allergenic activity of the 2 antigens.

c. Histamine releasing activity of Ra3 compared to antigen E was examined quantitatively by the in vitro human leucocyte technique

(collaborative studies with Dr. Lawrence M. Lichtenstein (1973)). Fig. 31 shows 3 patterns of histamine release observed when leucocytes from 23 patients were separately challenged with antigens Ra3 and E at concentrations ranging from 10^{-5} to 10^{-1} ug/ml. At one extreme were patients highly sensitive to E who showed no reactivity to Ra3, even at concentrations 10^3 to 10^4 times higher (Fig. 31a). More common were individuals who reacted to both allergens but were significantly less sensitive to Ra3 (Fig. 31b). Other patients were about equally or slightly more sensitive (3- to 10-fold) to Ra3 (Fig. 31c).

Table XI lists the concentrations of antigens Ra3 and E required for 50% histamine release. Of the 23 patients, the leucocytes of 6 did not respond to Ra3; 4 released histamine only with very high concentrations of the allergen; 8 reacted to both allergens but needed 3 to 40 times more Ra3; 4 responded more strongly to Ra3. Thus, no constant relationship was observed between sensitivity to these 2 antigens, as has been noted with cross-reacting antigens such as E and K (King et al., 1964, 1967).

Antigenic relationship was studied by determining the ability of homologous and heterologous antisera to inhibit Ra3- and E-induced leucocyte histamine release (Fig. 32). The absence of common antigenicity was implied by the finding that, in most patients, anti-E inhibited E-induced histamine release but not that caused by Ra3, while anti-Ra3 inhibited the response to Ra3 but not to E (Fig. 32a). However, in some patients unexpected patterns of inhibition were observed. Thus, anti-E

also caused inhibition of Ra3-induced histamine release (Fib. 32b) but only at antiserum concentrations 10^2 to 10^3 times higher than required to inhibit the homologous antigen. In 2 cases (Fig. 32c), anti-E inhibited E-induced histamine release and, at 10- and 50-fold higher concentrations, also blocked the response to Ra3, while anti-Ra3 was completely inactive. Anti-Ra3 did not in any case inhibit E-induced histamine release. (Discussion of the significance of these findings is reserved to a later section (D, III, 2.)).

2. Antigen Ra4

i. Molecular weight

The mol wt of a preparation of Ra4 containing 6.5% carbohydrate was determined by analytical equilibrium ultracentrifugation. The value for the slope of log c vs r^2 was calculated by least square analysis to be 0.157 (Fig. 33). The partial specific volume of 0.731 calculated from the amino acid analysis (Table XII) was adjusted for carbohydrate to 0.723. Substitution into the standard equation gave a mol wt of 22,700 daltons or 21,100 daltons for the protein moiety.

ii. Isoelectric point

A sample (7.0 O.D.$_{280nm}$ units) of Ra4 was examined in an LKB preparative electrofocussing column under the same conditions as described for Ra3-I. Using the LKB Ampholine carrier ampholytes in the range of pH 7-10, Ra4 was eluted as a sharp symmetrical peak with an isoelectric pH of 8.0 (Fig. 34).

iii. Absorption spectrum

The absorption spectrum of antigen Ra4 was determined in 0.05 M phosphate buffer, pH 6.8, from optical density readings in the wavelengths range 240-340 mu (Fig. 35). The O.D. profile was typical of proteins with peak absorption at 278 nm.

iv. Carbohydrate content

In preliminary studies, preparations of Ra4 were found to have variable and relatively high (> 30%) carbohydrate content. Repeated cycling of the preparations through molecular sieve or ion-exchange columns did not appear to reduce the carbohydrate content to any significant degree. On the other hand, the carbohydrate and protein moieties were readily dissociated on acrylamide gel disc electrophoresis at pH 4.3 (Fig. 36). However, attempts to utilize gel electrophoresis on a preparative scale for this purpose proved unsuccessful owing to an apparent deterioration (Fig. 37) and low yield of antigen.

A drastic reduction in carbohydrate content to the 5% level was achieved by precipitating twice at 0.6 saturation in ammonium sulfate as described in Section II, ii. No attempt was made to further reduce the carbohydrate content by additional precipitations, owing to diminishing recovery of antigen.

v. Amino acid composition

The amino acid composition of Ra4 (containing 6.5% carbohydrate) was determined from samples of 0.6 and 0.4 O.D.$_{280nm}$ units after 24, 48, and 72 hrs acid hydrolysis (Table XII). The number of residues was found by calculating the number of moles per 21,000 g of protein. All of the commonly present amino acids, including methionine, were found in Ra4 for a total of 189 residues. The number of tyrosine plus tryptophan residues was approximately double the number found for Ra3. In view of the

differences in mol wt of the 2 allergens, this finding suggested similar extinction coefficients and provided the basis for the assumption made in this study of an absorption coefficient of $E^{1\ cm,\ 1\ mg}_{280\ nm} = 1.0$ for Ra4.

vi. Free sulfhydryl

Free sulfhydryl content of Ra4 was determined by the method of Rohrbach et al. (1973) using BDC-OH at 2 to 7 nM/ml. The 612 nm absorbancy did not decrease with increase in protein concentration, indicating the absence of active sulfhydryl (Table VIII).

vii. Polypeptide chain structure

A sample (0.11 $O.D._{280nm}$ units) of Ra4 (6.5% carbohydrate) was incubated for 3 hr at 37°C in the medium of Virella and Parkhouse (1972) containing 1% DTT and 1% SDS. Aliquots of 0.05 to 0.1 ml were subjected to SDS-polyacrylamide gel electrophoresis in the presence of 0.1% DTT as for Ra3. Electrophoresis was stopped when the bromphenol blue marker had travelled a distance of 4.8 cm. Staining of the gels with Coomassie blue revealed a sharp major band and 3 faint bands of lower mobility (Fig. 38). The control reagent gel was devoid of detectable staining components.

An estimate of the size of the molecular species in the major band was obtained from a plot of log mol wt vs distance travelled by Ra3 and Ra5 in identical runs (Fig. 39). The mol wt, 22,900 daltons,

was in close agreement with the value derived by ultracentrifugal analysis, 22,700 daltons. Although the faint bands of lower mobility required explanation, the results were strongly indicative of a single polypeptide chain for the Ra4 molecule.

viii. N-Terminal amino acid sequence

Antigen Ra4 was degraded by the Edman procedure in a Beckman automated Sequencer, following reduction of the protein with mercaptoethanol and alkylation with iodoacetamide (collaborative studies with Dr. Capra and his associates). The sequence obtained (Capra, J. D., personal communication) for 22 residues in the N-terminal region is shown in Fig. 40. A single N-terminal acid, alanine, was found to be present, further evidence that the protein consisted of a single polypeptide chain.

ix. Immunological properties

a. Antigenic purity and specificity of Ra4 were studied by immunodiffusion with various antisera.

Single precipitin arcs were obtained in a reaction of complete identity on immunodiffusion of Ra4 with antisera to Ra4 and BPAR (Fig. 41), evidence for homogeneity of the Ra4 preparation and its (antigenic) equivalence with BPAR. In contrast, antiserum to Ra4 failed to react with antigens E, Ra3, and Ra5, while antisera raised to the latter antigens were inactive toward Ra4.

Single precipitin arcs were obtained for Ra4 and antigen E tested

with (NIH) antiserum to WSR (Fig. 42), antigenic cross-reaction was evident between the antigens, suggesting the presence of a common antigenic (structural) region in Ra4 and E (and the presence and absence of antibodies to this structural region in the anti-E and anti-WSR antisera, respectively).

b. Data on the incidence of allergenic (cutaneous) activity of Ra4 (obtained by Dr. John Santilli) are given in Table X. Although 80% of the ragweed pollen sensitive patients reacted to antigen E, only 20% of these were sensitive to Ra4, a cross-reacting antigen. It is noteworthy that one patient reacted to Ra4 but was insensitive to antigen E.

3. Antigen Ra5

i. Amino acid composition

Within experimental error, an identical residue composition was found for Ra5 isolated in the present study and that obtained by the earlier procedure of Lapkoff and Goodfriend (1974) (Table XIII).

ii. Isoelectric point

The isoelectric point of Ra5 was determined (Roebber et al., 1975) in an LKB preparative isoelectric focussing column. The sample (10 mg) of Ra5 was incorporated in a glycerol density gradient together with Ampholine Carrier ampholytes in the pH range 9-11. After application of 400 volts for 4 days at 5°C, the column contents were collected in 2.2 ml fractions. Ra5 was eluted as a sharp homogeneous peak (Fig. 43) at an isoelectric pH of 9.5.

iii. Free sulfhydryl

The assay for free -SH groups was performed by the method of Rohrbach et al. (1973) on solutions of Ra5 in the concentration range of 1 to 30 nM/ml (Table VIII). Active sulfhydryl could not be detected.

iv. Polypeptide chain structure

As detailed earlier (see part 1. of this Section), a single band was obtained on SDS-acrylamide gel disc electrophoresis of Ra5 after reduction with DTT (Fig. 29), evidence for a single polypeptide chain structure.

v. Immunological properties

a. The isolation of antigen Ra5 was monitored by immunodiffusion tests with a goat antiserum to Ra5-ovalbumin previously shown to have the same specificity to Ra5 as the anti-Ra5 antiserum used in the initial isolation (Lapkoff and Goodfriend, 1974) of the antigen. The final preparation gave a single precipitin arc with the antiserum (Fig. 44).

b. Table X shows the incidence of allergenic (cutaneous) activity of Ra5 (data obtained by Dr. John Santilli). An appreciably higher incidence (approx 40%) was found in this study than previously (20%; cf. (Marsh et al., 1973). It is noteworthy that, of 20 patients who failed to react to antigen E, 3 reacted to Ra5, underlining the allergenic independence of the 2 antigens.

PART D

DISCUSSION

I. Isolation of the 2 Major Forms of Antigen Ra3

Methodologically, the isolation of antigen Ra3 from fraction C of the aqueous ragweed extract described in Part C, I, was a variant of that employed by Underdown and Goodfriend (1969). Thus, a change was made in the Sephadex G type used for molecular sieving: from G-100 to G-50m. As a result, higher hydrostatic pressures and flowrates were achieved as well as sharper and more reproducible eluate peak patterns. In addition, in the final ion exchange step, DEAE-Sephadex replaced DEAE-cellulose and the eluting Tris buffer was reduced from 0.002 to 0.001 M. This step in the isolation of Ra3 proved to be critical and difficult. Critical, because any contaminants still present following DEAE-Sephadex chromatography (Fig. 3) invariably appeared in the final Ra3 preparation obtained by G-50s chromatography. Difficult, because the separation of the slightly retained Ra3 peak from the unretarded peak (Fig. 3) appeared to be appreciably affected by very minor changes in the procedure and was likely also sensitive to batch differences in DEAE-Sephadex as well as in the start-up pollen.

The 2 forms of antigen Ra3 were numbered according to the order of their elution from Sephadex G-50s. The yield of Ra3-I plus -II was generally 100-120 mg/kg pollen, i.e., similar to (although somewhat higher than) the yields reported in the initial study by Underdown and Goodfriend (1969). It seems likely that the antigen Ra3 prepared by these workers was also a 2-component isolate. Detection of the 2 forms

had to await improvement in the technique of polyacrylamide gel disc electrophoresis and the use of Sephadex G-50s as a final purification step.

II. Development of a More Integrated Method of Isolation of Antigens Ra3, Ra4, and Ra5.

1. The method

A feature of the procedure for isolation of the 3 allergens was omission of the step of ether extraction of the ragweed pollen conventionally used in the preparation of WSR. The extract prepared from the untreated pollen contained, of course, considerable amounts of pigment and lipid. However, pigment and probably also lipid were partially eliminated by DEAE-Sephadex column chromatography. Column size and buffer molarity were chosen so as to allow passage of the known ragweed pollen allergens (including antigens E and K) and retain unwanted (colored) components. Partial depigmentation was previously achieved by other workers (King and Norman, 1962) and early in the present study by passage of the aqueous extract through large Pharmacia columns (K11/100) of Sephadex G-25. However, this gel tended to adsorb the basic protein allergens resulting in trailing of the unretarded A-peak into the retarded B-fraction (Roebber, 1970), and its use was therefore abandoned.

For molecular size separation of Ra3, 4 and 5, Biogel P-10 was selected in preference to Sephadex G-50m because the former retained pigment somewhat longer than the latter, allowing the Ra5 to elute in a pigment-free fraction. While antigens Ra3 and 4 eluted as overlapping fractions from P-10, no better resolution was observed using Sephadex G-50m.

Antigen Ra4 had a tendency to drag on gel filtration unless high salt concentrations were employed. This was achieved by incorporation of 0.2 M ammonium sulfate in the eluting (Tris) buffer. It is noteworthy, that the bulk of the Ra4 was confined to the descending limb of the symmetrical peak eluted from Sephadex G-75.

The procedure for isolation of antigens Ra3-I and II incorporated a DEAE ion exchange step which was similar to that previously employed (Underdown and Goodfriend, 1969) except that the cellulose ion exchanger was replaced by the Sephadex ion exchanger. The difficulty of this chromatographic step noted with the earlier procedure was again observed and may be apparent from the chromatographic pattern shown in Fig. 13a. It was frequently necessary to repeat the DEAE step once or twice to achieve highly homogeneous preparations. The degree of homogeneity of the preparations thereby attained was somewhat surprising considering the fact that only partial depigmentation and rather crude size separation was achieved by filtration through Biogel P-10: virtually all contaminating components in the P-10 fraction were separated from Ra3 by the DEAE-Sephadex step. Some low level contamination with molecular species different in

size but similar in charge properties may have been removed by the subsequent Sephadex G-50s filtration step to separate the 2 forms of antigen Ra3.

Almost all non-Ra5 components in the P-10 fraction were confined to the unretarded fraction eluted from CM-Sephadex. Antigen Ra5 was well retained on CM-Sephadex and eluted rather slowly in a large volume of the single eluant buffer. The final gel filtration steps through Biogel P-10 and P-4 were used to remove baseline contaminants.

Simplification of the method of isolation probably accounts for the appreciably higher yields of antigens Ra3 (150 mg/kg pollen) and Ra5 (75 mg/kg) than formerly achieved (100 and 15 mg/kg, respectively). The increased yields obtained for the low molecular proteins Ra3 and Ra5 run contrary to the concept that such components are breakdown products of larger native structures of the ragweed pollen grain (Goldfarb, 1968). The observation that Ra5 appeared in considerable quantity in a 20 min aqueous extract is also contraindicatory (Marsh; Goodfriend and Lapkoff; unpublished observations). In general, pollen allergens appeared to pass quite readily into the borate buffered physiological saline employed in the first extraction step (Section C, II, 1.) and, as found by Johnson and Thorne (1958) with grass pollen, the cell wall of the pollen grain remained unimpaired on microscopic inspection.

2. Difficulties in the method

The overlap of Ra3 and Ra4 in the Biogel P-10 chromatogram (Fig. 9) led to lower yields than might otherwise be possible but this could not be remedied by simple changes. Cuts were therefore made containing the 2 allergens in one fraction and the allergens separated by a subsequent CM-Sephadex step (Section C, II, 4.; Fig. 10). Ra3 was quantitatively obtained from the unretarded CM-Sephadex fraction II-1 and further purified in the usual way.

The method of isolation of antigen Ra3 has become routine. The only difficulty lies in the separation of the 2 Ra3 forms (Ra3-I and Ra3-II) by Sephadex G-50s chromatography (Section C, II, 5.; Fig. 14). Complete separation was achieved with considerable losses in material and time until it was established that the 2 species are essentially identical for the purpose of most of the chemical and immunological studies.

The isolation procedure for Ra4 has yet to become routine. This allergen appeared in several fractions on salt gradient elution from CM-Sephadex when a concentration gradient was used lower than the one employed by Griffiths and Brunet (1971). As already noted, it was confined to a symmetrical peak on Sephadex G-75 chromatography (Fig. 11) or rather to the descending branch of the peak, indicating inhomogeneity. Use of Sephadex G-75 superfine to resolve this peak was not attempted because early in this study it was observed that Ra4 absorbed very strongly to this gel. Recently, however, we have used filtration through Sephadex G-75

superfine in the presence of 0.2 M ammonium sulfate and have resolved the symmetrical peak from Sephadex G-75 into 2 peaks, the second of which contained all the Ra4. It is anticipated that use of this step will enable us to obtain Ra4 in homogeneous form in appreciably higher yields than the current levels of 20-25 mg/kg pollen.

Of the 3 allergens, Ra5 has proven to be the easiest to isolate in a highly homogeneous state. Recently, with technical improvement, the yield for Ra5 increased to 100 mg/kg pollen, still appreciably short of theoretical. It has been suggested (Roebber et al., 1975) that the presence of a number of components in WSR cross-reacting with Ra5 may partially explain the discrepancy between the Ra5 content of aqueous pollen extract as determined by radioimmunodiffusion (Marsh, 1973) and the yield of the allergen obtained. Similar quantitative measurements are planned for Ra3 and Ra4 using the RAST assay (Ceska et al., 1972) which has been recently introduced in our laboratory for antigen Ra3 (Goodfriend and Lundkvist, unpublished observations).

III. Chemical and Immunological Properties

1. Chemical properties

i. The molecular weights of the 2 forms (I and II) of antigen Ra3 were found to be identical by analytical ultracentrifugation, viz, 12,000 daltons. The preparations contained approx 8% carbohydrate, so that the weight of the

protein moieties may be estimated as 11,000 daltons.

As the amino acid compositions of Ra3-I and -II were also identical within the variation of the method, it is unclear what difference(s) in physicochemical properties cause the 2 forms to separate by acrylamide disc electrophoresis as well as by Sephadex G-50s column chromatography. Charge difference due to unequal amide content may account for the differential mobility, as found for the various forms of antigen E. Differences in carbohydrate content may play a major role, though it was not clearly established whether and to what extent the carbohydrate detected formed an integral part of the molecule. As the carbohydrate content could be reduced to the same low level of approx 3% by acrylamide gel disc electrophoresis, it seems likely that Ra3 is not, in fact, a glycoprotein. It will be of interest to determine if quantitative removal of carbohydrate can be effected by the simpler expedient of ammonium sulfate precipitation and, if so, what effect this would have on the disc electrophoretic migration of the 2 Ra3 forms.

ii. The Ra4 isolates contained high amounts of carbohydrate (> 30%), most of which at least, was loosely bound and could be removed by ammonium sulfate precipitation. However, the stability of the preparations appeared to decrease with loss of carbohydrate as evidenced by formation of visible aggregates and reduction in precipitin formation on immunodiffusion test, and no attempt was made to completely remove carbohydrate by further salting-out with ammonium sulfate.

