

Rapidly released allergens from short ragweed pollen

I. Kinetics of release of known allergens in relation to biologic activity*

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Study of the kinetics of in vitro release of known antigens from short ragweed pollen revealed a slow release of AgE (1.5% to 4% release after 16 min) but a rapid release of other highly allergenic components. The rapidly released allergens in 16-min pollen extracts were found to be mainly highly basic proteins including the well-characterized Ra5 molecule along with several hitherto uncharacterized components. Considering previous data that suggest that particles of a similar size to ragweed pollen grains remain in the nose only about 6 to 8 min following inhalation, it is difficult to explain the apparent anomaly between the high biologic potency of 16-min extracts and their low AgE content. Increase in the pH of the extracting buffer from the physiologically normal nasal pH of 5.5 to 6.5 to about pH 8.0 to 8.5 (characteristically found in rhinitis) increased the proportion of AgE released after 16-min extraction by about 10-fold, suggesting that response to allergens in ragweed and other inhalants may enhance AgE release and thereby aggravate allergic symptoms. Detailed comparison of the allergenic activity of AgE and of 16-min and 4-day ragweed extracts in 38 ragweed-sensitive subjects suggested that allergens other than AgE were together more important than AgE in causing ragweed allergy in most patients, although large patient-to-patient differences in relative response were observed. Our data emphasize the need to reevaluate materials used in both diagnosis and treatment of ragweed allergy. The importance of AgE relative to other ragweed pollen components with respect to induction of allergic symptomatology deserves to be placed in a more balanced perspective.

Pollen grains are highly complex plant organisms containing a large array of macromolecules that are antigenic in mammals and potentially allergenic in humans. The allergenic importance of each of the antigens is a function of its immunogenicity and concentration in the pollen and of the genetic capacity of the host to recognize the antigen as foreign and to synthesize IgE antibody.¹ A further important factor,

which has previously been largely overlooked, is the relative rate of release of each of the antigens following inhalation of the pollen grains. It seems likely that allergens that are eluted rapidly from the pollen grains on contact with the moist nasal mucosa would be among those having greatest relevance in causing allergic symptomatology. Study of the rates at which different pollen allergens are released are therefore also relevant in defining the most appropriate extracts for testing and treating allergic patients.

The present study utilizes the pollen of short ragweed (*Ambrosia elatior*) as a model for studying the kinetics of antigen release in comparison with the release of allergenically reactive material. The short ragweed model is particularly useful because several highly purified allergens have been isolated (antigen E, antigen K, Ra3, Ra4, and Ra5).

Bridger and Proctor² showed that, after inhalation, ⁹⁹Tc-labeled albumin spheres (15 to 25 μ m in diame-

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ter) of similar size to ragweed pollen grains (18 μm in diameter) were cleared from the nasal mucosa in about 6 to 8 min. The spheres rapidly migrated to the larynx, where they remained for about 20 min before being swallowed. It would therefore be reasonable to assume that the ragweed antigens provoking allergic symptoms would have to be extracted in substantial amounts within the first few minutes after inhalation of the pollen.

A further factor that might possibly affect the rates of antigen release could be variations in the pH of the nasal mucosa. Fabricant³ observed that the normal pH of the nose, in the absence of rhinitis and physical stimuli such as cold or heat, ranged from about pH 5.5 to 6.5. On the other hand, symptoms of rhinitis caused the pH to become slightly alkaline (pH 7.2 to 8.3).

Our experiments therefore explore the rates of release in vitro of known ragweed allergens under different pH conditions and compare these rates with the rate of release of allergenically active material from the pollen. In particular we focus on the rate of release of antigen E (AgE), which has been considered to be the major allergen in short ragweed pollen extract⁴⁻⁷ and which has been usefully employed in the standardization of ragweed extracts.⁸ Finally, we provide evidence for the existence of important new allergens in material that is rapidly extracted from the pollen.

MATERIALS AND METHODS

Allergens

Four batches of dried pollen of *Ambrosia elatior* (short ragweed), Lots No. 18-56-70, 18-56-71, 18-56-74, and 18-56-P9, were purchased from Greer Laboratories within 6 mo after collection and were stored at -20°C in airtight containers until used.

Highly purified short ragweed pollen allergens, antigen E (AgE),^{4,5} Ra3,⁹ and Ra5¹⁰ used in skin testing, histamine release, and antigen quantitation were kindly supplied by Dr. T. P. King, Rockefeller University and Dr. L. Goodfriend, McGill University. An exception was the AgE used for immunization of rabbits, which was purified by Sephadex G75 gel filtration from material supplied by the Research Resources Branch, National Institute of Allergy and Infectious Diseases, through the courtesy of Dr. John Nutter.

Extractions

To investigate in detail the rates of allergen extraction at a pH characteristic of the normal physiologic condition of the nose, we performed a series of extractions of pollen Lot No. 18-56-70 in physiologic borate-buffered saline,¹¹ using a ratio of one part pollen to ten parts buffer (10% w/v). Extractions were performed in quadruplicate for 1, 4, 16, 32, 64, 256, and 1024 min at $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with continuous slow magnetic stirring or for 4 days at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with

gentle rocking. The initial buffer pH was 8.4 but fell to 6.3 to 6.35 within the first minute of extraction as a result of the strong buffering action exerted by the ragweed components; the pH remained within the range of 6.2 to 6.35 throughout the extractions. These extracts will be referred to as pH 6.3 extracts. Immediately after each extraction period, the supernatant was separated from the pollen by centrifugation for approximately 1 min. The supernatants were rapidly removed, centrifuged at 4°C for 15 to 30 min (to remove remaining pollen fragments) and stored at -20°C , at which temperature no detectable change in biologic potency or antigenic composition was observed.

When comparing the properties of different extracts of Lot No. 18-56-70 on a pollen equivalent basis, extract concentrations were expressed in milligrams pollen equivalents per milliliter (mg PE/ml). For example, the supernatant from an extract of 1 mg pollen with 10 ml buffer prepared after extracting for 4 min at 23°C is referred to as a 4-min extract at 10^{-1} mg PE/ml. Solutions prepared by extracting for different times usually contained different amounts of pollen components, but the pollen equivalent concentration remained the same.

To examine lot-to-lot variation, the three other lots of pollen were similarly extracted at about pH 6.3 for 16 min at 23°C and for 4 days at 4°C . We also examined the concentrations of AgE released after 16 min at a series of pHs ranging from about pH 5.5 to 8.5 for two lots of pollen. Each extract was adjusted to the desired pH (about 5.5, 6.5, 7.5, or 8.5) by addition of small measured volumes of 2N HCl or 2N NaOH immediately after the pollen had been fully wetted with the extracting buffer (about 1 min). To minimize possible degradation of AgE at low pH, the extract prepared at pH 5.5 was adjusted to pH 7.5 immediately after extraction. King et al.⁵ have observed that AgE is stable between pH 6 and pH 8.5; therefore no adjustment of other extracts was deemed necessary. All of the experiments just described were performed in duplicate or quadruplicate.

Quantitative antigenic analysis

Rabbit anti-AgE was obtained from two rabbits hyperimmunized with repeated doses of AgE emulsified in complete Freund's adjuvant. Monospecific rabbit anti-Ra3 and anti-Ra5 (from single rabbits) were kindly supplied by Dr. Lawrence Goodfriend, McGill University. The contents of ragweed AgE and Ra3 in the various ragweed extracts were determined by radial immunodiffusion utilizing the monospecific antisera and purified antigens. The methodology was analogous to that described previously for grass group I analyses.¹² In later analyses of AgE contents (Fig. 1), the "slow rocket" procedure¹³ was utilized in the place of radial immunodiffusion. In all cases the antigenic contents per gram pollen were computed. It should also be noted that the anti-AgE serum used reacted weakly with the cross-reactive AgK, which could be detected when present in relatively high concentrations, although it could not be accurately quantitated.

The anti-Ra5 serum available at the time was not sufficiently potent for use in radial immunodiffusion analysis; hence Ra5 contents were determined by an inhibi-

tion radioimmunoassay similar to that commonly used for the measurement of IgE.¹⁴ Briefly, Ra5 was labeled with ¹²⁵I using the chloramine T procedure. The Ra5 content of each ragweed extract was determined by studying the extract's ability to inhibit the binding of ¹²⁵I-Ra5 to rabbit anti-Ra5 in comparison with an unlabeled Ra5 standard. Following a 4-hr incubation at room temperature, the resultant antigen-antibody complexes and carrier rabbit IgG were induced to precipitate by addition of a slight excess of goat anti-rabbit IgG. After overnight incubation at 4° C, the precipitates were washed and counted and the Ra5 concentrations calculated.

Estimation of nondialyzable solids

Duplicate portions of the various short ragweed extracts (at pH 6.3) from Lot No. 18-56-70 were dialyzed for a total of approximately 3 days in Visking size 18 tubing,* using 200-fold excess volumes of 0.002 M NH₄HCO₃ for each of the first four changes and deionized water for the last two. The resultant dialyzed extracts were lyophilized to constant weight (a minimum of 48 hr). The quantities of nondialyzable solids extracted per gram pollen were computed taking into account any volume changes that occurred during dialysis.

Patients

A total of 38 ragweed-sensitive adults (aged 18 to 55 yr) were tested with the various antigens. Fifteen patients (8 men, 7 women; 13 whites, 2 blacks), who had asthmatic symptoms as well as severe hay fever during the ragweed pollen season, are described in detail elsewhere.¹⁶ The remaining 23 patients (16 men, 7 women; 22 whites, 1 black) reported complete absence of asthmatic symptoms but had severe hay fever during the ragweed pollen season. Ten of the nonasthmatics had received immunotherapy with alum-precipitated ragweed extract during the previous 3 yr¹⁷ and were currently undergoing therapy with aqueous ragweed antigen. These subjects will be referred to as "treated for hay fever." About half of each of the asthmatic and remaining nonasthmatic patients had never received immunotherapy, and the balance had received immunotherapy at some time in the past but not in the previous 3 yr. These subjects will be referred to as "untreated asthmatic" and "untreated for hay fever," according to whether they had asthma.

Skin testing

Quantitative intradermal skin-test titrations were performed on the forearms of the allergic subjects, using serial tenfold dilutions of the purified allergens (AgE, Ra3, and Ra5) and the crude 4-, 16-, and 64-min and the 4-day extracts of Lot No. 18-56-70 (prepared at pH 6.3) using meth-

odology previously described in detail.¹⁸ We commenced testing the purified allergens at 10⁻⁴ µg/ml and the crude extracts at a 10⁻⁸ mg PE/ml. The concentrations of further test solutions were increased or decreased as appropriate until 2+ (0.8- to 1-cm wheal with erythema) skin-sensitivity end points were obtained. If necessary, 2+ end points were interpolated from allergen concentrations that produced larger and smaller reactions.

Skin testing of eluates from the agarose gel experiment (see below) was performed by the prick-test method,¹⁹ utilizing 26-gauge disposable hypodermic needles, with the modification that tests were performed at single rather than a series of antigen concentrations.

Leukocyte assays

Leukocyte histamine-release assays were performed *in vitro* according to the method of Lichtenstein and Osler²⁰ as modified by May et al.,²¹ using AgE, and the 16- and 64-min and the 4-day ragweed extracts of Lot No. 18-56-70 (pH 6.3). The leukocyte sensitivity of an individual to a particular preparation was expressed as the concentration of allergen (mg PE/ml) required to induce 50% histamine release from the leukocytes. When analyzing the biologic activities of different ragweed preparations relative to AgE, most comparisons were made on an AgE equivalent basis, i.e., in terms of the content of AgE in the extract.