The mol wt of Ra4 (6% carbohydrate) was found by analytical equilibrium ultracentrifugation to be 23,000 daltons. Taking the carbohydrate content into account, the mol wt of the protein moiety comes close to that of the heavy chain of antigen E, 21,800 daltons (King, 1973). This lends credence to the possibility that Ra4 constitutes free heavy chain of antigen E. However, it should be noted that appreciable differences in amino acid composition were found for Ra4 (Table XIV) and published data for antigen E heavy chain. If these differences are confirmed, it would seem more likely that Ra4 and antigen E heavy chain share only certain regions along the polypeptide chain.

iii. Determination of the isoelectric points of Ra3, Ra4, and Ra5 showed that the "basic protein allergen" was not the most anionic molecular species obtained from ragweed pollen extract (pI = 8.0). In fact, the pI of Ra3 with an isoelectric point of 8.6 and Ra5 with 9.5, lay somewhat higher in the basic region, though CM-Sephadex chromatography did not conform to these results.

iv. Evidence for homogeneity of the antigen preparations was obtained by various physicochemical criteria.

All final column chromatographic runs for separation by size as well as by charge yielded single symmetrical peaks. (Retention of Ra5 on Biogel P-4 (exclusion limit: 3,600) was likely due to adsorption of the

basic protein to the gel in the presence of a low ionic strength buffer.).

Analysis of antigens Ra3-I, Ra3-II and Ra4 by analytical equilibrium ultracentrifugation gave linear plots of log c vs r^2, showing monodispersity of the preparations.

Ra3-I, Ra4 and Ra5 migrated as a single band on SDS polyacrylamide gel disc electrophoresis, indicating size homogeneity. The very faint slower migrating minor bands in the electropherogram for Ra4 which were present in the reagent blank gel are not believed to be contaminants as they exhibit very different size and/or charge properties. They might consist of carbohydrate with small amounts of attached protein.

Purification was usually monitored by polyacrylamide gel disc electrophoresis at pH 4.3. The presence of only a single electrophoretic band was one of the criteria for homogeneity of the final preparations. The pH 6.6 buffer system did not result in improved resolution and was therefore discontinued; basic systems could not be used because the allergens did not enter the gel under these conditions.

On isoelectric focussing in a glycerol density gradient, single well-defined peaks were obtained for Ra3-I, Ra4 and Ra5, showing charge homogeneity.

2. Immunological properties

i. On Ouchterlony tests, the 3 allergens under study reacted with the corresponding specific rabbit antisera, and Ra3 and Ra4 with rabbit antisera to WSR, with formation of single precipitin arcs. Ra5 did not

precipitate with anti-WSR. This was observed previously (Lapkoff, 1974) and we have in fact not succeeded in preparing a rabbit anti-WSR antiserum which also contained antibodies to Ra5, nor to our knowledge has any other laboratory. It seems likely that too much of this small, weakly antigenic protein has been lost during conventional ether extraction of the pollen prior to preparation of the aqueous extract used for immunization.

The 3 antigens showed no antigenic cross-reactivity, since rabbit antiserum to any of the 3 antigens failed to give immunodiffusion arcs to the remainder. It is recognized, however, that detection of cross-reactivity in these tests is strongly dependent on reaction conditions and the nature (immune or hyperimmune) of the antiserum.

ii. The data presented in Figs. 31 and 32 and Tables X and XI demonstrate that Ra3 is a distinct allergen having essentially no cross-reactivity with the major ragweed pollen allergen, antigen E. There is no relationship between the sensitivity to the 2 allergens in the 23 patients assayed by leucocyte histamine release. In terms of relative activities in different allergic patients, there is more than a 100,000-fold spread. For example, the concentrations of Ra3 and antigen E required for 50% histamine release in patient 3 were 1.2 and 11 x 10^{-4} ug per milliliter, respectively, so that this patient was about ten times <u>more</u> sensitive to Ra3 than antigen E. At the other extreme, the same figures for patient 17 were 14,000 and 1.8 x 10^{-4} ug/ml, i.e., this patient was about 10,000 times <u>less</u> sensitive

to Ra3 than to antigen E. This more than 10^5-fold ratio of sensitivities to Ra3 and E at the same time demonstrates that the Ra3 preparation was free from any significant contamination with E.

In studying the immunological relationship of antigens E and Ra3 by inhibition of leucocyte histamine release with specific rabbit antisera, the expected pattern was observed, i.e., antigen E induced histamine release was inhibited by anti-E antiserum and not by antiserum to Ra3, and vice versa. However, in 2 patients, antiserum to antigen E also inhibited Ra3 activity, and in one of those patients the homologous antiserum was not effective at all (Figs. 32b and c).

These findings were somewhat surprising and require an explanation. The data, while limited, were well reproducible in these two patients. The anti-E antiserum concentration that gave a significant inhibition of Ra3 activity was 10^3 and 10^2 times higher than was needed to obtain the same degree of inhibition of the homologous allergen. The observed inhibition of the heterologous allergen may therefore well be due to contamination in the immunizing material with either Ra3 or some Ra3 cross-reacting component. The data shown in Fig. 32c, in which the anti-Ra3 has no ability to inhibit Ra3-induced histamine release cannot be explained in this way.

One possibility is that the rabbit anti-Ra3 antiserum, which was not hyperimmune, may not have contained antibodies to some less immunogenic

determinants on the Ra3 molecule. These determinants may have been the same, or topographically close to the allergenic determinants detected by the cell-bound IgE antibodies of these two particular patients. Findings paralleling this phenomenon have been reported in studies on enzyme activity in presence of specific anti-enzyme antibodies (Cinader, 1967). Thus, neutralizing and non-neutralizing rabbit antibodies to RNase were shown to exist; indeed, one rabbit consistently produced antibodies which activated the enzyme. At present it cannot be excluded that similar activating, or at least non-neutralizing antibodies may be produced in the rabbit on immunization with Ra3 - and perhaps other allergens as well.

However, another possibility which cannot be excluded is that the Ra3 sensitizing preparation contained a minor allergenic contaminant not detected by the physicochemical techniques employed, one that did not cross-react with antigen Ra3 and cross-reacted weakly with antigen E. Further investigation, including the use of absorption with specific antibody, are required to discriminate between these possible explanations.

iii. The incidence of patient sensitivity to Ra3, Ra4, and Ra5 was determined by skin puncture test. For the assays, a single concentration of allergen, 30 ug/ml, was employed, and patients were considered insensitive to a particular allergen if they were skin test negative at this concentration. For this reason, the results obtained in these studies must be considered approximate only, however, they are likely to be close approximations in

view of the good correlation found between data obtained by skin puncture and intradermal tests performed simultaneously on individual patients (Dr. J. Santilli, personal communication). The advantage of the method used was that a large number of patients could be screened for sensitivity to several allergen preparations simultaneously without too high an expenditure of time and effort.

As expected, the largest number of people were allergic to antigen E (79%)(Table X), but 21% did not react to the major allergen of ragweed pollen. Sensitivities to Ra3 and Ra5 were equally distributed (39% in each case) indicating that the 2 allergens are important contributors to the total patient symptoms. Somewhat surprising was the low incidence of sensitivity to Ra4 (20%) in light of its antigenic cross-reaction with antigen E. Of the 95 WSR sensitive patients tested, 16% failed to react to any of the purified preparations, a result warranting further search for the component(s) of ragweed responsible for these patients' allergy.

There is no obvious linkage of sensitivities to any grouping of the allergens tested. Table XV was constructed to elucidate this type of relationship, listing all possible combinations of sensitivities. At one extreme were patients sensitive to all test allergens (13%); most Ra4 sensitive patients belong to this group. At the other extreme were patients insensitive to any of the test allergens (16%). The largest proportion of the patient population was allergic to a single antigen, antigen E (30%); only 2 patients were sensitive to Ra3 alone, one to

Ra5 alone. Almost all the other possible combinations are present, except (Ra3-Ra4) (Ra3-Ra5) and (Ra4). The latter would likely be found if a larger number of patients had been used, because sensitivities not involving antigen E were present in only a relatively small group (21%). The distribution of sensitivities suggests that each allergic person has his own (genetically defined?) spectrum of sensitivities to the different allergens he encounters in his environment, and though he may be exposed to all the allergens that are components of ragweed pollen, he selectively responds only to a certain number of them.

IV. Structure and Function of Protein Allergens

As discussed in the Introduction, a long-range aim of the present studies was to assist in the development of our knowledge of the structure of protein allergens which mediate their allergenic function. Recently, Stanworth has advanced the concept that the "bridge" mechanism of interaction of allergens with adjacent pairs of cell-fixed IgE antibodies for mediator release requires that protein allergens possess a 2-polypeptide chain structure. However, the results of the present study do not appear to conform to this "requirement". Thus, antigens Ra3 and Ra4 were shown to have single chain structures, as previously found for Ra5. Antigens Ra3 and Ra5 have not been observed to dimerize, while Ra4 had a tendency to aggregate but only in a low ionic strength buffer environment or following removal of carbohydrate to the 5% level. While dimerization of these

allergens at the (mast) cell surface cannot be excluded, there is no compelling reason why a single polypeptide chain carrying multiple allergenic determinants (identical or otherwise) cannot "bridge" adjacent IgE antibodies directed to these determinants.

Several other structural features have been proposed to be necessary for proteins to function as allergens but, except for a relatively small size (determined by limitation in permeability through mucosal membrane barriers), none could be shown to be a common property for this class of proteins. Recently, the complete amino acid sequence of 2 allergens has become available. The first allergen to be sequenced (although retrospectively) was phospholipase A from bee venom; the second was ragweed antigen Ra5. In addition, N-terminal sequences were determined for three other allergens; TM2 from cod white muscle (17 residues), antigen Ra3 (25 residues), and Ra4 (22 residues). Elsayed and Sletten (1972) pointed out that a salient feature of the TM2 primary structure was the occurrence of Arg-Ala (x 1) and Lys-Ala (x 2).

The doublet Arg-Ala also appears in Ra3 and Ra5 and there, interestingly, with Tyr as the next residue (Fig. 45). The cod allergen has Phe following one Lys-Ala doublet. Another triplet which is identical in Ra3 and Ra5 is Ser-Asp-Pro. A very similar triplet Thr-Asp-Ala occurs in TM2 and phospholipase A and a version of this Ile- Asp-Pro is found in Ra4.

Another striking feature is the association of Tyr with Cys/2 as direct neighbors. This occurs 3 times in Ra5, once in phospholipase A and once in Ra4. Ra3 and cod TM2 have no Cys/2 in the presently known part of their sequence. Cys-Cys bonding in Ra5 would necessarily juxtapose the 2 aromatic rings, a doublet Phe-Tyr is found once, and one Tyr-Phe twice in the sequence of phospholipase A. If some of the doublets and triplets mentioned above should be involved in the formation of an active site, it can be visualized that the activity would be lost, for instance with tryptic hydrolysis at (Arg-Ala-Tyr) and with reduction of cystine which may separate Tyr rings from one another.

The N-terminal sequence constitutes a potentially important region in the primary structure of an allergenically active molecule (carrier region?). The corresponding N-terminal sequences for 4 allergens are compared in Fig. 46. The allergenically active TM2 fragment was enzymatically split from the native N-terminal region of the M allergen. It is noteworthy that all N-terminal amino acids are aliphatic. Val occurs close to residue 1, partially replaced by Leu in Ra5 (micro-heterogeneity) and Ile in phospholipase A. The first Tyr in the amino acid sequence is located very close to the N-terminal accompanied by a Val in Ra3 and Ra4, and by an Ile in phospholipase A. It is possible that Trp is taking the place of Tyr in Ra5, and should the first Cys/2 bind to the second one in the sequence, a Val would be positioned close to this Trp.

Except in Ra3, the first Cys/2 occurs early in the sequence, and the first Lys (the second in Ra3) is at a comparable place in all 4 allergens.

Chemically "equivalent" amino acids are often substituted for one another by point mutations in the genetic code. Val and Leu appear at the same position 2 in Ra5. One of the codons for Val, G-U-U, could have changed to the C-U-U codon for Leu. Interchanges of Ser and Thr and Ala and Pro could occur by G-C mutations. Phe and Tyr are coded for by the triplets U-U-U and U-A-U, respectively. A question which might be asked is whether T-cell receptors have accepted these "replacements" or if a consequence of such mutations could be the loss of biological activity in cessation of IgE antibody biosynthesis.

The foregoing discussion of similarities in the primary structures of 5 allergens was presented as an attempt to indicate the possibilities which would open up with increasing availability of sequence structural information for related and unrelated protein allergens.

SUMMARY

1. Two disc electrophoretic forms of Ra3 (I and II) were isolated by column chromatography in yields of 30 and 10 mg/kg pollen, respectively. The 2 forms had identical size and amino acid composition and displayed complete antigenic identity on immunodiffusion against specific and polyvalent antisera.

2. A column chromatographic method was developed for the sequential isolation of antigens Ra3, Ra4, and Ra5 from non-defatted short ragweed pollen. The allergens were obtained in highly purified states as judged by a number of physicochemical and immunological criteria and in appreciably higher yield for the 2 smaller proteins: from 10-15 mg to 75-100 mg/kg pollen for Ra5; from 100 mg to 175 mg/kg for Ra3 (I and II).

3. The variable carbohydrate content of antigen Ra3 was reduced from 7-20% to 3% by preparative acrylamide gel electrophoresis, and of Ra4 from 30% to 6% by ammonium sulfate precipitation.

The mol wt of antigens Ra3 (containing 8% carbohydrate) and Ra4 (containing 6% carbohydrate) were determined by equilibrium ultracentrifugation to be 12,000 and 23,000 daltons, respectively. The isoelectric points were determined by isoelectric focussing to be pH 8.6 and 8.0, respectively.

4. On immunodiffusion analysis, no cross-antigenicity was detected between antigens Ra3, Ra4, Ra5, nor between Ra3, Ra5, and E; however,

antigens Ra4 and E exhibited partial antigenic identity.

In a collaborative study, the activities of Ra3 and E in releasing histamine from leucocytes of ragweed sensitive patients were compared in the absence and presence of homologous or heterologous rabbit antiserum. The results demonstrated that about half the antigen E sensitive patients were also sensitive to Ra3. In most cases studied, Ra3 and E induced leucocyte histamine release was inhibited by the homologous but not heterologous antisera, demonstrating antigenic independence of Ra3 and E. However, in 2 cases, anomalous results were obtained in that Ra3 induced release was not inhibited by anti-Ra3 antiserum.

In a second collaborative study, the incidence of cutaneous activities of Ra3, Ra4, and Ra5 were compared with that of E in ragweed sensitive patients. The results indicated that the 4 antigens were allergenically independent.

5. Antigens Ra3 and Ra4 were shown to consist of single chain polypeptide structures, as demonstrated earlier for antigen Ra5. In collaborative studies, the sequences of 25 and 22 N-terminal amino acid residues were determined for Ra3 and Ra4, respectively. Comparison of these sequences with those of Ra5, bee venom phospholipase A, and the 17 N-terminal sequence of cod TM2 revealed the presence of similar triplets

of amino acids: Lys / Arg Phe / Tyr Ser / Thr Pro / Ala , Ala / Asp in 5 of the allergens, the occurrence of certain amino acid pairs (Tyr-Cys) and similar amino acids at or close to the N-terminal.

CLAIMS TO ORIGINALITY

1. A column chromatographic method was developed for the sequential isolation of antigens Ra3, Ra4, and Ra5 from non-defatted, short ragweed pollen.

2. The 2 major forms of antigen Ra3 (I and II) were separated and shown to be identical by physicochemical and immunological criteria.

3. The chemical properties of antigens Ra3 and Ra4 were determined; mol wt, carbohydrate content, isoelectric point, chain structure, amino acid composition and (in a collaborative study) the N-terminal amino acid sequence.

4. The high and variable content of carbohydrate of antigens Ra3 and 4 were shown to be capable of reduction to relatively low levels by physicochemical methods, indicating that the term "glycoprotein" may not be appropriate for these 2 allergens.

5. The immunological relationship between antigens Ra3 and E was determined in a collaborative study using the human leucocyte histamine release assay.

6. In a collaborative study, the incidence of cutaneous activity of Ra3 and Ra4 was determined in a population of ragweed sensitive patients utilizing a skin puncture method of assay.

TABLE I

AMINO ACID COMPOSITION OF ANTIGEN Ra3-I

Amino Acid	µmoles 24 hr	µmoles 48 hr	µmoles 72 hr	Average or Extrapolated µmoles
Lysine	0.122	0.126	0.134	0.127
Histidine	0.047	0.050	0.054	0.050
Arginine	0.075	0.083	0.084	0.081
Aspartic Acid	0.165	0.159	0.160	0.161
Threonine	0.133	0.125	0.118	0.137
Serine	0.090	0.072	0.060	0.095
Glutamic Acid	0.198	0.196	0.196	0.197
Proline	0.133	0.129	0.131	0.131
Glycine	0.197	0.178	0.179	0.185
Alanine	0.133	0.122	0.123	0.126
Cystine/2	0.050	0.051	0.048	0.050
Valine	0.125	0.126	0.127	0.126
Methionine	-	-	-	-
Isoleucine	0.052	0.056	0.053	0.054
Leucine	0.146	0.145	0.145	0.145
Tyrosine	0.054	0.054	0.056	0.055
Phenylalanine	0.130	0.134	0.134	0.133

TABLE II

AMINO ACID COMPOSITION OF ANTIGEN Ra3-I

Amino Acid	Moles/11,000g Protein	No. of Residues/11,000g Protein
Lysine	6.6	7
Histidine	2.6	3
Arginine	4.2	4
Aspartic Acid	8.4	8
Threonine	7.2	7
Serine	5.0	5
Glutamic Acid	10.3	10
Proline	6.9	7
Glycine	9.7	10
Alanine	6.6	7
Cystine/2	2.6	3
Valine	6.6	7
Methionine	-	-
Isoleucine	2.8	3
Leucine	7.6	8
Tyrosine	2.9	3
Phenylalanine	7.0	7
Tryptophan	2.1*	2
		101

* Determined spectrophotometrically.

TABLE III

COMPARISON OF THE AMINO ACID COMPOSITIONS OF TWO Ra3 PREPARATIONS

Amino Acid	No. of Residues/Mole Protein*	
	Ra3-I containing 7% carbohydrate**	Ra3-I containing 20% carbohydrate**
Lysine	6.5	6.5
Histidine	2.7	3.0
Arginine	3.9	4.0
Aspartic Acid	8.2	8.8
Threonine	7.2	7.6
Serine	5.6	5.0
Glutamic Acid	11.0	11.0
Proline	8.1	7.9
Glycine	9.7	9.0
Alanine	7.1	6.9
Cystine/2	2.6	2.4
Valine	7.0	6.9
Methionine	-	-
Isoleucine	2.7	2.2
Leucine	7.9	7.5
Tyrosine	4.0	3.9
Phenylalanine	7.0	7.0

*Assuming 7.0 residues of phenylalanine.

**Based on amino acid analyses of 24 hr hydrolysates.

TABLE IV

CARBOHYDRATE CONTENT OF THREE Ra3 PREPARATIONS

Arabinose Standards (µg)	OD 500	Carbohydrate Content µg	%
5	0.019		
10	0.050		
20	0.090		
30	0.122		
40	0.164		
Ra3 (I + II)*			
0.29 mg	0.122	29.0	10.0
in duplicate:	0.123		
Ra3-I**			
0.35 mg	0.047	10.5	3.0
in duplicate:	0.048		
Ra3-II**			
0.21 mg	0.030	7.0	3.3
in duplicate:	0.029		

* From an Ra3 preparation after DEAE-Sephadex chromatography (Fraction III-2).

** From preparative polyacrylamide gel electrophoresis of fraction III-2.

TABLE V

PARTIAL AMINO ACID COMPOSITION OF PERFORMIC ACID OXIDIZED Ra3-I

Amino Acid	µmoles*	No. of Residues/ Mole of Protein **	Nearest Integer
Aspartic Acid	0.204	7.9	8
Glutamic Acid	0.294	11.4	11
Proline	0.192	7.5	8
Glycine	0.250	9.7	10
Alanine	0.169	6.6	7
Cysteic Acid	0.073	2.8	3
Valine	0.173	6.7	7
Methionine Sulfone	-	-	-
Isoleucine	0.079	3.1	3
Leucine	0.222	8.6	9
Phenylalanine	0.180	7.0	7

*Based on amino acid analyses of 24 hr hydrolysates.

**Assuming 7.0 residues of phenylalanine.

TABLE VI

COMPARISON OF THE AMINO ACID COMPOSITIONS OF Ra3-I AND Ra3-II

Amino Acid	No. of Moles/11,000g* Ra3-I	Ra3-II
Lysine	6.4	7.2
Histidine	2.5	2.7
Arginine	4.0	4.2
Aspartic Acid	8.7	8.6
Threonine	7.0	7.0
Serine	4.7	4.9
Glutamic Acid	10.5	9.9
Proline	7.1	8.2
Glycine	10.4	9.1
Alanine	7.0	6.4
Cystine/2	2.0	2.8
Valine	6.6	6.3
Methionine	-	-
Isoleucine	2.7	3.5
Leucine	7.7	7.6
Tyrosine	2.8	2.9
Phenylalanine	6.9	6.2

*Based on amino acid analyses of 24 hr hydrolysates.

TABLE VII

SULFHYDRYL CONTENT OF Ra3-I

	Protein Concentration pM/ml	OD_{412}	Free Sulfhydryl M/M
BSA	10.3	0.087	0.64
Ra3	16.7	0.026	0.15

TABLE VIII

DETERMINATION OF SULFHYDRYL CONTENT

Protein	Protein Concentration nM/ml	Optical Density at 612 nm
BSA	0	1.050
	4	0.938
	8	0.757
	12	0.618
	16	0.415
Ra3	0	0.813
	2	0.772
	4	0.820
	6	0.796
	8	0.768
	10	0.800
Ra4	0	0.995
	2	1.060
	3	1.040
	5	0.984
	7	1.100
Ra5	0	0.549
	3	0.551
	5	0.542
	10	0.539
	15	0.540
	20	0.556

TABLE IX

PARTIAL AMINO ACID COMPOSITION OF REDUCED AND ALKYLATED Ra3-I

Amino Acid	μmoles*	No. of Residues/ Mole of Protein**	Nearest Integer
Aspartic Acid	0.093	9.4	9
Threonine	0.068	6.8	7
Serine	0.041	4.1	4
Glutamic Acid	0.102	10.3	10
Proline	0.064	6.4	6
Glycine	0.096	9.8	10
Alanine	0.073	7.4	7
Carboxymethyl Cysteine	0.039	3.9	4
Valine	0.066	6.7	7
Methionine	-	-	-
Isoleucine	0.033	3.4	3
Leucine	0.079	8.0	8
Tyrosine	0.028	2.8	3
Phenylalanine	0.069	7.0	7

*Based on amino acid analysis of a 24 hr hydrolysate.

**Assuming 7.0 phenylalanine residues.

TABLE X

SKIN PUNCTURE TEST OF 95 RAGWEED SENSITIVE PATIENTS WITH PURIFIED ALLERGENS

Allergen*	Number of Patients with Positive Skin Reactions **	%
AgE	75	79
Ra3	37	39
Ra4	19	20
Ra5	37	39

*Each allergen was tested at a concentration of 30 µg/ml.

**Of the 95 patients who were skin test positive to aqueous ragweed extract, 15 (i.e. 16%) did not react to any of the purified allergens.

TABLE XI

LEUCOCYTE HISTAMINE RELEASE IN RESPONSE TO ANTIGENS E AND Ra3

Donor	Antigen Concentration for 50% Histamine Release x 10^{-4} µg/ml	
	Ag Ra3	AgE
8	Not sensitive	5.2
9	" "	4.0
16	" "	1.6
15	" "	1.4
14	" "	1.0
11	" "	0.33
17	14,000.0	1.8
19	1,600.0	50.0
7	810.0	11.0
4	220.0	0.3
21	50.0	3.0
18	45.0	0.14
6	17.0	1.0
23	15.0	1.2
2	10.0	4.5
13	10.0	0.32
5	1.0	2.1
1	4.8	10.0
10	3.8	0.35
22	2.0	0.68
3	1.2	11.0
12	0.44	0.92
20	11%*	25%*

* Maximal histamine release.

TABLE XII

AMINO ACID COMPOSITION OF ANTIGEN Ra4

Amino Acid	Moles/21,000g 24 hr	48 hr	72 hr	Average or Extrapolated Moles	Nearest Integer
Lysine	17.3	15.8	16.3	16.4	16
Histidine	3.7	3.5	3.6	3.6	4
Arginine	8.2	7.3	7.1	7.5	8
Aspartic Acid	25.1	25.0	22.8	24.3	24
Threonine	11.0	10.7	10.6	11.2	11
Serine	9.7	10.4	9.4	9.8	10
Glutamic Acid	13.6	13.6	13.7	13.7	14
Proline	6.3	7.7	7.6	7.2	7
Glycine	14.5	14.7	14.6	14.6	15
Alanine	10.9	11.4	10.9	11.1	11
Cystine/2	4.3	3.0	4.3	3.9	4
Valine	11.1	12.1	13.3	12.3	12
Methionine	4.1	3.0	3.2	4.1	4
Isoleucine	10.6	12.2	13.0	13.0	13
Leucine	12.7	12.8	12.8	12.8	13
Tyrosine	9.1	9.7	9.7	9.5	10
Phenylalanine	8.3	8.3	9.1	8.6	9
Tryptophan				4.0 *	4

*Spectrophotometrically determined.

TABLE XIII

AMINO ACID COMPOSITION OF ANTIGEN Ra5

Amino Acid	No. of Residues/Mole Protein	
	Recent Isolate*	Reported**
Lysine	3.8	3.7
Histidine	0	0
Arginine	2.4	1.9
Aspartic Acid	1.7	1.8
Threonine	0	0
Serine	3.8	3.9
Glutamic Acid	4.5	3.9
Proline	3.4	3.0
Glycine	4.0	3.7
Alanine	2.7	2.8
Cystine/2	6.7	5.9
Valine	3.4	3.2
Methionine	0	0
Isoleucine	0.9	0.9
Leucine	1.3	1.3
Tyrosine	2.8	2.8
Phenylalanine	0	0

*Values were averaged from analysis of 48 and 72 hr acid hydrolysates, assuming 4 glycines per mole Ra5.

**From Lapkoff and Goodfriend (1974).

TABLE XIV

COMPARISON OF THE AMINO ACID COMPOSITION OF ANTIGEN Ra4 AND THE α-CHAIN OF ANTIGEN E

Amino Acid	Ra4 Moles/21,000g	α-chain Moles/21,800g*
Lysine	16.4	7.1
Histidine	3.6	3.8
Arginine	7.5	9.1
Aspartic Acid	24.3	25.4
Threonine	11.2	10.6
Serine	9.8	23.1
Glutamic Acid	13.7	16.8
Proline	7.2	11.4
Glycine	14.6	21.7
Alanine	11.1	20.2
Cystine/2	3.9	5.0
Valine	12.3	13.3
Methionine	4.1	3.5
Isoleucine	13.0	8.7
Leucine	12.8	14.1
Tyrosine	9.5	2.1
Phenylalanine	8.6	7.9
Tryptophan	4.0	3.2

*From King et al., 1973.

TABLE XV

COMBINATIONS OF SKIN SENSITIVITIES TO 4 ALLERGENS OF RAGWEED POLLEN IN 95 RAGWEED ALLERGIC PATIENTS

Allergen Combinations	Number of Patients with Positive Skin Reactions
E, Ra3, Ra4, Ra5	12
E, Ra3, Ra4	2
E, Ra3, Ra5	12
E, Ra4, Ra5	2
Ra3, Ra4, Ra5	1
Ra3, Ra4	0
Ra3, Ra5	0
Ra4, Ra5	1
E, Ra3	8
E, Ra4	3
E, Ra5	7
E	29
Ra3	2
Ra4	0
Ra5	1
None	15

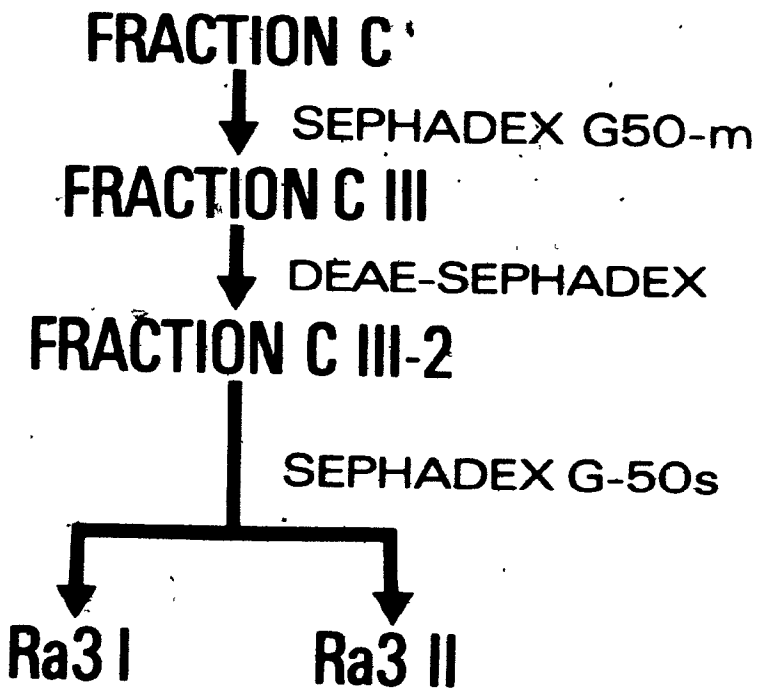

Fig. 1. Isolation of antigens Ra3-I and Ra3-II from DEAE-cellulose fraction C.

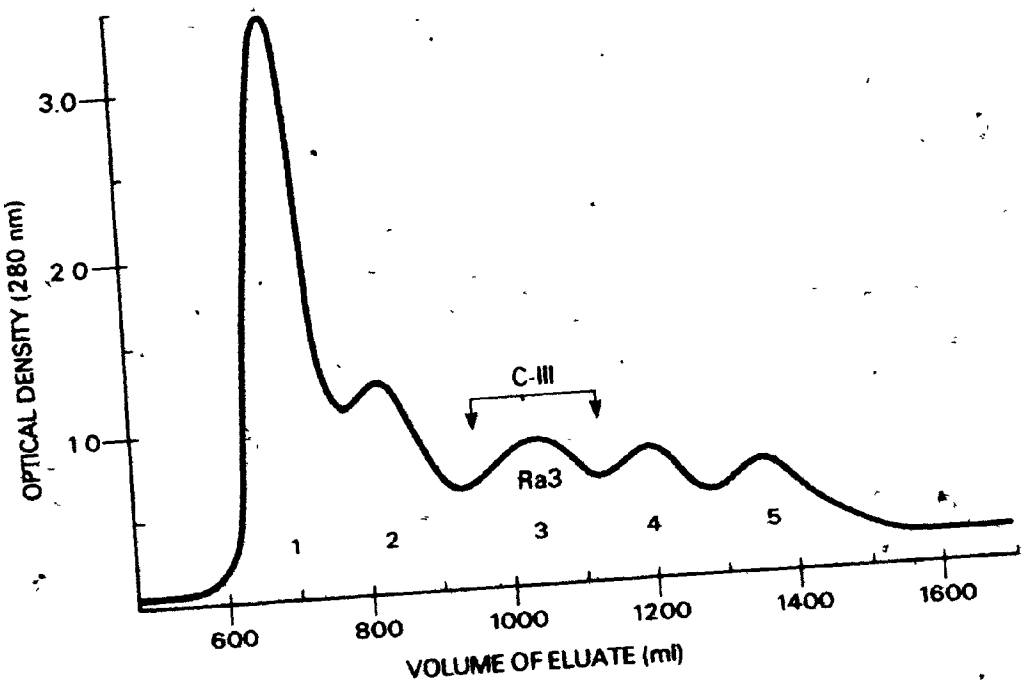

Fig. 2. Molecular sieving of fraction C through Sephadex G-50 medium.

Ra3 was localized to the third chromatographic peak (C-III). The column bed was 5 x 95 cm, the eluting buffer was 0.05 M Tris HCl, pH 7.6 and the flowrate was 60 ml/hr.

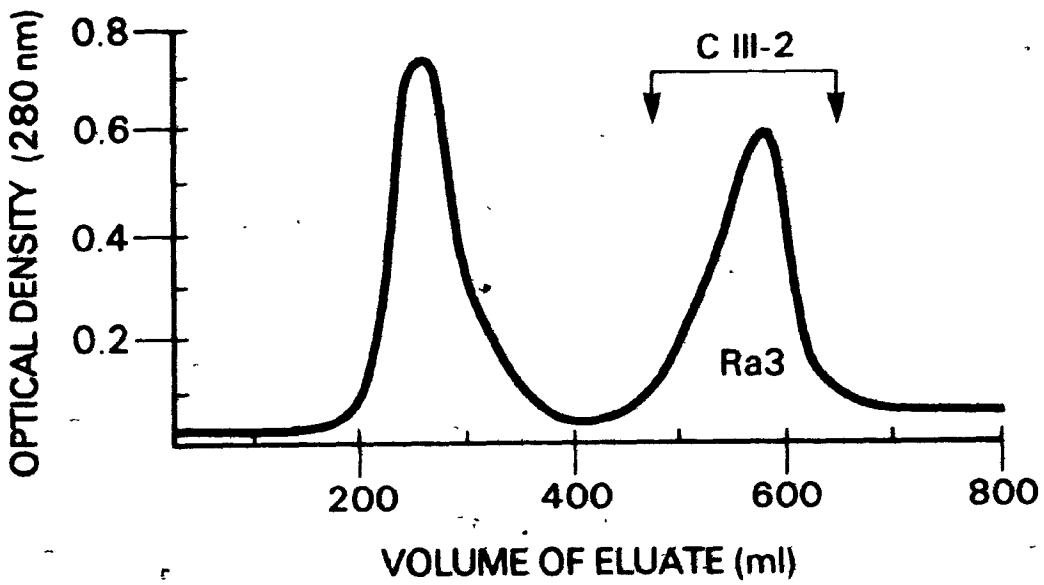

Fig. 3. DEAE-Sephadex chromatography of fraction C-III.

Ra3 was localized to peak 2 (C-III-2). The column bed was 5 x 25 cm, the eluting buffer was 0.001 M Tris-HCl, pH 7.6 and the flowrate was 120 ml/hr.

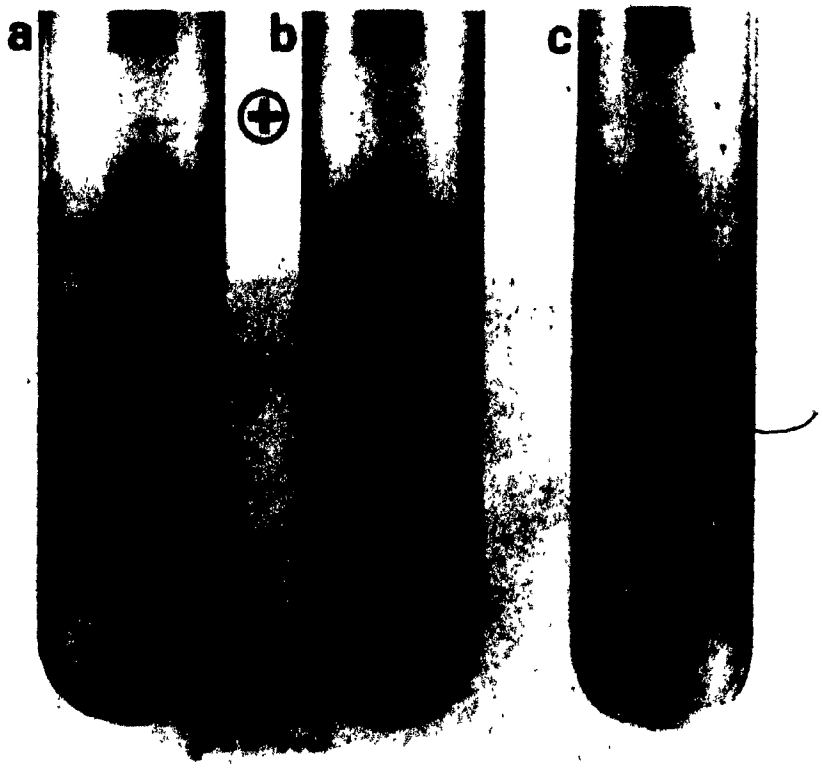

Fig. 4. Polyacrylamide disc electrophoresis of antigen Ra3.

a. Ra3 (I + II) from fraction C-III-2.
b. Ra3-I, fraction C-III-2a.
c. Ra3-II, fraction C-III-2b.

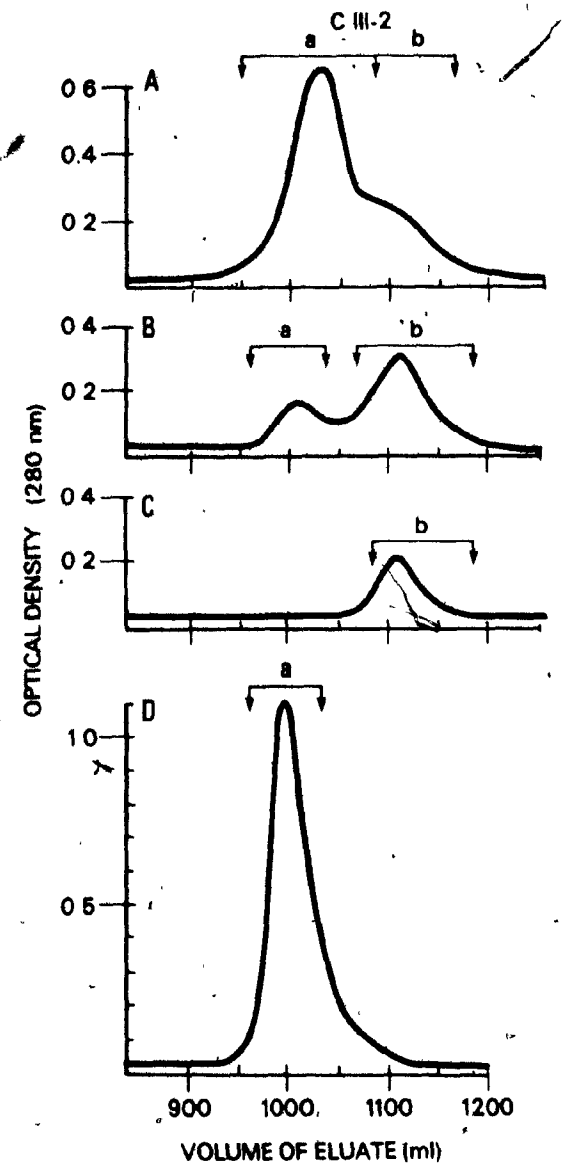

Fig. 5. Separation of Ra3-I from Ra3-II by gel filtration through Sephadex G-50 superfine.