Agarose gel electrophoresis

Analytical agarose gel electrophoresis was performed at pH 8.6 for 50 min at 15 V/cm using a water-cooled apparatus similar to that described by Laurell²² (cf. reference 13). Sixteen-minute and 4-day extracts of Lot No. 18-56-70 (pH 6.3) were first concentrated 10-fold (to 1,000 mg PE/ml) by pressure dialysis at 4° C through an Amicon UM2 membrane (molecular cutoff approximately 2000 daltons). Four-microliter samples of the concentrated extracts were applied in sample slits in the gel. After completion of the electrophoresis, the protein bands were fixed with picric acid-acetic acid solution for 15 min. Excess picric acid was removed by washing with 95% ethanol for 5 min, and the gels were dried and stained with Coomassie blue.¹³ Since Ra5 was not fixed in agarose gels by picric acid (nor by a variety of combinations of trichloroacetic acid, sulphosalicylic acid and phosphotungstic acid that we tried), the position of Ra5 was determined in eluates of portions of the gel using the radioinhibition method already described.

To examine the distribution of allergenic material in the 16-min extract, we carried out a separate agarose electrophoresis experiment using conditions similar to those described above. Instead of using a series of individual sample slits, 100 µl 10-fold concentrated 16-min extract were applied into a wide sample slit cut in the central part of the gel. After completing the electrophoresis, the two outer edges of the gel containing electroseparated samples of the extract were fixed, dried, and stained to locate the protein-staining bands. The remaining section of the gel containing the separated fractions (from 80 µl 10-fold concentrated 16-min extract) was cut into 0.5-cm strips perpendicular to

*Experiments by Craig and King¹⁵ and in our laboratory (unpublished) have shown that the size 18 tubing gives smaller losses of low molecular weight peptides, such as the B chain of insulin and Ra5 (mol wt 3600 and 5000 daltons, respectively) as compared with Visking "dialysis" tubing, size 20, and Spectrapore 3 (Spectrum Medical Industries).

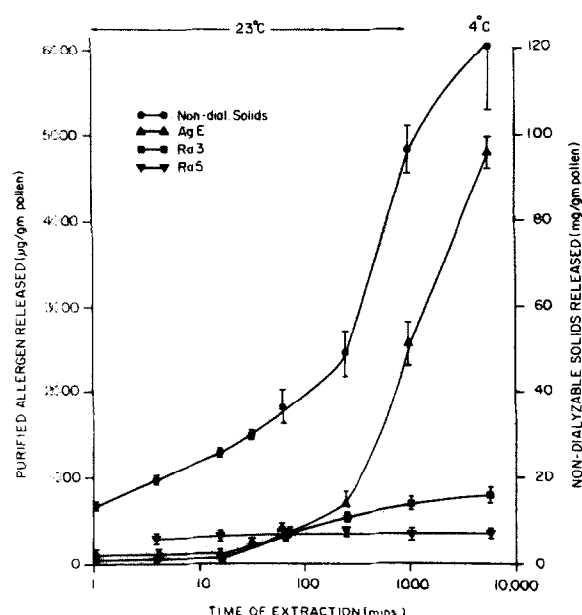


FIG. 1A. Kinetics of release of nondialyzable pollen solids, AgE, Ra3, and Ra5 from nondefatted short ragweed pollen extracted in vitro for between 1 and 1,024 min at 23° C or for 4 days (5,760 min) at 4° C. Total amount of each allergen released ($\pm$ SD) is shown.

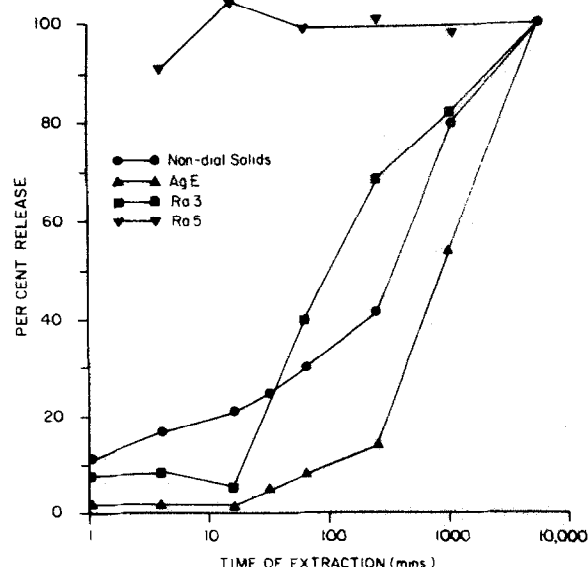


FIG. 1B. Kinetics of release of nondialyzable pollen solids, AgE, Ra3, and Ra5 from nondefatted short ragweed pollen extracted in vitro for between 1 and 1,024 min at 23° C or for 4 days (5,760 min) at 4° C. Percentage of each allergen released relative to the 4-day value is shown.

TABLE I. Lot-to-lot variation in concentrations of antigen E in 16-min and 4-day extracts (at approximate pH 6.3)

Lot number	16-min extracts (23° C)		4-day extracts (4° C)	
	pH during extraction*	AgE extracted (μ g/gm pollen) [†]	pH during extraction [†]	AgE extracted (μ g/gm pollen) [†]
18-56-70	6.30 – 6.35	70.7 $\pm$ 1.1	6.20 – 6.35	4765 $\pm$ 157
18-56-71	6.30 – 6.35	93.7 $\pm$ 1.3	6.18 – 6.35	4715 $\pm$ 76
18-56-74	6.35 – 6.54	189 $\pm$ 17	6.16 – 6.49	4672 $\pm$ 231
18-56-P-9	6.25 – 6.41	193 $\pm$ 16	Not determined	

*Measurements were made at 2 and at 16 min. The lower value was always at 16 min.