The column bed was 5 x 95 cm, the buffer was 0.025 M Tris-HCl, pH 7.6 and the flowrate was 20 ml/hr. A-C represent consecutive steps in the purification of Ra3-II (fraction C-III-2b). D shows the final cycling of Ra3-I (fraction C-III-2a) through the same column.

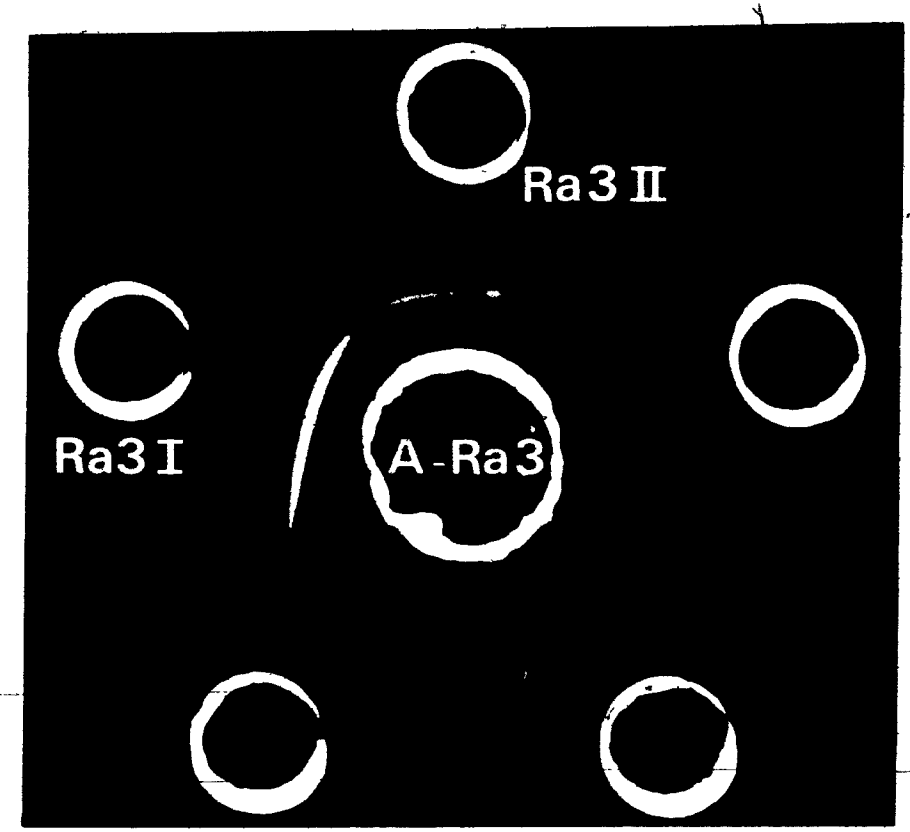

Fig. 6. Immunodiffusion analysis of antigens Ra3-I and Ra3-II.

The antigen concentration was 0.20 mg/ml.

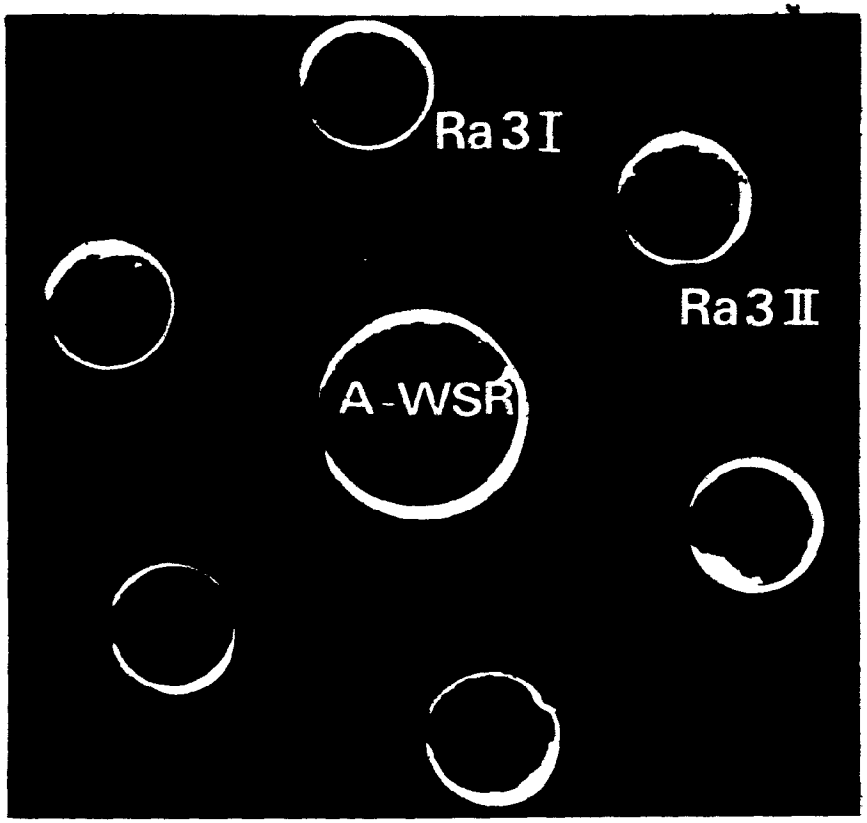

Fig. 7. Immunodiffusion analysis of antigens Ra3-I and Ra3-II

The antigen concentration was 0.10 mg/ml.

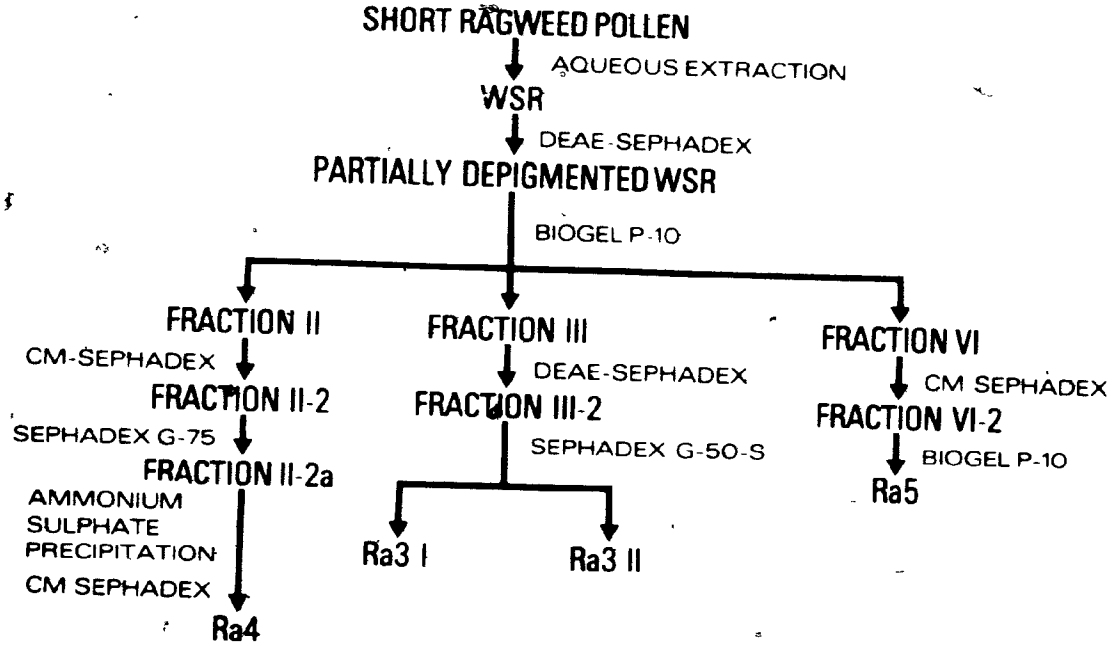

Fig. 8. Chromatographic procedures for the isolation of antigens Ra3, Ra4 and Ra5.

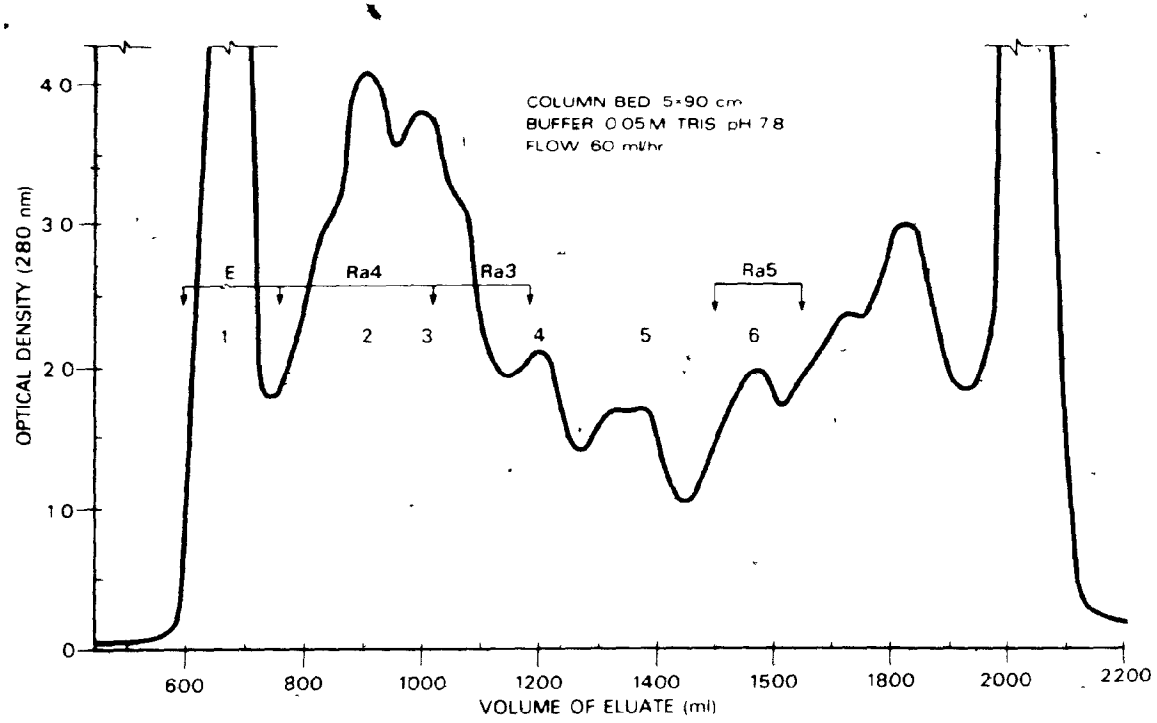

Figure 9. Filtration of WSR (after partial depigmentation) through Biogel P-10.

Specific antisera were used to localize antigens to their respective peak fractions.

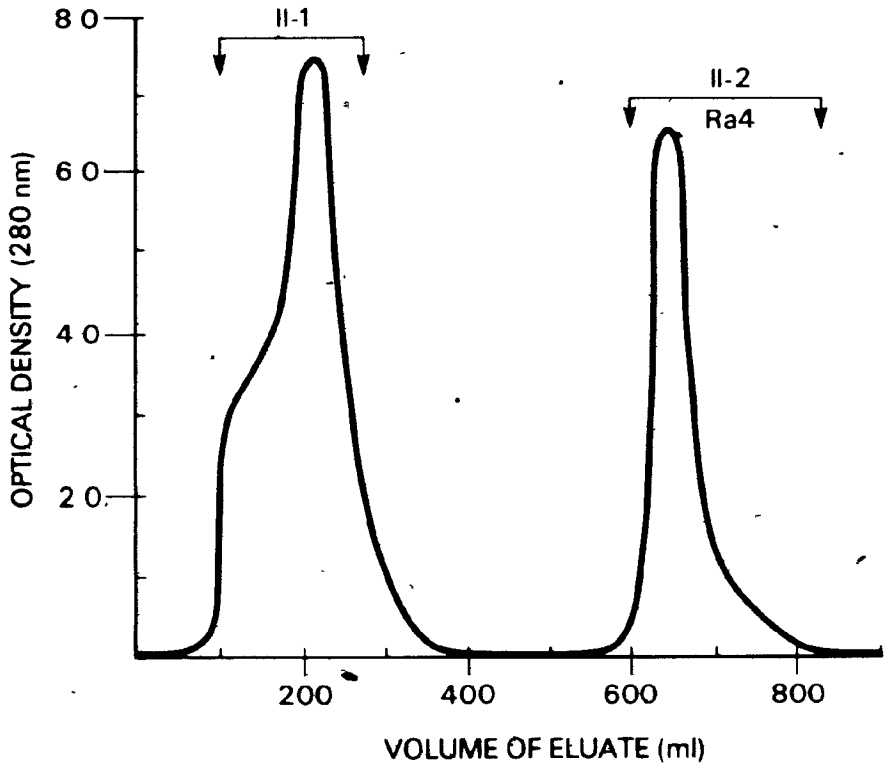

Figure 10. CM-Sephadex chromatography of Fraction II-2.

The column bed was 2.5 x 30 cm, the buffer was 0.05 M phosphate, pH 6.8; 0.35 M NaCl were incorporated in this buffer for the second chromatographic step. The flowrate was 100 ml/hr.

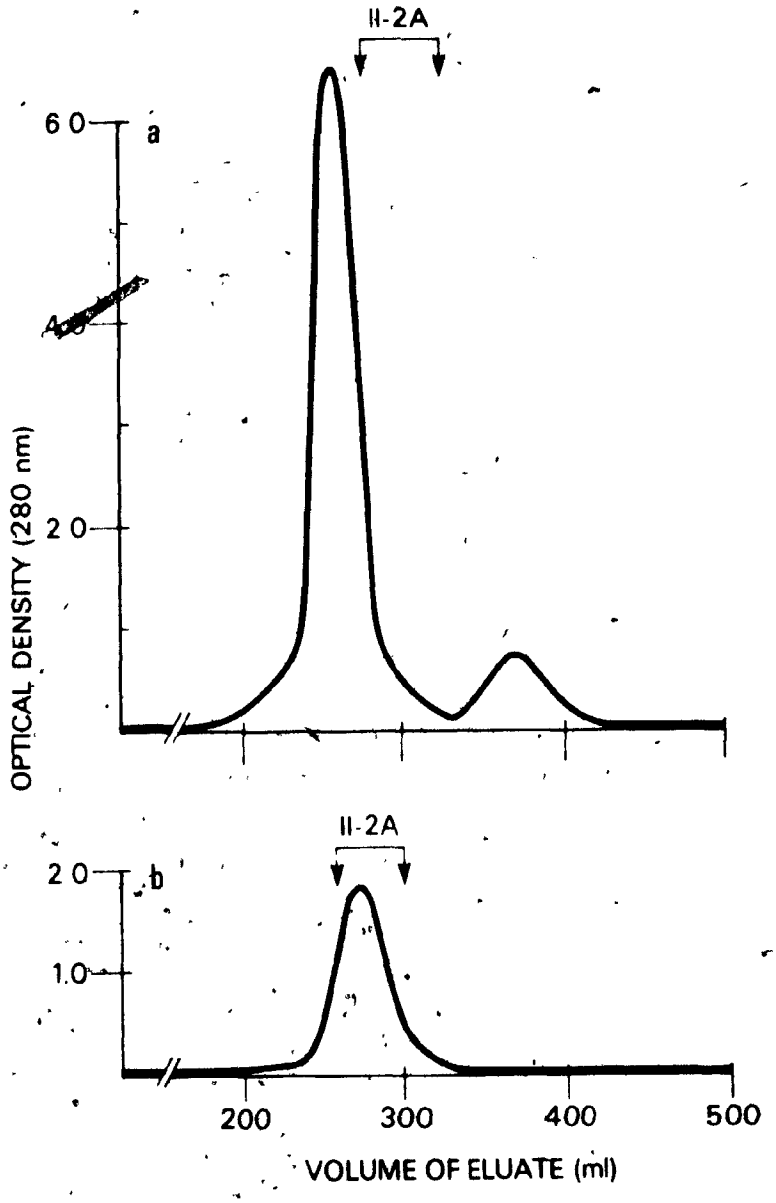

Fig. 11. Gel filtration of fraction II-2 through Sephadex G-75 medium.

The column bed was 2.5 x 90 cm, the buffer was 0.05 M Tris HCl, pH 7.6 containing 0.2 M $(NH_4)_2 SO_4$. The flow was 15 ml/hr. Ra4 was localized to the descending branch of the main peak. b. shows the last chromatographic run of a pool of 3 cuts (a.) through the same column.

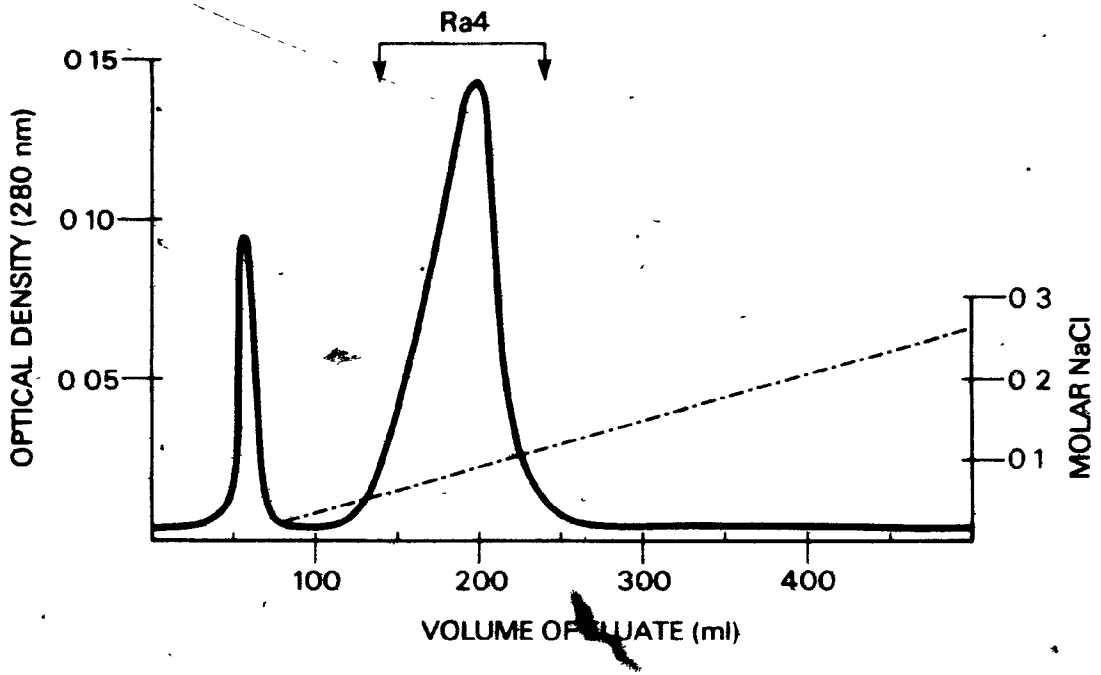

Fig. 12. Salt gradient elution of fraction II-2A (after ammonium sulfate precipitation) from CM-Sephadex.

The column bed was 1.5 x 27 cm, the starting buffer was 0.05 M phosphate pH 6.8. The gradient consisted of 0.5 l of starting buffer and 0.5 l of the same buffer containing 0.5 M NaCl. The flowrate was 28 ml/hr.

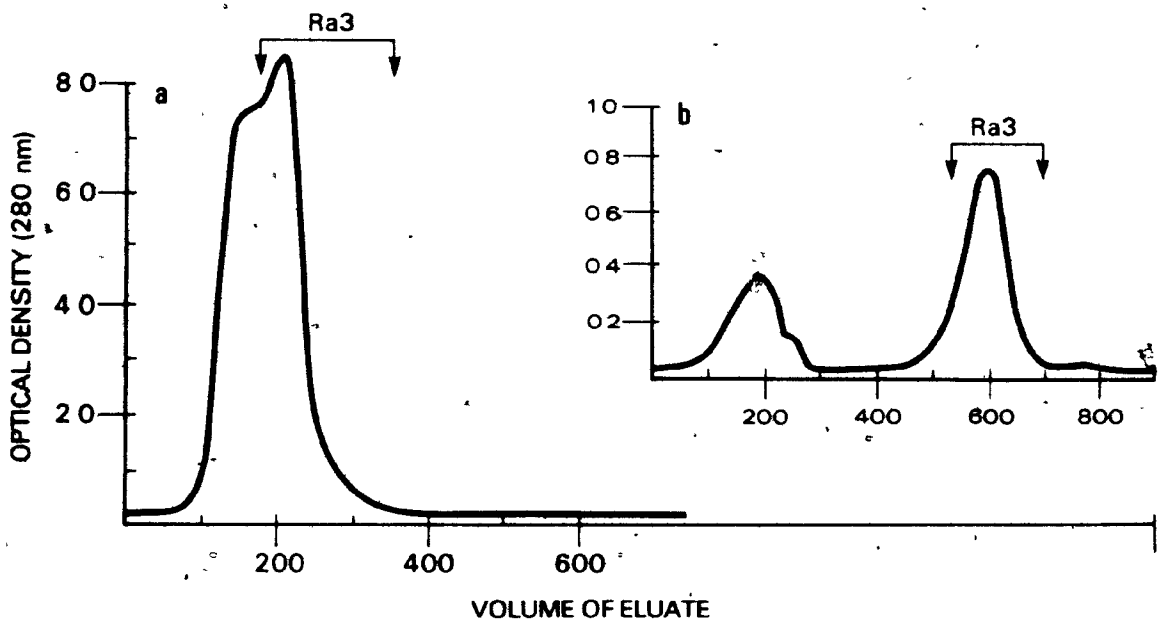

Fig. 13. DEAE-Sephadex chromatography of fraction III.

The column bed was 2.5 x 30 cm, the eluting buffer was 0.001 M Tris-HCl, pH 7.8 and the flowrate was 60 ml/hr. The cut in "a" was recycled through the same column and eluted as a separate peak (b).

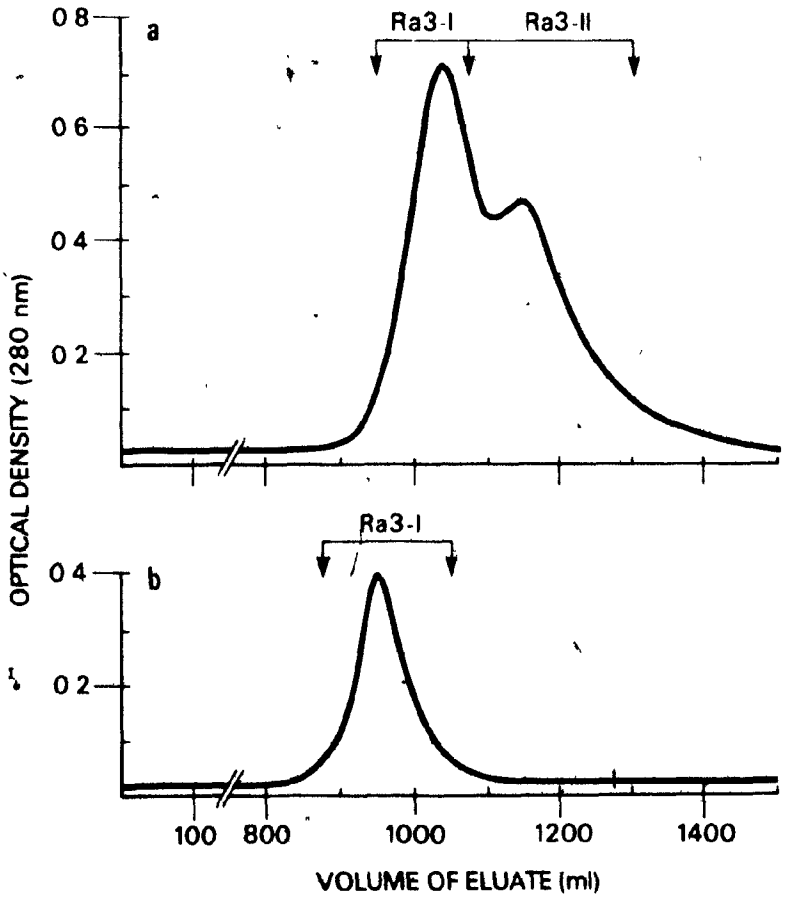

Fig. 14. Gel filtration of fraction III-2 through Sephadex G-50 superfine.

The column bed was 5 x 90 cm, the eluting buffer was 0.025 M Tris-HCl, pH 7.6 and the flowrate was 25 ml/hr. The Ra3-I cut (a) was recycled twice on the same column to obtain a symmetrical peak (b).

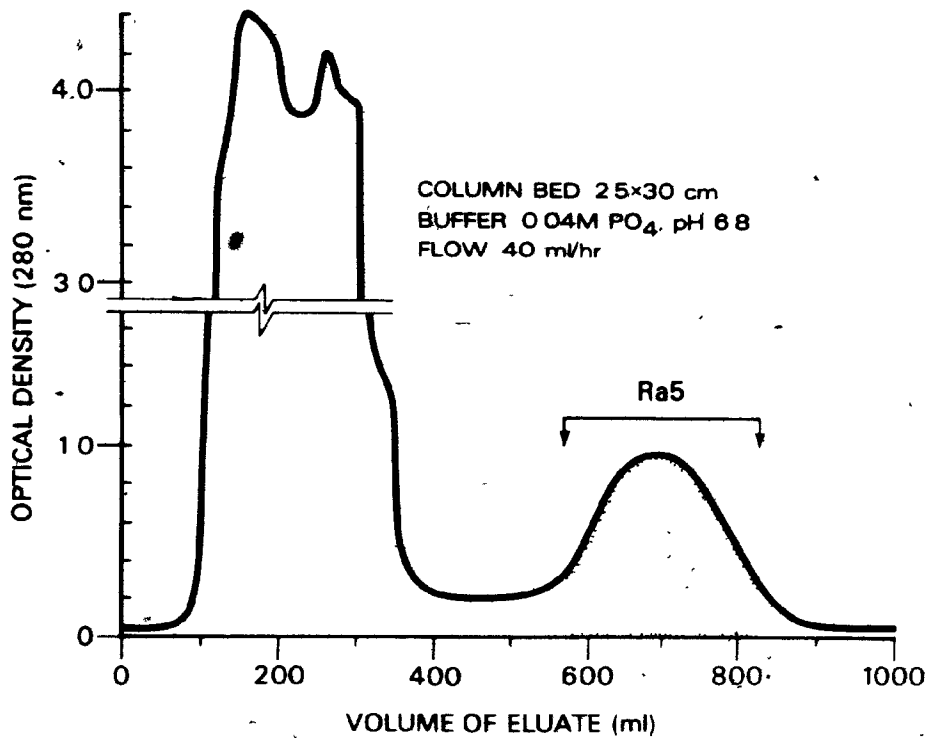

Fig. 15. CM-Sephadex chromatography of Fraction VI.

Ra5 was localized to the second peak fraction (VI-2).

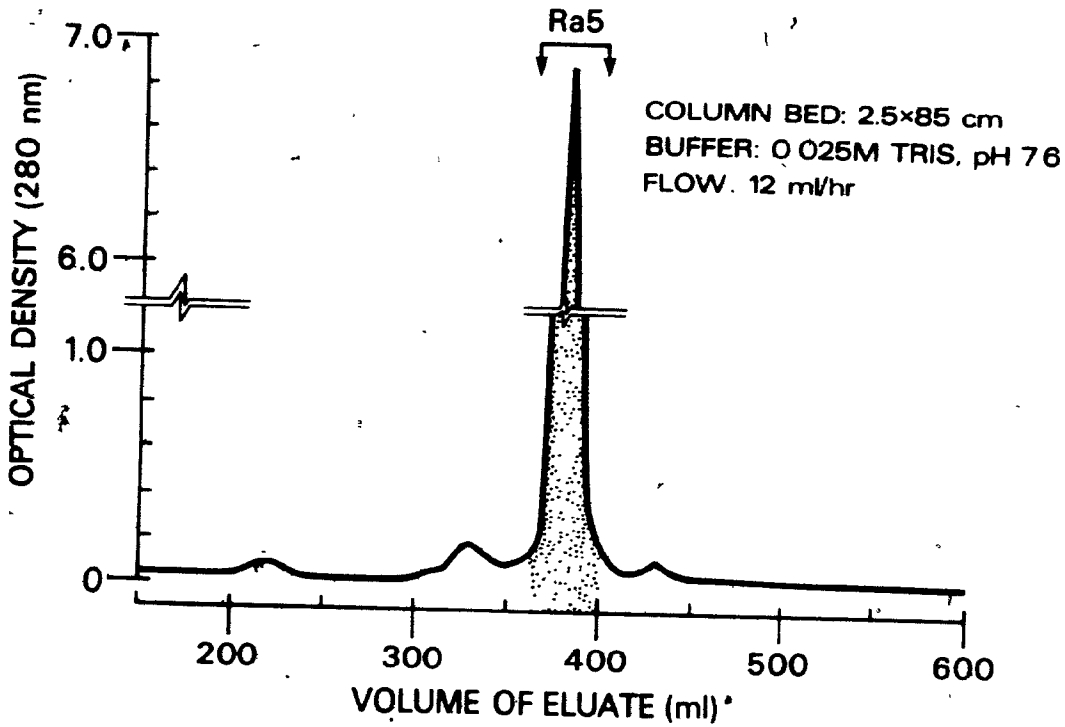

Fig. 16. Gel filtration of fraction VI-2 on Biogel P-10.

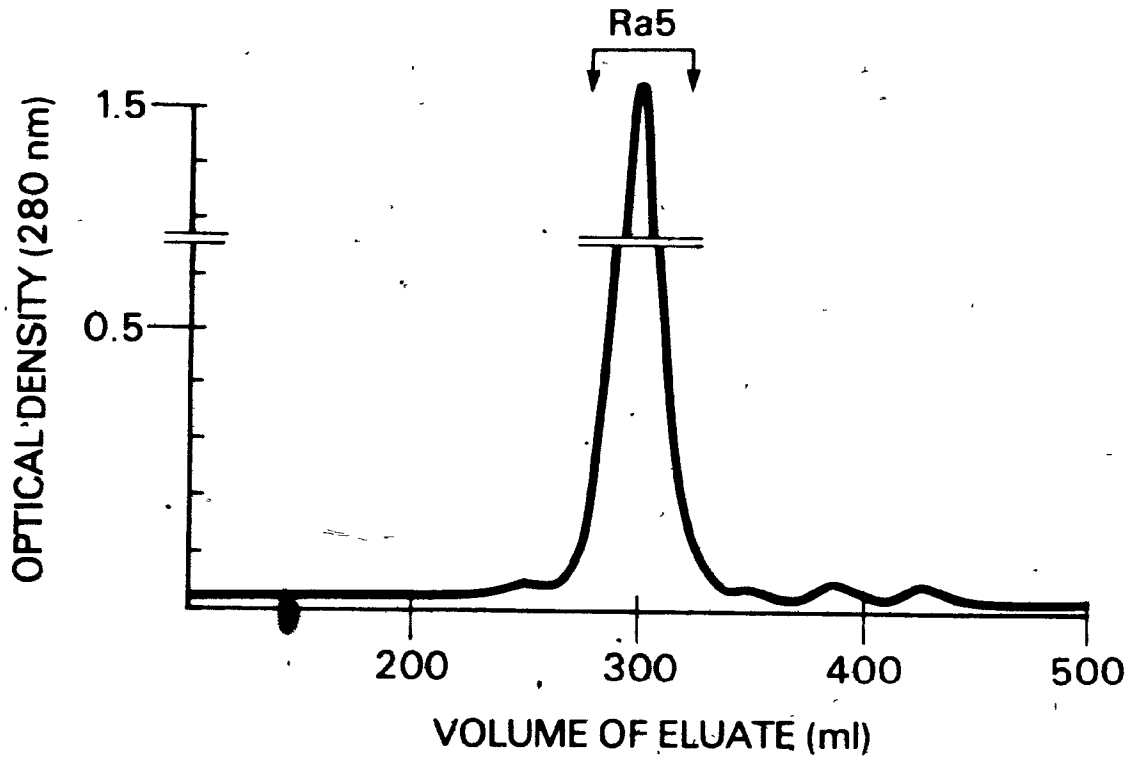

Fig. 17. Gel filtration of Ra5 on Biogel P-4.

The column bed was 2.5 x 90 cm, the eluting buffer was 0.025 M Tris-HCl pH 7.6 and the flowrate was 25 ml/hr.

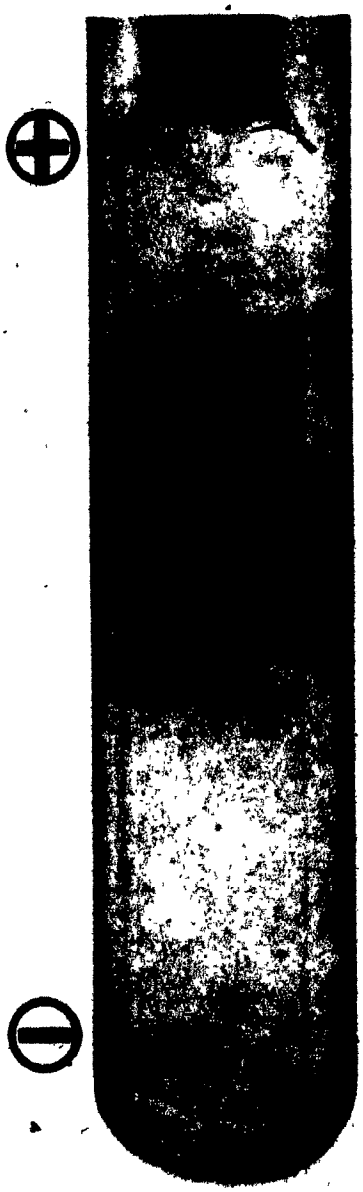

Fig. 18. Polyacrylamide gel disc electrophoresis at pH 4.3 of antigen Ra5.

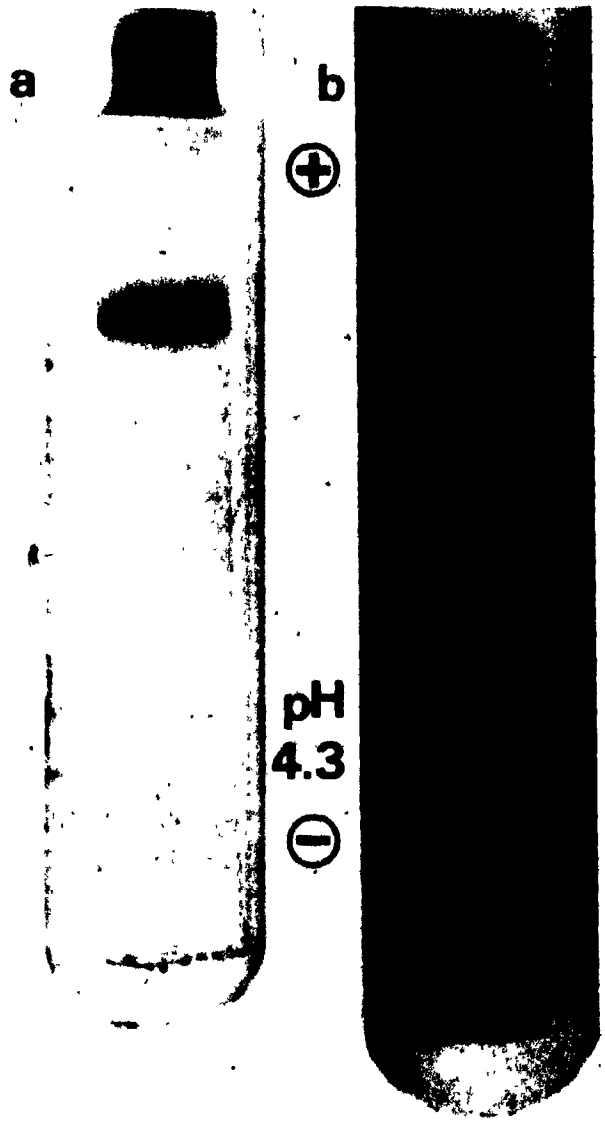

Fig. 19. Polyacrylamide gel disc electrophoresis at pH 4.3 of antigen Re4.

A sample (15 ug) from a 60% ammonium sulfate precipitate was run on the gel to the left; the same amount of a sample from a 70% ammonium sulfate precipitate was run on the gel to the right.

LEU VAL PRO CYS ALA TRP ALA GLY ASN VAL
LEU

CYS GLY GLU LYS ARG ALA TYR CYS CYS SER

ASP PRO GLY ARG TYR CYS PRO TRP GLN VAL

VAL CYS TYR GLU SER SER GLU ILE CYS SER

LYS LYS CYS GLY LYS

Fig. 20. The amino acid sequence of antigen Ra5.