[†]Means ($\pm$ SD) from two to four different extracts. Measurements were made at 2 min and at 1, 2, 3, and 4 days. Lowest value was always at 4 days and highest at 2 min.

the electric field. The strips were separately frozen overnight at -20° C, allowed to thaw, and centrifuged. The resultant supernatants (approximately 0.5 ml per strip) were used for Ra5 analyses and prick skin testing of seven ragweed-sensitive individuals.

RESULTS

Fig. 1 shows the kinetics of release of AgE, Ra3, and Ra5 and total nondialyzable solids from nondefatted short ragweed pollen (Lot No. 18-56-70) at pH 6.3. Fig. 1, A, shows the data in terms of the total amounts of materials released, and Fig. 1, B as percentage release, taking the values for the 4-day extract as 100%. It was of great interest to find that neither AgE (considered to be the major ragweed allergen) nor Ra3

were rapidly released from the pollen under slightly acidic extraction conditions resembling those found in the nose under normal physiologic conditions in the absence of rhinitis.³ Assuming that the 4-day extract contained the maximum extractable proportions of the antigens (4732 μ g AgE and 810 μ g Ra3 per gram native dry pollen), only 1.5% of the AgE and 7.5% of the Ra3 in the pollen had been extracted in the first 16 min. On the other hand, all of the Ra5 had been released by this time. After a 16-min "lag-period," AgE and Ra3 were released at somewhat different relative rates, with AgE being released more slowly. Although the AgE concentration was higher after extraction for 4 days at 4° C than after extraction for 1024 min at 23° C, this could well be the result of thermal or

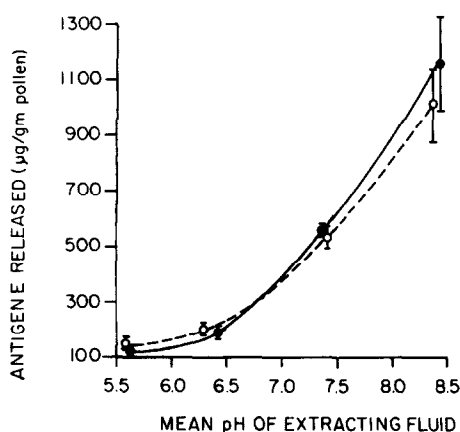


FIG. 2. Release of ragweed AgE ($\pm$ SD) from short ragweed pollen following extraction for 16 min at 23° C as a function of the pH of the extracting fluid. Solid line, data for pollen lot 18-56-74; dashed line, data for lot 18-56-P9.

enzymatic degradation of the AgE at 23° C. Large amounts of AgK could be found in the 4-day extract, but none could be detected in the 16-min extract using the cross-reacting anti-AgE serum. Analysis of the nondialyzable solids showed that about 50 mg/gm pollen had been released at 16 min and 120 mg/gm pollen after 4 days. Subsequent analyses have shown that 95 to 125 mg/gm pollen is the normal concentration range of nondialyzable solids in 1- or 4-day extracts prepared at 4° C.

To examine AgE release in different lots of pollen, three further lots of ragweed pollen were extracted at about pH 6.3 for 16 min and 4 days. The results (Table I) show some lot-to-lot variation in the AgE contents of the 16-min extracts, with Lot Nos. 18-56-70 and 18-56-71 averaging about 40% of the AgE content of the other two lots. However, the AgE contents of the 4-day extracts were similar, indicating that these differences did not primarily result from a large intrinsic variation in the total AgE in the different lots of pollen. The maximum release of AgE after 16 min of extraction, seen in Lot Nos. 18-56-74 and 18-56-P9, was only about 4% of the 4-day value. Variation in the proportion of AgE released after only 16 min is not unexpected in view of probable lot-to-lot variation in wetting properties of the dried pollen.

Our detailed study of the effect of pH on the release of AgE over the range of pH 5.5 to 8.5 (Fig. 2) showed an increasingly more rapid release of AgE with increasing pH. For the two lots of pollen studied, the relative rates of release were 7- to 8-fold higher at pH 8.5 than at pH 5.5. Although Ra5 was not measured in these particular experiments, a series of subsequent experiments has shown that 280 to 350 μ g Ra5 per gram

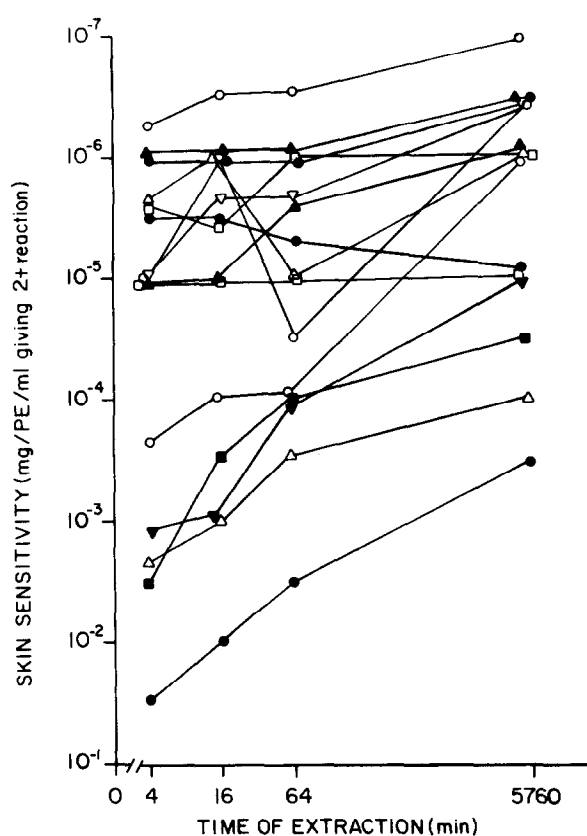


FIG. 3. Intradermal skin test sensitivity to 4-, 16-, 64-, and 5,760-min extracts of short ragweed pollen in the 15 untreated asthmatic patients.