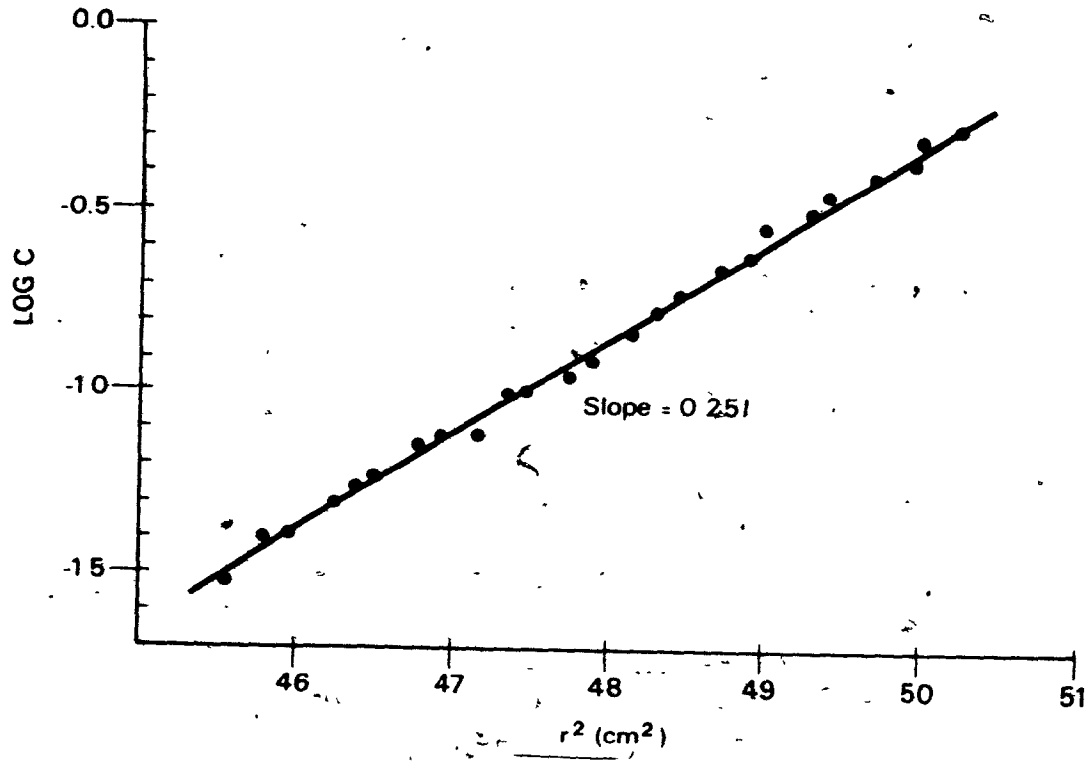

Fig. 21. Plot of log Ra3-I concentration (c) vs square of distance from rotor axis (r^2).

The slope was calculated by least squares analysis.

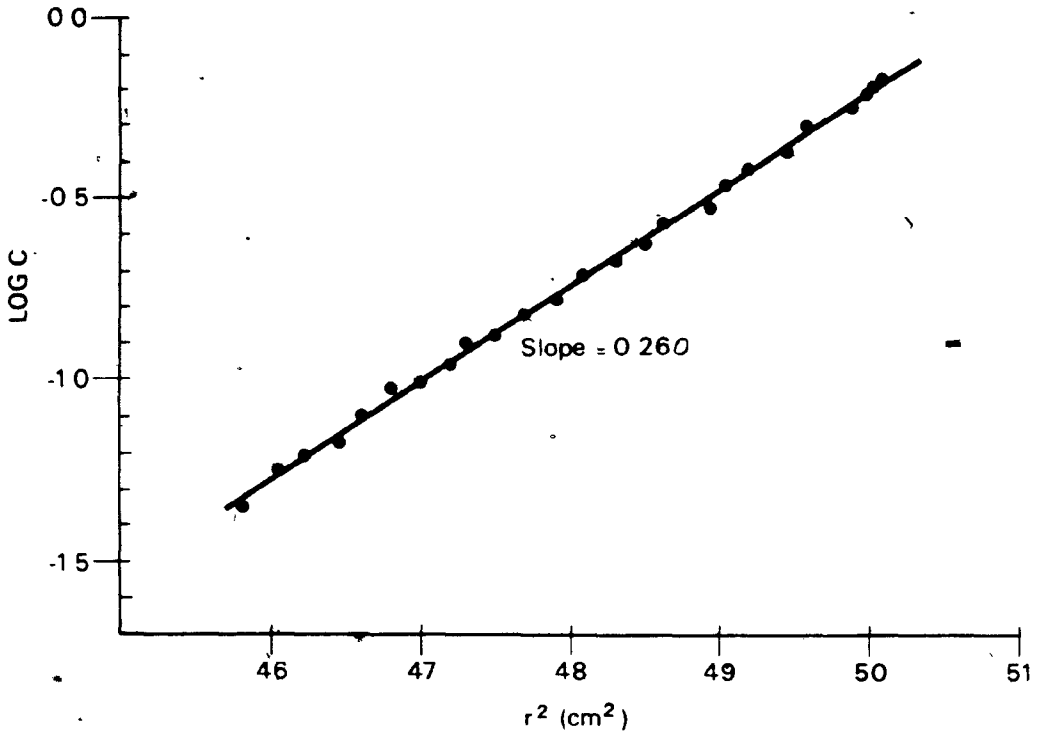

Fig. 22. Plot of log Ra3-II concentration (c) vs square of distance from rotor axis (r^2).

The slope was calculated by least squares analysis.

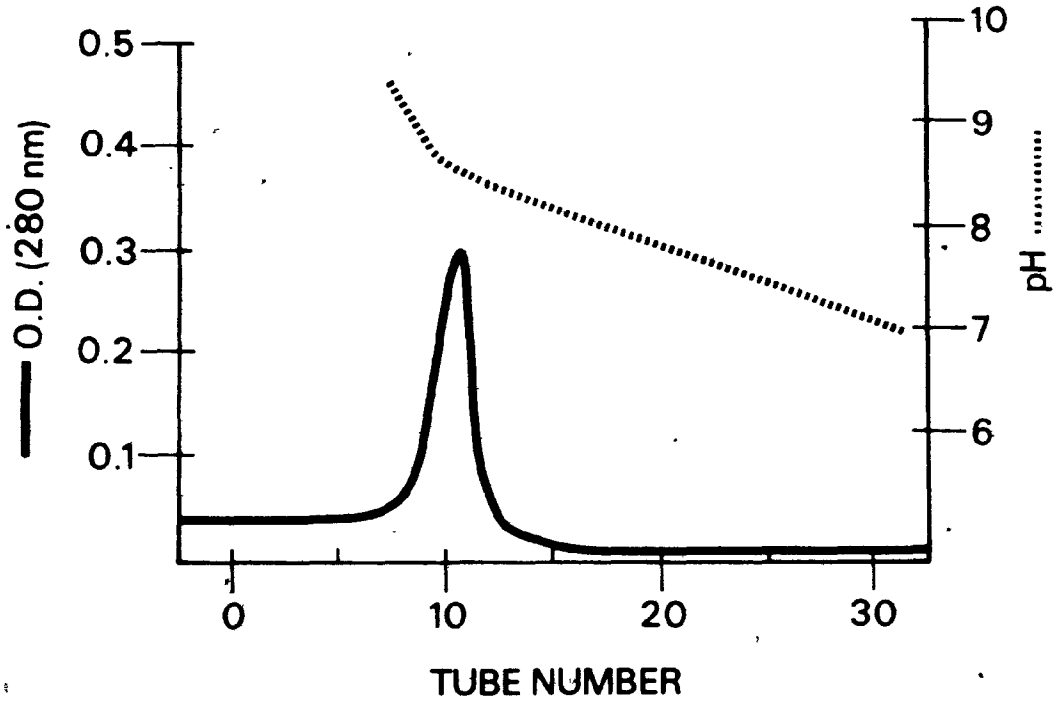

Fig. 23. Isoelectric focussing of antigen Ra3-I.

Shown is the elution pattern (%T converted to O.D.) from a glycerol density gradient column. The sample load was 10 mg, the volume/tube was 2.5 ml, the ampholine range was pH 7-9 and the focussing time was 4 days at 600 volts.

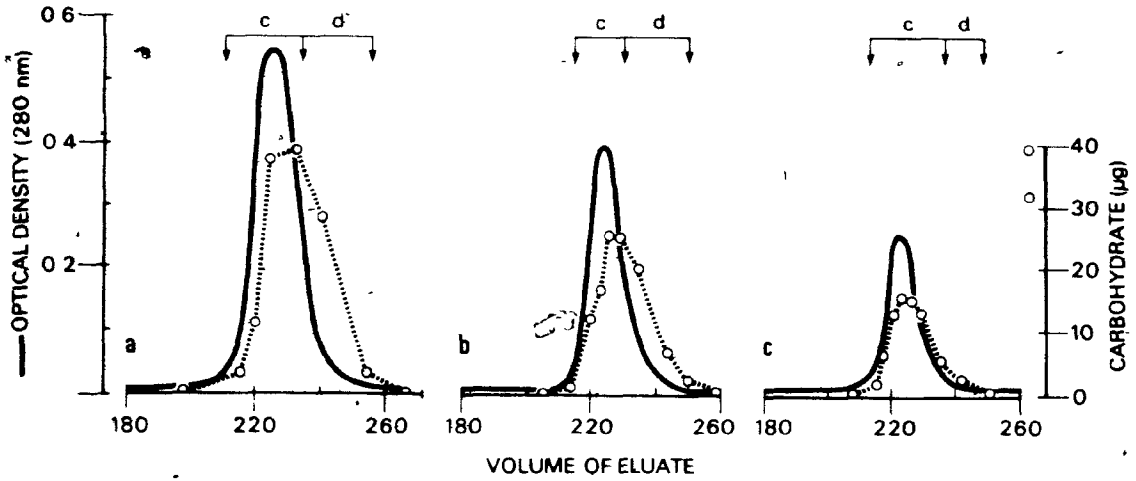

Figure 24. Biogel P-30 chromatography of an Ra3-I preparation.

The column bed was 2.5 x 90 cm, the eluting buffer was 0.025 M Tris-HCl, pH 7.6. Recycling of cut c (a) through the same column resulted in chromatogram "b". Recycling of a similar cut from "b" yielded chromatogram "c".

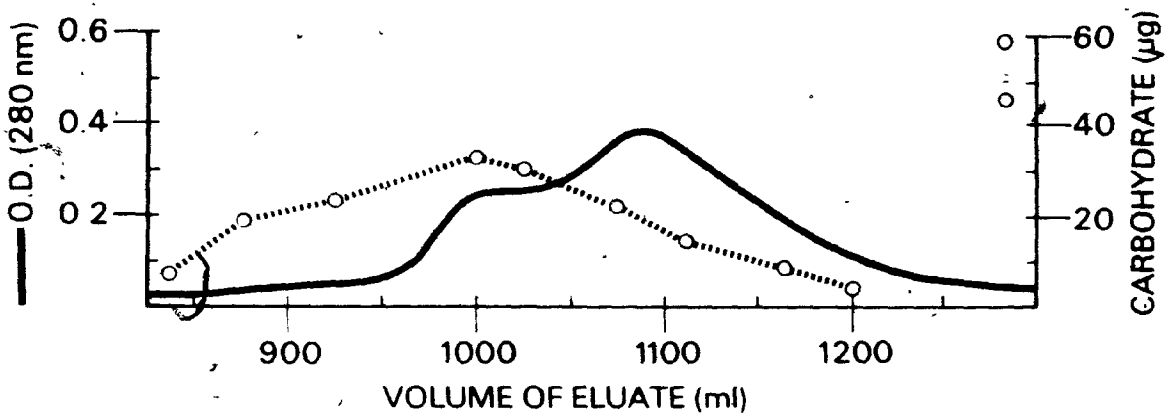

Fig. 25. Gel filtration of fraction III-2 through Sephadex G-50 superfine.

The column bed was 5 x 90 cm, the eluting buffer was 0.025 M Tris-HCl, pH 7.6.

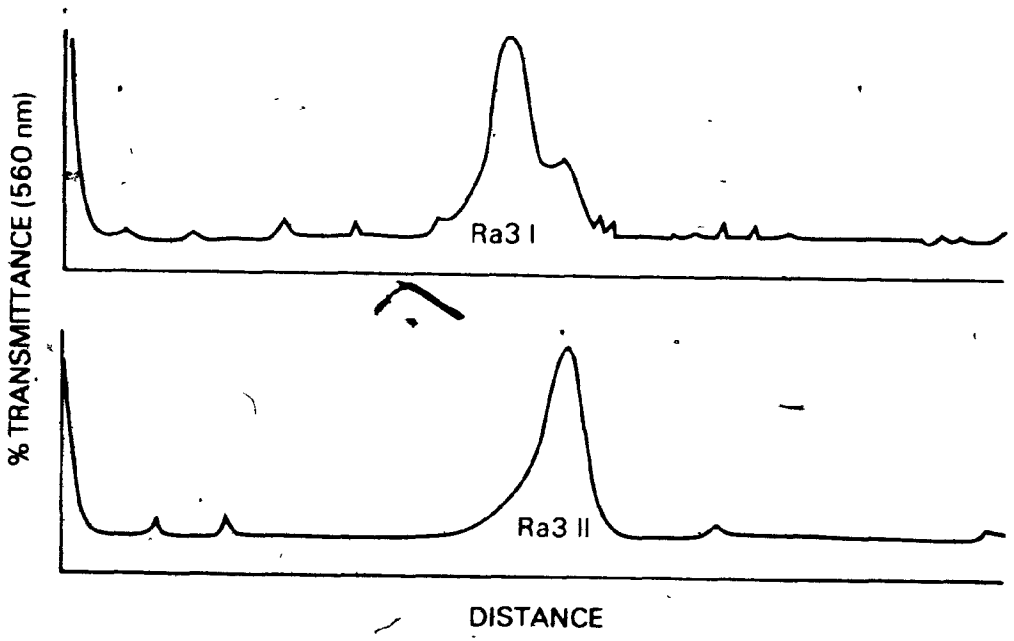

Fig. 26. Densitometer traces of 2 polyacrylamide electropherograms.

A sample (10 µg each) of an Ra3-I (above) and an Ra3-II (below) isolate from preparative polyacrylamide electrophoresis were analyzed by the routinely used pH 4.3 disc electrophoretic system. The Coomassie blue stained gels were used to obtain densitometer traces.

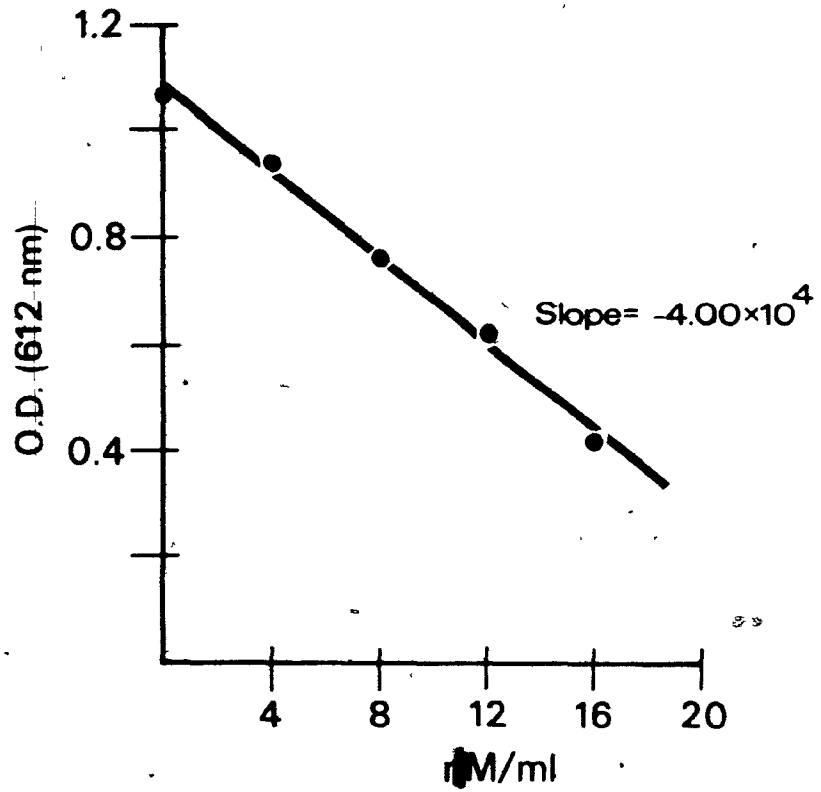

Fig. 27. Determination of the sulfhydryl content of BSA.

Shown is a plot of the $O.D._{612}$ as a function of the concentration of BSA that was reacting with BDC^+.

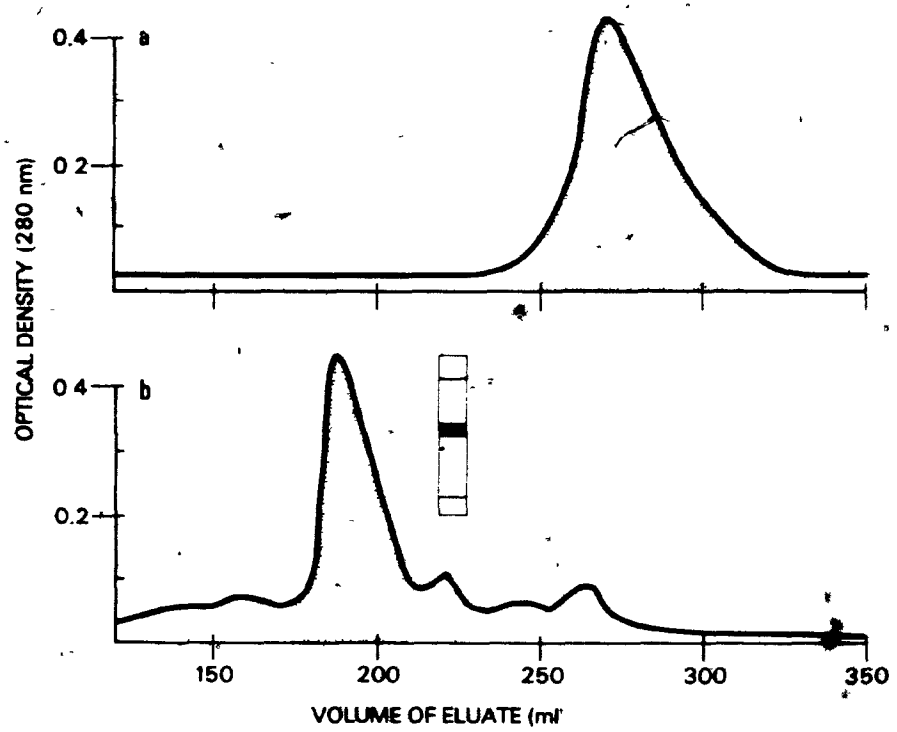

Fig. 28. Sephadex G-50 superfine chromatography of (a) native and (b) reduced and alkylated Ra3-I.

The column bed was 2.5 x 90 cm, the buffer in "a" was 0.01 M phosphate, pH 7.1. The buffer in "b" contained 0.1% SDS. Also shown is the electropherogram of an SDS polyacrylamide gel run of a sample (20 µg) from the shaded peak fraction (b).