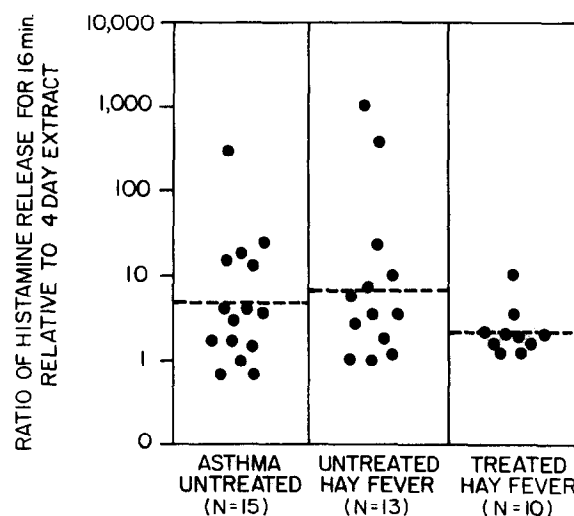


FIG. 4. Comparison of leukocyte histamine-release sensitivity ratios, 16-min/4-day, in the three groups of patients studied. In determining these ratios, the concentrations of both 16-min and 4-day ragweed extracts required for 50% histamine release were expressed in terms of pollen equivalents per milliliter.

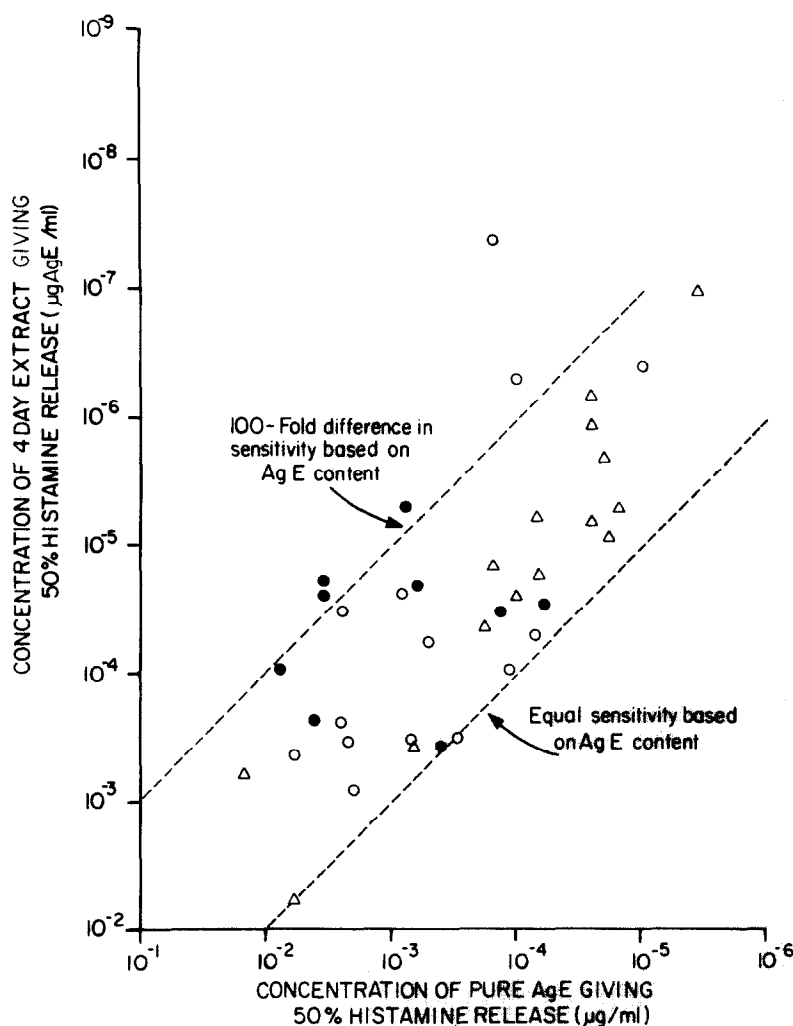


FIG. 5. Comparison of patient histamine-release sensitivity to 4-day short ragweed pollen extract and ragweed AgE. The concentration of the 4-day extract is expressed in terms of its AgE content. ○, Patients untreated for hay fever; ●, patients treated for hay fever; △, untreated asthmatic subjects.

pollen are released under a wide variety of short- and long-term extraction conditions for different lots of pollen.

We investigated the kinetics of release of allergenically active material by studying skin-test response in 15 asthmatic patients to the identical 4-, 16-, and 64-min and the 4-day extracts of Lot No. 18-56-70 used for the detailed studies of the kinetics of antigen release at pH 6.30 to 6.35. Most of the patients showed increasing skin-test sensitivity on a pollen equivalent (PE) basis to extracts prepared using increasing periods of extraction (Fig. 3). However, one patient showed no detectable increase in response to the 4-day as compared with the 4-min extract, and another showed a slight but not significant decrease. Six other patients exhibited only a 1 $\log_{10}$ or lower

increase in sensitivity to the 4-day as compared with the 4-min extract. Surprisingly, two patients showed a decreased sensitivity to the 64-min extract as compared with the 16-min and 4-day extracts, suggesting that these patients may be highly sensitive to allergen(s) that are rapidly released and subsequently degraded. One of the latter patients consented to donate blood samples for two histamine-release experiments, both of which confirmed our skin-test results. Finally, it is worth noting that the greatest increases in patient sensitivity [$\log_{10}$ (sensitivity to 4-day extract) minus $\log_{10}$ (sensitivity to 4-min extract)] occurred in those patients who were least sensitive to the 4-min extract.

Because the increases in patient response to 4-day as compared with 4-min extracts were often quite

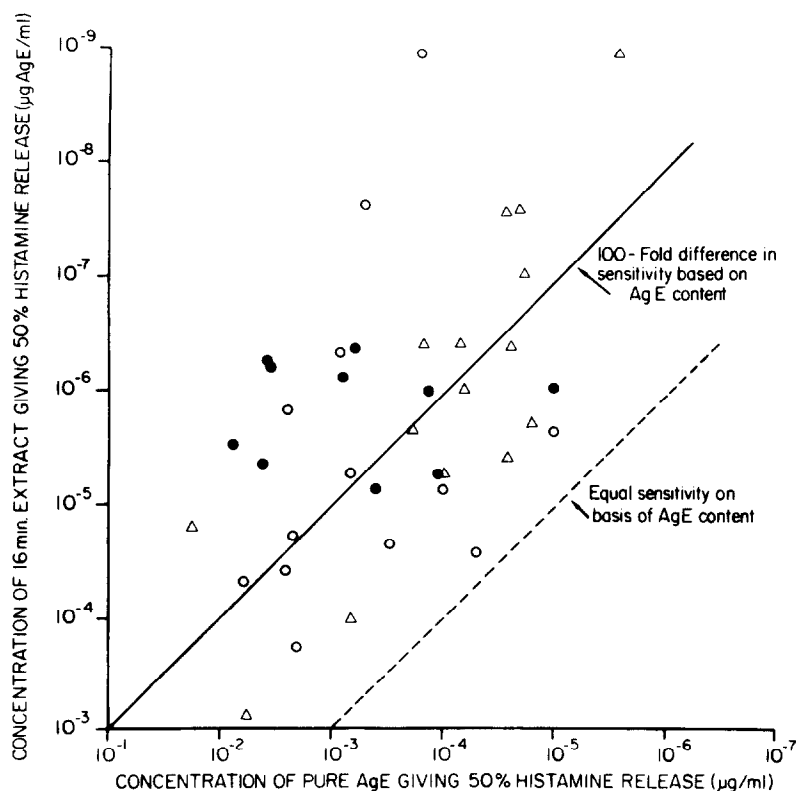


FIG. 6. Comparison of patient histamine-release sensitivity to 16-min short ragweed pollen extract and ragweed AgE. The concentration of 16-min extract is expressed in terms of its AgE content. $\circ$, Patients untreated for hay fever; $\bullet$, patients treated for hay fever; Δ , untreated asthmatic patients.

small, despite the enormous difference in AgE content, we made a more quantitative study of the relative allergenic activities of 16-min and 4-day crude ragweed pollen extracts by leukocyte histamine-release assay in all three patient groups (Fig. 4). The sensitivity ratios (16-min/4-day) most commonly lay in the range of 1 to 10 with geometric means of 4.8, 7.8, and 2.1 for patients with untreated asthma, untreated hay fever, and treated hay fever, respectively. There were no significant differences between any of those geometric means by *t* test analysis. Thus the 4-day extract was usually not dramatically more active than the 16-min extract on a pollen equivalent basis, despite the fact that the 4-day extract contained almost 70 times more AgE than the 16-min extract. Thus these findings completely confirm the skin-test data. Linear regression analysis of $\log_{10}$ sensitivities to 16-min and to 4-day extracts in each of the three patient groups showed no significant differences in the slopes of the regression lines. The correlation between $\log_{10}$ sensitivities to 16-min and 4-day extracts in the combined patient groups was excellent ($r = 0.81$, $p < 0.001$), with a slope of 0.76.

We next investigated by histamine-release assay

the contribution of AgE to the allergenic activities of the 16-min and 4-day extracts. Patient sensitivities to AgE and to each of the two extracts were compared on an AgE equivalent basis (i.e., extract concentrations were expressed *only* in terms of their AgE content). There was a significant correlation between sensitivities to AgE and to the 4-day extract ($r = 0.71$, $p < 0.001$, with a slope, $b = 0.87$; Fig. 5); however, it is apparent from the figure, as well as from the intercept of the regression line ($a = -1.51$ on the $\log_{10}$ scale), that most allergic subjects respond to allergens other than AgE. The correlation between sensitivities to AgE and to the 16-min extract was not as striking ($r = 0.46$, $p < 0.001$, with a slope, $b = 0.64$; Fig. 6). Both from examination of the figure and from the intercept of the regression line ($a = -3.26$ on a $\log_{10}$ scale), it is clear that AgE contributes only a small fraction of the total biologic activity of the 16-min ragweed extract, the fraction varying considerably from person to person.

We made a preliminary comparison of the protein components and allergenically active materials in the 16-min and 4-day extracts following separation by agarose gel electrophoresis (Fig. 7). When tested at

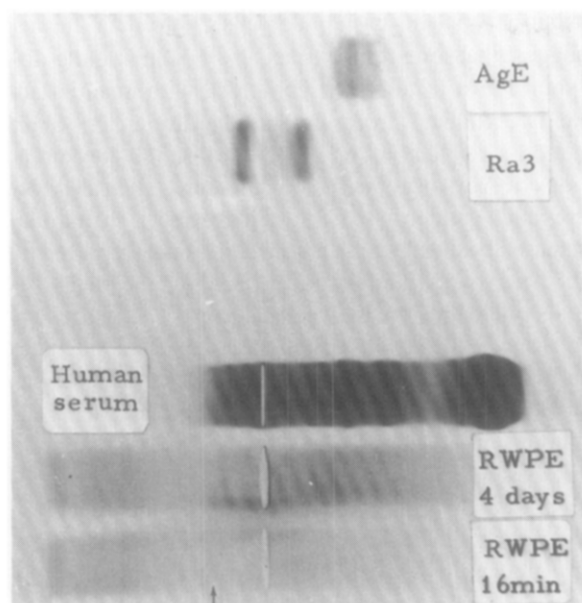


FIG. 7. Agarose gel electrophoresis (at pH 8.6) of 16-min and 4-day ragweed pollen extracts (RWPE), human serum (4 μ l), and ragweed AgE (20 μ g) and Ra3 (20 μ g) (NIH Research Reference Reagents). Arrow, Ra5 (not fixed by picric acid in the gel).