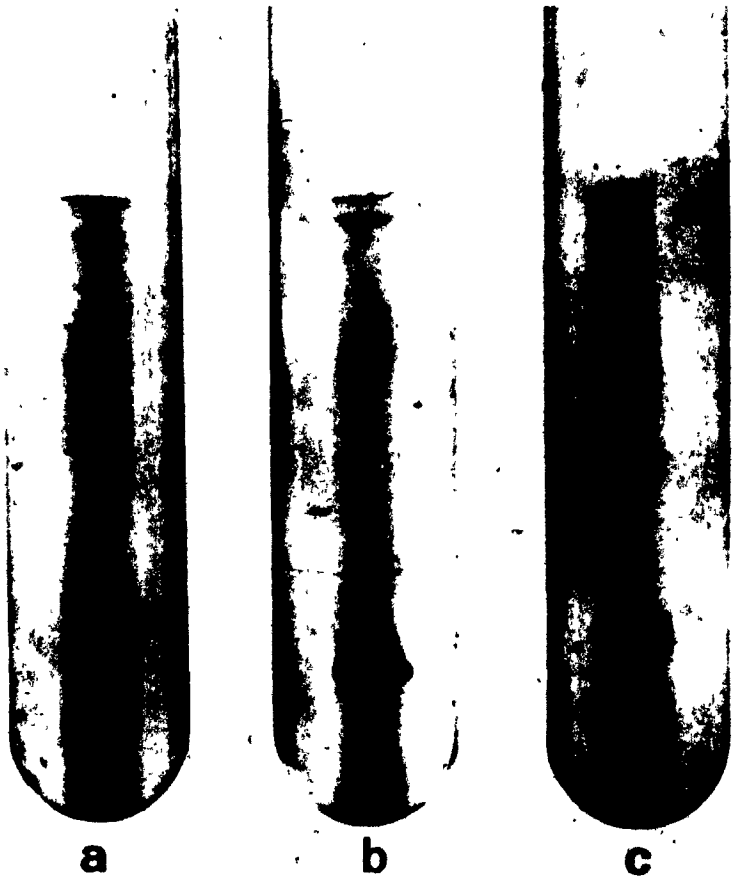

Fig. 29. SDS-polyacrylamide gel electrophoresis of antigens Ra5(b) and Ra3(c) in dithiothreitol.

The gel in "a" was used for the reagent blank run. Sample loads were approx 20 ug.

GLY LYS VAL TYR LEU VAL GLY GLY PRO GLU

LEU GLY GLY TRP LYS LEU GLN SER ASP PRO

ARG ALA TYR ARG LEU

Fig. 30. The N-terminal amino acid sequence of antigen Ra3.

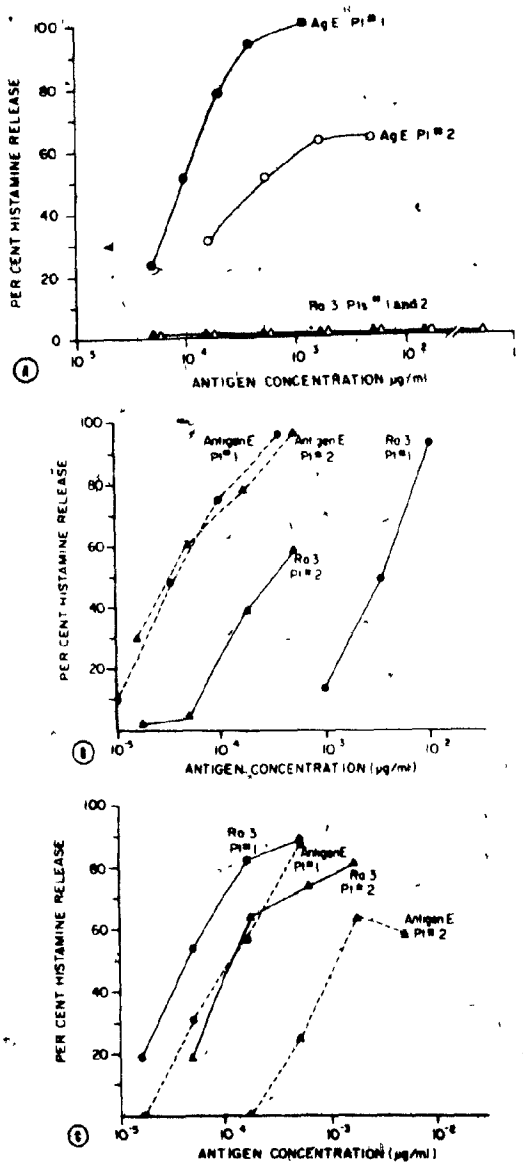

Fig. 31. Patterns of histamine release on challenge of the leucocytes of different patients with ragweed antigens E and Ra3.

A. Two patients sensitive to antigen E but totally unreactive to antigen Ra3.
B. Two patients sensitive to both antigens but more highly sensitive to antigen E.
C. Two patients more sensitive to antigen Ra3 than to antigen E.

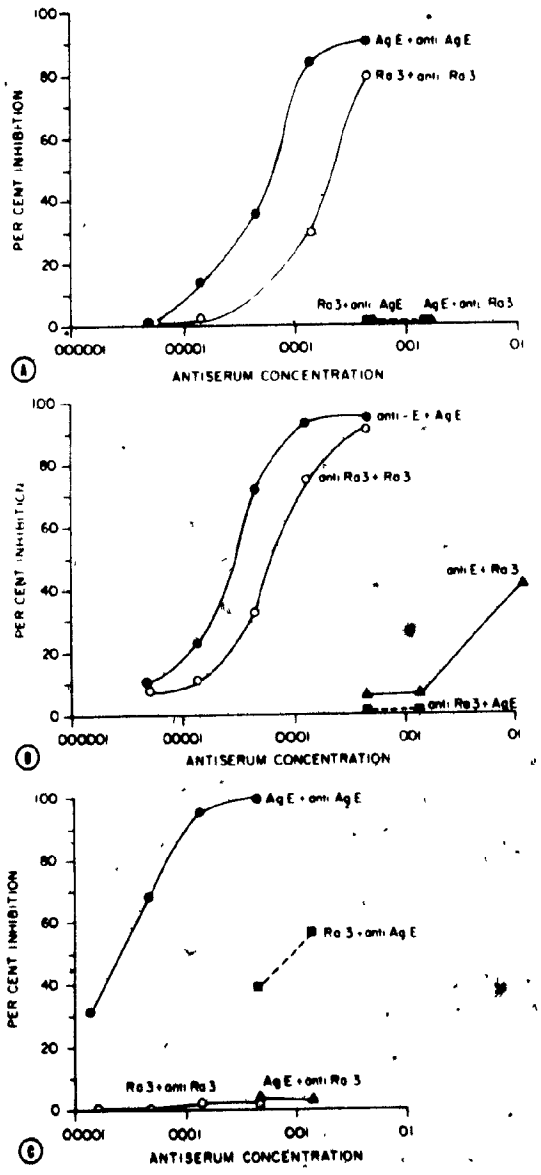

Fig. 32. Inhibition of antigen E and Ra3 histamine release by rabbit antisera.

A. Specific inhibition by rabbit antisera to antigens Ra3 and E.
B. The same inhibition pattern as A, but demonstrating some cross-reactivity between rabbit anti-antigen E and antigen Ra3.
C. The failure of anti-Ra3 to inhibit antigen Ra3-mediated histamine release together with its inhibition by anti-antigen E.

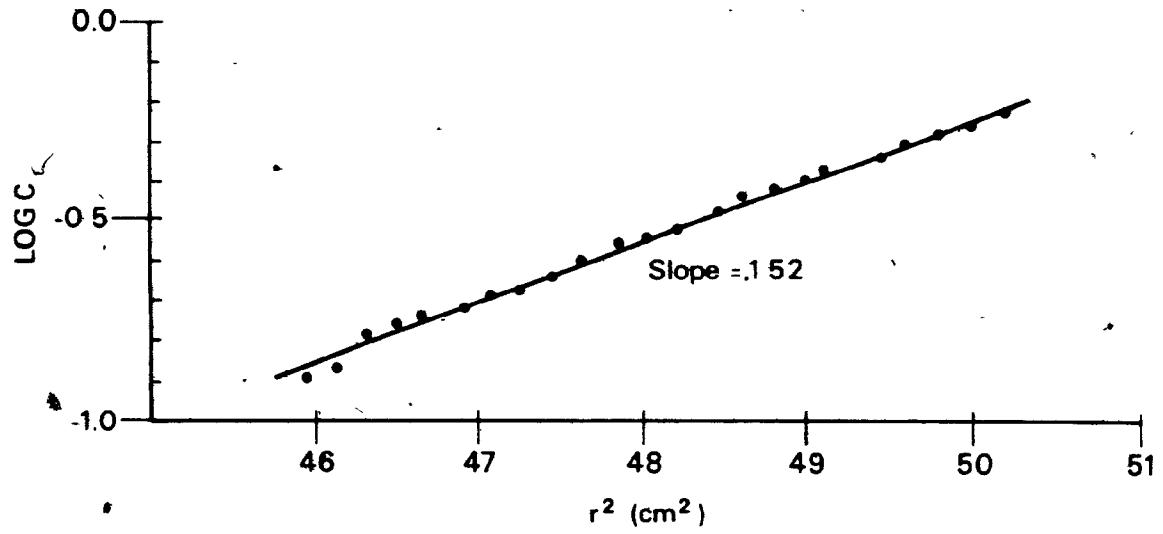

Fig. 33. Plot of log Ra4 concentration (C) vs square of distance from rotor axis (r^2).

The slope was calculated by least squares analysis.

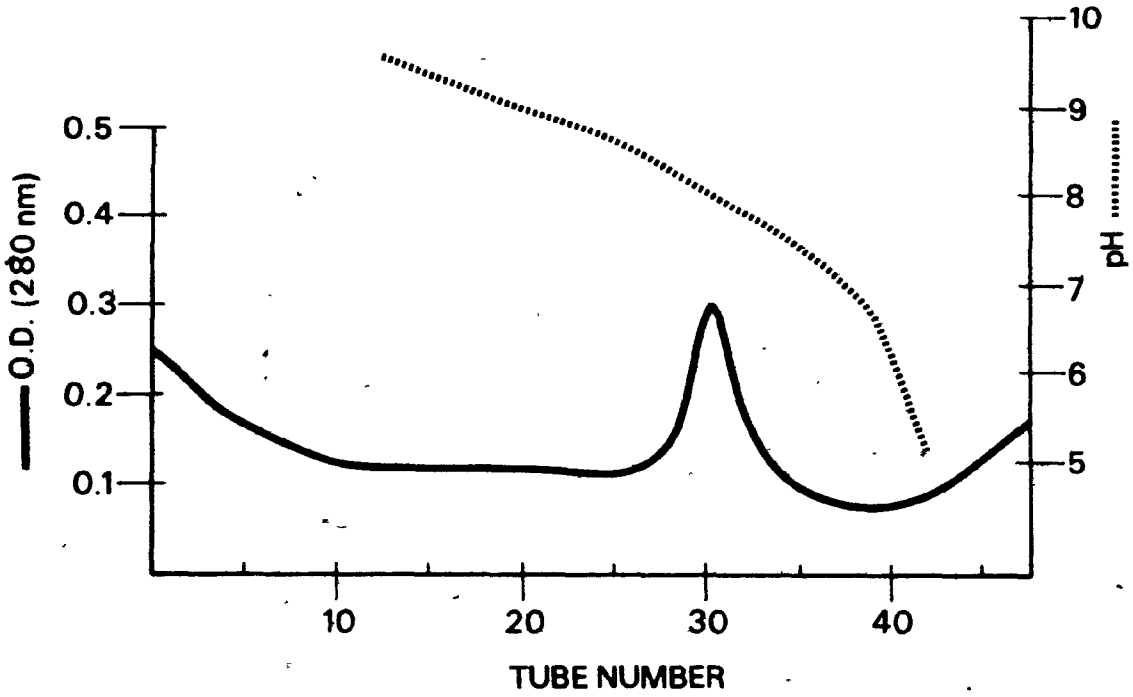

Fig. 34. Isoelectric focussing of antigen Ra4.

Shown is the elution pattern (O.D. was directly determined) from a glycerol density gradient column. The sample load was 7 mg, the volume/tube was 2.5 ml, the ampholine range was 7-10 and the focussing time was 4 days at 600 volts.

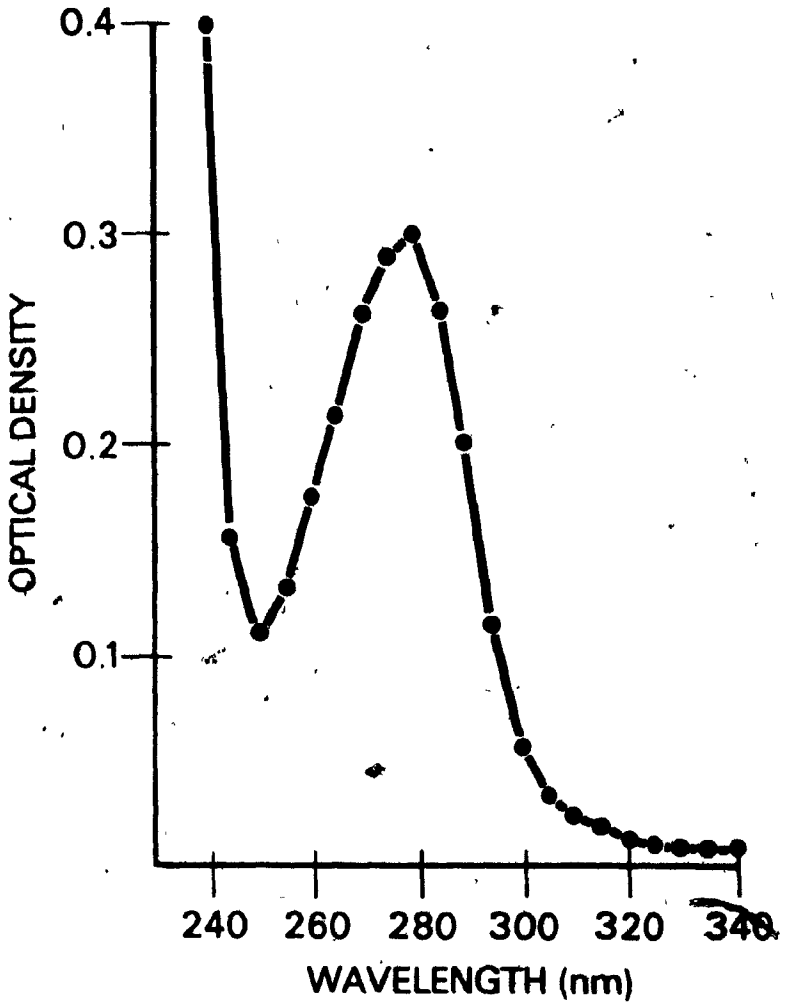

Fig. 35. Absorption spectrum of antigen Ra4.

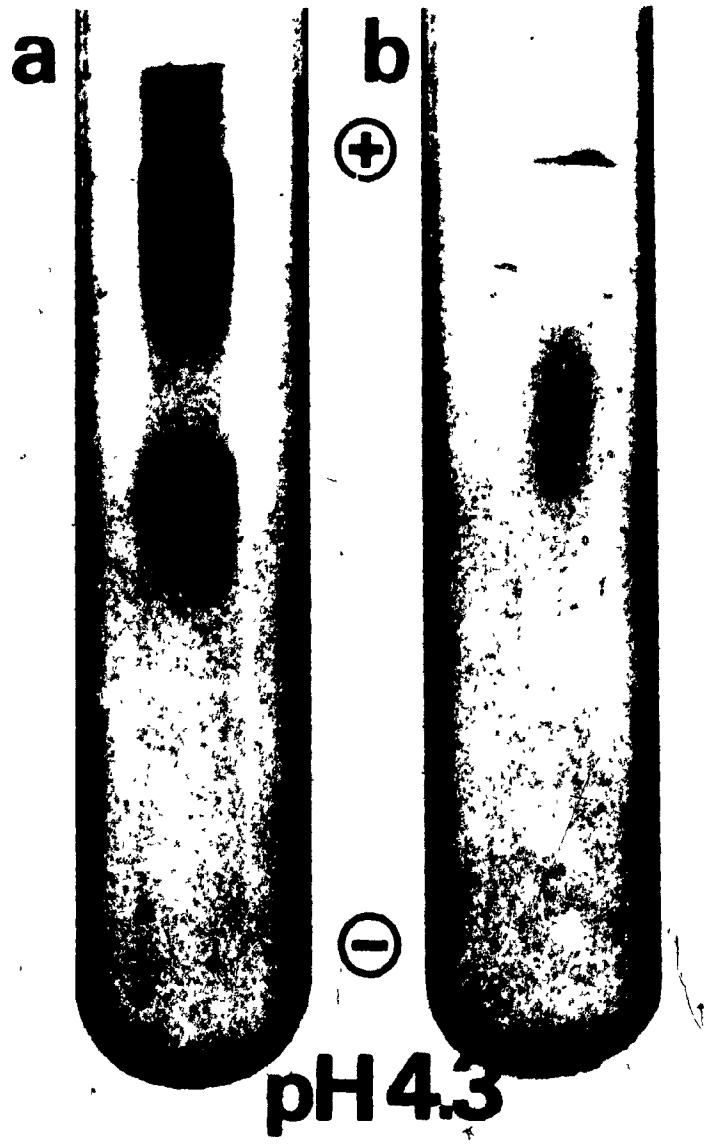

Fig. 36. Polyacrylamide gel disc electrophoresis at pH 4.3 of an Ra4 preparation.

The left hand gel was stained with Coomassie blue, the gel to the right with a PAS stain for carbohydrate.