the same pollen equivalence, both fractions showed similar compositions of cathodic protein bands. However, anodic bands were seen almost exclusively in the 4-day extract. By simultaneous gel analyses of AgE and Ra3 standards, the major bands of these allergens could be preliminarily identified in the pattern of the 4-day extract, identification being confirmed by immunodiffusion analysis of portions cut from untreated gels. Because Ra5 did not precipitate with picric acid, it was localized by radioimmunoassay, and its position in the gel is indicated on the figure. Allergens AgK and Ra4 could not be unequivocally located in this electrophoretogram, because of the unavailability of monospecific antisera toward these components. It should, however, be noted that AgK is an acidic allergen ($pI = 5.9$ for the "A" form) that migrates slightly slower than AgE at pH 8.6, and that such an acidic band(s) is absent on the 16-min but present in the 4-day extract. The pI of Ra4 has been found to be 8.0,²³ which would indicate that it should migrate slightly toward the anode at pH 8.6. Thus most of the rapidly extractable cathodic components appeared to be unrelated to any previously identified allergens with the notable exception of Ra5.

Seven ragweed-allergic subjects were scheduled for skin testing with the electrophoresed components of the 16-min and 4-day extracts. First, their skin sensitivity to the unfractionated 16-min in relation to the

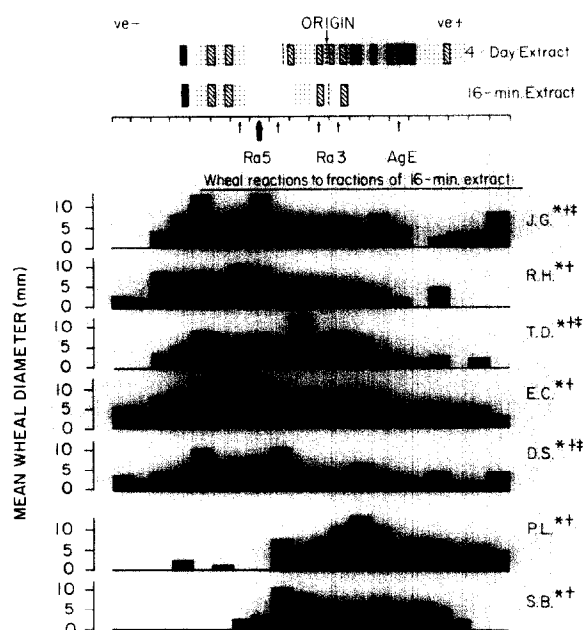


FIG. 8. Patterns of response of seven ragweed-sensitive patients following prick testing with electrophoresed fractions of 16-min ragweed pollen extract. Wheal diameters of 2 mm or greater indicate significantly positive reactions. Stained patterns of electrophoresis runs of 16-min and 4-day extracts, together with the location of ragweed AgE, Ra3, and Ra5, are indicated at the top of the diagram. Patient sensitivity, determined by intradermal end-point skin testing to highly purified allergens, is indicated as follows: *AgE, †Ra3, and ‡Ra5.

4-day extract was estimated by end-point prick testing using serial 10-fold dilutions of both extracts, diluted on the basis of pollen equivalents per milliliter. The first five subjects were found to be similarly sensitive to the crude 16-min and 4-day extracts at the same pollen equivalence (sensitivity ratios, 16-min/4-day, were between 1 and 0.2). The last two individuals were about ten times more sensitive to the 4-day extract. The patterns of their prick-test responses (millimeter wheal diameter) to electrophoresed fractions of the 16-min extract (Fig. 8) show marked patient-to-patient differences. Of particular interest, the response patterns clearly reflect a markedly higher frequency and degree of response to the basic components in the first five patients as compared with the last two. A schema of the electrophoretic patterns of 16-min and 4-day extracts, together with the location of allergens AgE, Ra3, and Ra5, is included for comparison. The sensitivities of individual patients to AgE, Ra3, and Ra5, determined by previous intradermal skin testing, are reflected in the sensitivities of the patients to the specific electrophoresed fractions that contain these components in a partially purified state.

DISCUSSION

We have demonstrated that antigens that are rapidly released from short ragweed pollen (within 4-to 16-min extraction *in vitro*) are highly allergenic in most ragweed-sensitive individuals. In view of the high allergenic activity of short-term extracts, it is surprising that only 1.5% to 4% of the available AgE in the pollen is released by extracting with physiologic saline for 16 min at pH 6.3. Studies by Belin and Rowley²⁴ and Belin²⁵ showed that the major allergen is extracted very rapidly from birch pollen. Similarly, Brown and Thantrey²⁶ found that essentially all of the allergenic activity present in several different grass pollens is released during a 15-min extraction, and we have found that the major (group I) rye grass pollen allergen is essentially completely extracted within 16 min (unpublished observations). It would appear therefore that the release of ragweed AgE is exceptionally slow by comparison, in view of its reported major importance in ragweed allergy.⁴⁻⁷ Our data are also of interest in regard to the biology of pollen fertilization and are more compatible with the localization of AgE primarily but not exclusively in the intine (inner cell wall) rather than the exine of the ragweed pollen grain.^{27, 28}

Although AgE and Ra3 were released quite slowly, Ra5 had been completely released from the pollen within 4 min. However, rapid release of Ra5 could not explain the high allergenicity of short-term (relative to long-term) extracts in many subjects (Figs. 3 and 4), because only 13% of our study group were sensitive to Ra5 (subset of results reported in reference 29). These results therefore suggest the presence of further important and as yet uncharacterized allergens in ragweed pollen, as previously emphasized by Goldfarb.³⁰ Our preliminary skin-test results using fractions of 16-min extract separated by agarose gel electrophoresis at pH 8.6 show that most of the rapidly released allergens are basically charged proteins (Fig. 8).