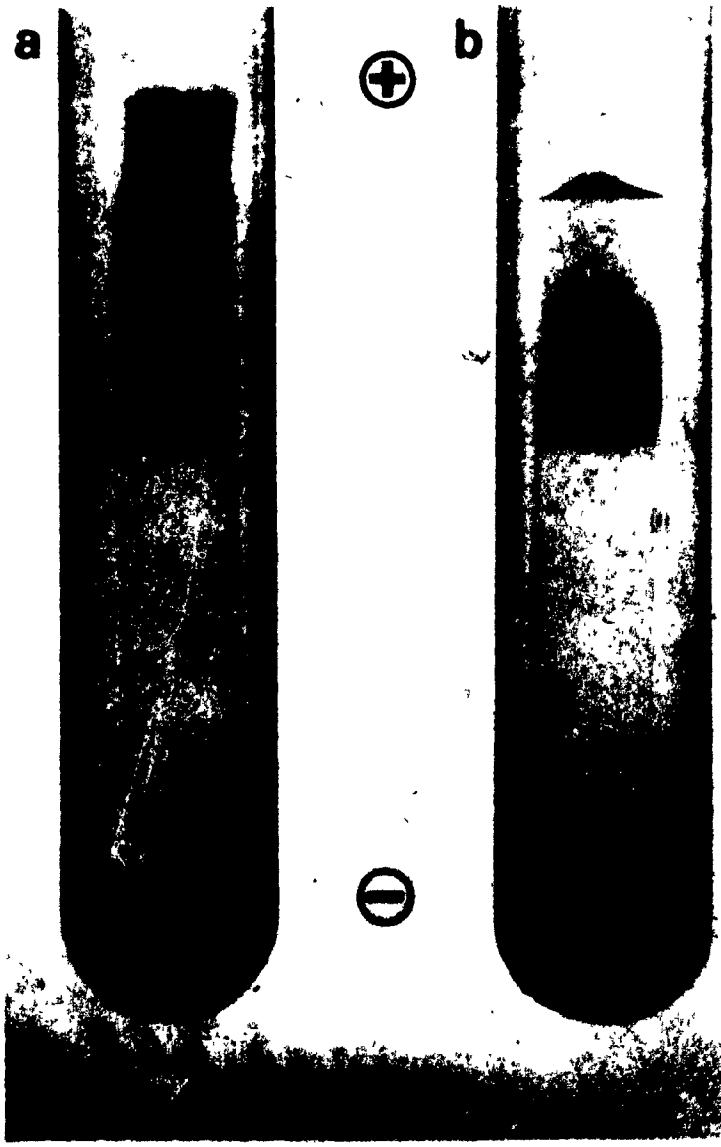

Fig. 37. Polyacrylamide gel disc electrophoresis at pH 4.3 of an Ra4 isolate from preparative acrylamide electrophoresis.

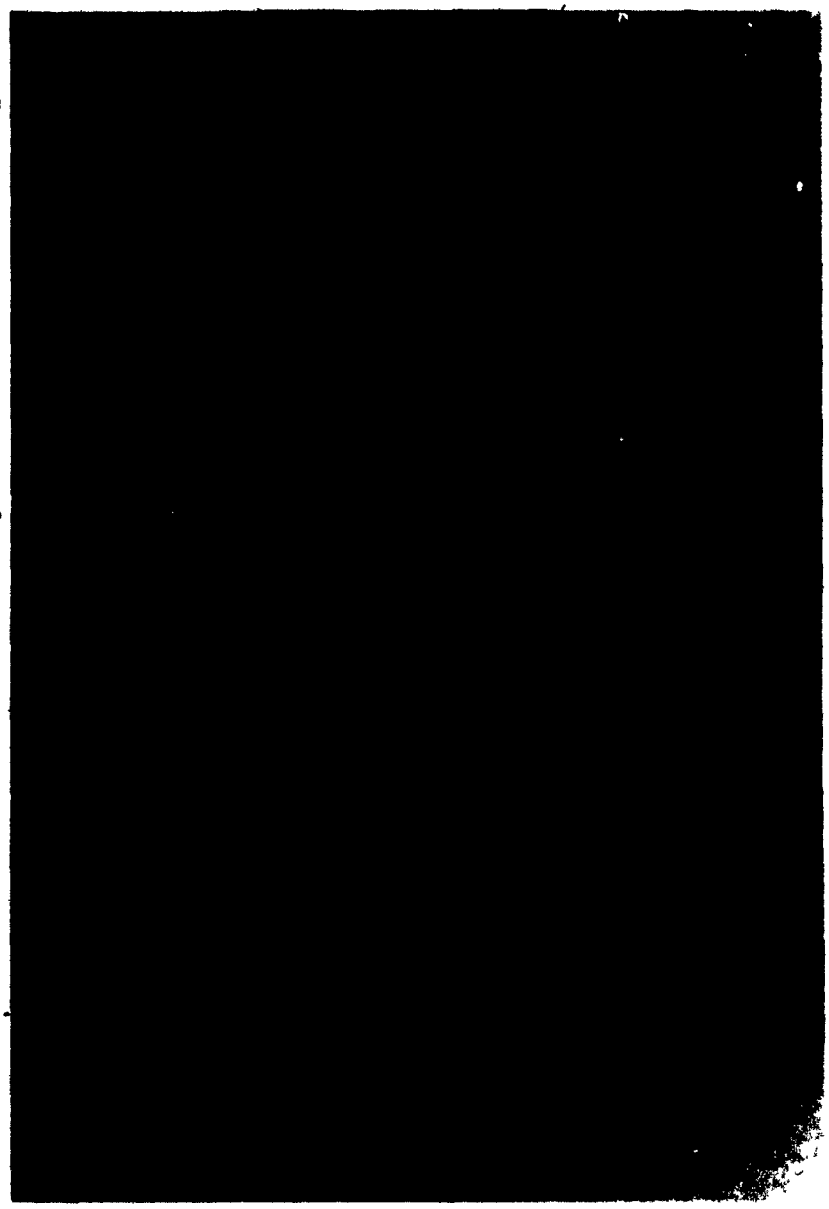

Fig. 38. SDS polyacrylamide gel electrophoresis of antigen Re4 in dithiothreitol.

The sample load was approx. 10 µg.

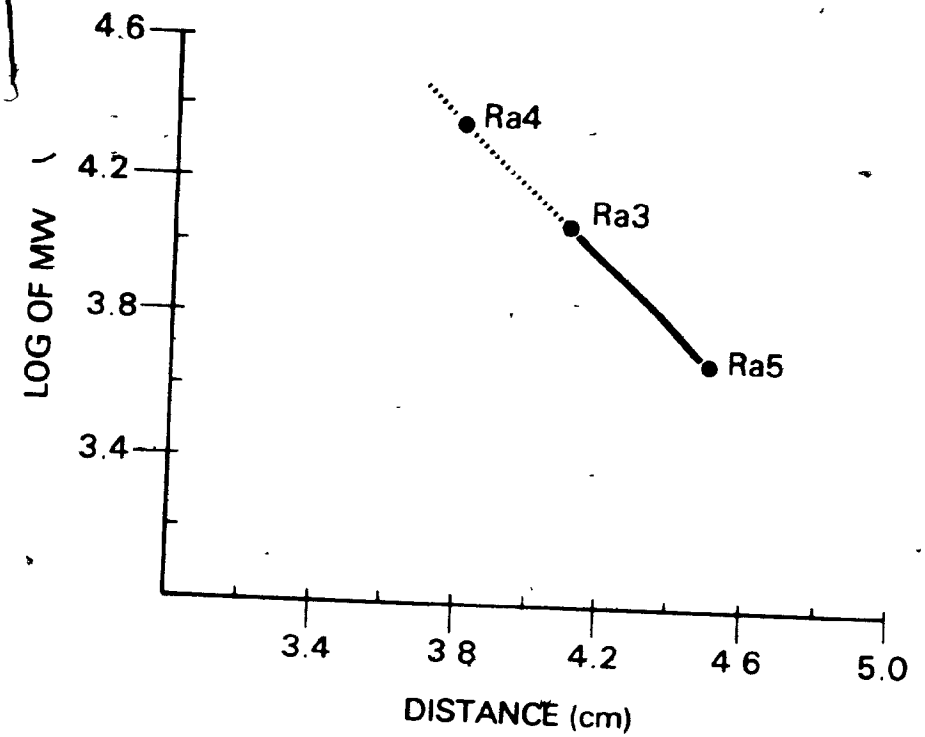

Fig. 39. Plot of log mol wt vs distance travelled on an SDS polyacrylamide gel.

The bromphenol blue marker was at 4.8 cm at the end of the run.

ALA CYS TYR VAL VAL ASP TRP LYS MET ASP

ILU ASP PRO HIS LEU ILU LYS GLY ILU LYS

PHE VAL

Fig. 40. The N-terminal amino acid sequence of antigen Ra4.

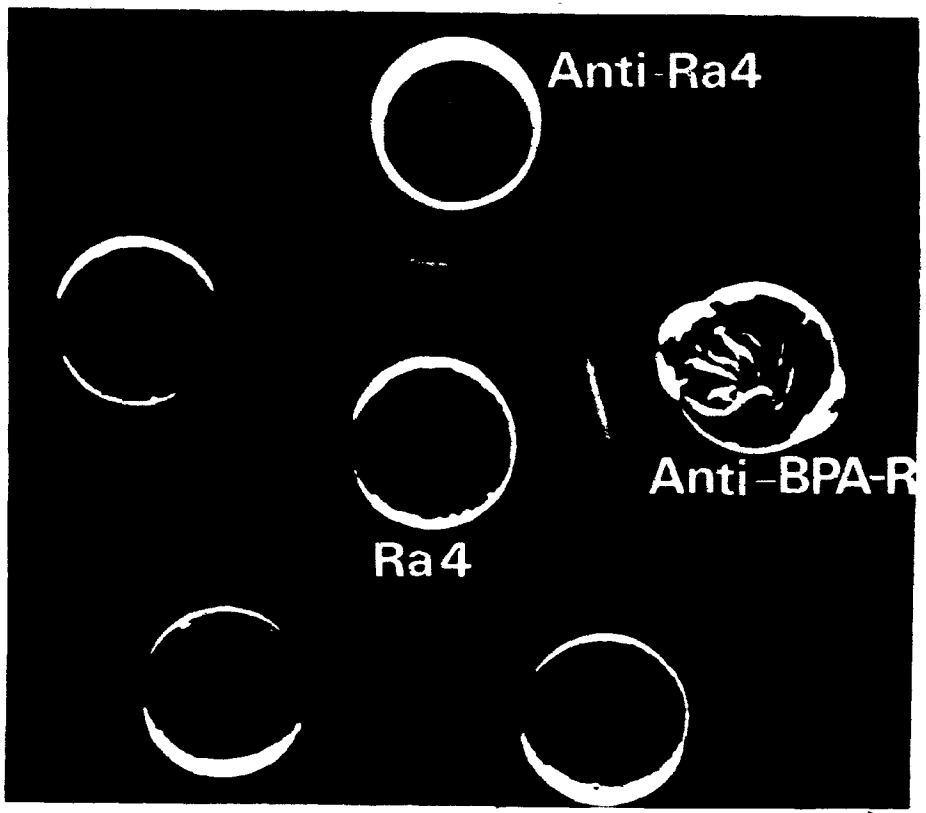

Fig. 41. Immunodiffusion of antigen Ra4 (0.20 mg/ml) against specific rabbit anti-Ra4 antiserum and rabbit antiserum to BPA-R.

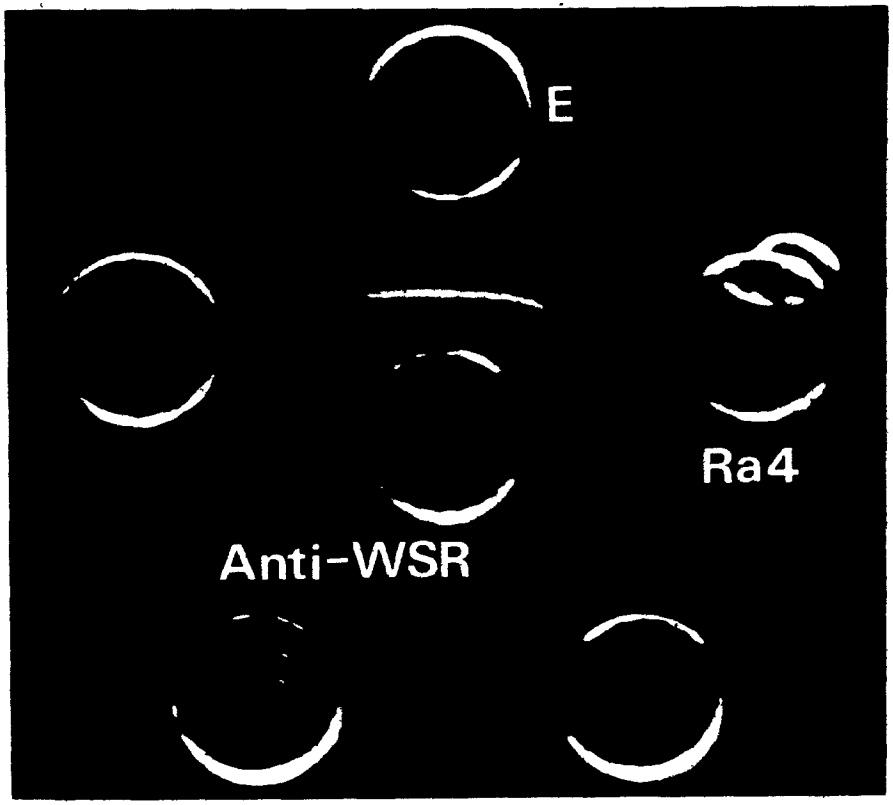

Fig. 42. Immunodiffusion of antigen E (0.10 mg/ml) and antigen Ra4 (0.10 mg/ml) against a rabbit antiserum to WSR

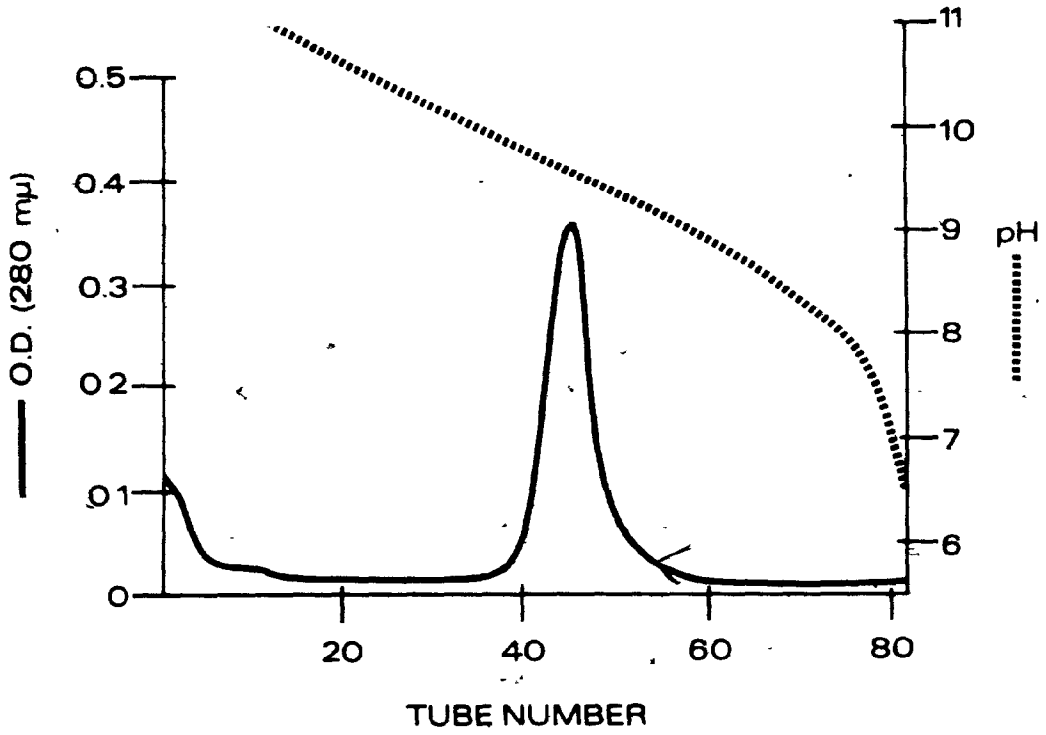

Fig. 43. Isoelectric focussing of antigen Ra5.

Shown is the elution pattern (%T converted to O.D.) from a glycerol density gradient column. The sample load was 10 mg, the volume/tube was 2.2 ml, the ampholine range was pH 9-11 and the focussing time was 4 days at 400 volts.

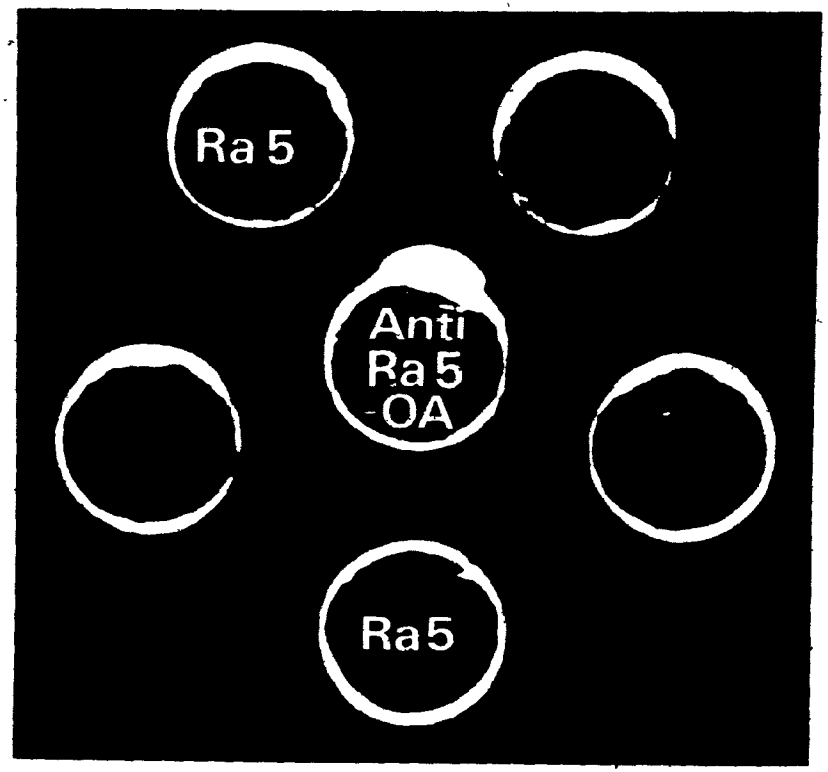

Fig. 44. Immunodiffusion of antigen Ra5 (0.05 mg/ml) against a goat antiserum to Ra5-ovalbumin conjugate.

Ra3	Ra4	Ra5	TM2	P-Lipase A
Arg-Ala-Tyr 21 22 23		Arg-Ala-Tyr 15 16 17	Lys-Ala-Phe 8 9 10	
			Arg-Ala-Leu + 1 2	
	Lys-Gly 17 18		Lys-Ala 12 13	
Ser-Asp-Pro 18 19 20	(Ile)-Asp-Pro 11 12 13	Ser-Asp-Pro 20 21 22	Thr-Asp-Ala 3 4 5	Thr-Asp-Ala 27 28 29
		Tyr-Cys 17 18		
		Tyr-Cys 25 26		
	Cys-Tyr 2 3	Cys-Tyr 32 33		Cys-Tyr 90 91
				Phe-Tyr 65 66
				Tyr-Phe 76 77
				Tyr-Phe 82 83

+ C-Terminal of TM1

Fig. 45. Homologous regions in the amino acid sequence of 5 allergens.

	Ra3	Ra4	Ra5	P-Lipase A
N-terminal	Gly	Ala	Leu	Ile
1st valine in position	3	4 + 5	2(+ Leu)	(2 = Ile)
1st Tyrosine in position	4	3	(6 = Trp)	3
1st Cysteine in position	-	2	4	9
1st Lysine in position	2 15(2nd Lys)	8	14	14

Fig. 46. Comparison of the N-terminal regions of 4 allergens.

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