We also found that the rate of release of AgE markedly increases by about 10-fold with increasing pH over the range of pH 5.5 to 8.5 (Fig. 2). Because persons suffering from rhinitis have nasal pHs at the higher end of this range,³ concurrent sensitivity toward other allergens (in ragweed pollen or other allergenic materials present in the atmosphere) could well cause an increase in the amount of AgE extracted and lead to greater symptomatology.

Insofar as extraction *in vitro* at pH 5.5 to 6.5 reproduces the circumstances where native pollen is extracted in the nose under normal physiologic conditions, our data suggest that the most potent ragweed allergen, AgE, is much less rapidly extracted than a

group of potent basic allergens. Our data further suggest that long-term extracts may contain quite different ratios of allergens compared with material actually extracted from the pollen during the short period it remains in the nose. Because the concentrations of AgE and other acidic allergens, such as AgK, are much greater in the long-term extracts, the rapidly released basic allergens may well be underrepresented in the extracts commonly utilized for the diagnosis and treatment of ragweed allergy. In particular, treatment dosages of the basic allergens would tend to be limited by the large local and systemic reactions induced by the larger amounts of acidic allergens administered. This could lead to inadequate immunizing dosages of basic allergens in many allergic subjects who are highly sensitive to these components. In view of these considerations, it is perhaps not surprising that treatment of allergic people with partially purified AgE (and its acidic protein contaminants)³¹ is almost as effective as treatment with conventional ragweed extracts,^{32, 33} especially when treated individuals are selected for their high sensitivity to AgE.

King et al.⁵ reported a 40-fold geometric mean decrease in eight patients' skin-test responses after ragweed pollen extract was treated with excess anti-AgE. This result is not incompatible with our data (Fig. 5) for *some* but certainly not *most* patients. Possibly their use of excess concentrated anti-AgE sera, which may not have been entirely monospecific, led to neutralization of allergens other than AgE, overemphasizing its role as an allergen.

AgE measurement appears to provide a useful standard of potency of conventional ragweed extracts, largely because there is a relatively good correlation between the allergenic reactivity of long-term ragweed extracts and their AgE content.⁸ This generally accepted conclusion is completely compatible with our data in this study, where the correlation coefficient between leukocyte response to AgE and the 4-day extract was 0.71. Norman and Lichtenstein³⁴ reported a similar correlation coefficient of 0.73 between leukocyte responses to AgE and a commercial ragweed extract. However, it is clear from Fig. 5 and the statistical evidence presented in Results that most patients respond strongly to other allergens in the 4-day extract and particularly to those also found in the 16-min extract (Fig. 6). Statistical analysis of the log₁₀-log₁₀ sensitivity data presented in Fig. 5 also reveals a roughly one-to-one relationship (slope of 0.87) between allergic response to AgE and response to the 4-day extract (where the concentration of this extract is expressed in terms of its AgE content). However, the intercept of the regression line (where sensitivity to AgE is 10⁰ µg/ml) is at a sensitivity to

the 4-day extract of $10^{-1.50}$ μg AgE equivalents/ml, clearly reflecting a much greater overall response to the combination of non-AgE allergens than to AgE itself.* It is, however, worth pointing out that average patient response to AgE may well have been greater than the average response to *individual* non-AgE allergens.

Thus, while most (over 90%) ragweed-allergic subjects develop good IgE responses and become sensitive to the highly immunogenic AgE molecule, they also become roughly *proportionally* more sensitive to the sum total of the other allergens in the 4-day extract.† These data neither refute the current use of AgE as a standard for *conventional* (long-term) ragweed extracts, nor its use in radioimmunoassays to provide an index of immune response following immunotherapy. Rather, they serve to emphasize the need for a fundamental reappraisal of the optimal composition of ragweed (and other antigen) extracts used in diagnosis and immunotherapy in relation to a broad spectrum of patient antibody responses before and after therapy. Our studies therefore clearly point to the need to identify and characterize the rapidly released ragweed allergens in order to advance our understanding of the complex problem of ragweed allergy and to improve both diagnostic and treatment materials.

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*From the regression line, the sensitivity to 4-day extract is $10^{-6.75}$ μg /ml at a sensitivity to AgE of 10^{-6} μg /ml. Thus, between the range of sensitivity to purified AgE of 10^0 to 10^{-8} μg /ml, the sensitivity to 4-day extract (on an AgE equivalent basis) ranges between 30- and 5.6-fold *higher* than toward AgE itself.

†Response to AgE is also significantly, albeit weakly ($r = 0.46$), related to response to the 16-min extract. This result is not surprising among subjects selected for their high sensitivity to ragweed, in view of the points raised in the previous paragraph. Likewise, sensitivity to grass and ragweed tends to be weakly correlated in people selected for reactivity to both allergens.

REFERENCES

- Marsh DG: Allergens and the genetics of allergy, in Sela M, editor: The antigens. New York, 1975, Academic Press, p. 271.
- Bridger GP, Proctor DF: Laryngeal mucociliary clearance. *Ann Otol Rhinol Laryngol* **80**:445, 1971.
- Fabricant ND: Significance of the pH of nasal secretions in situ. *Arch Otolaryngol* **33**:150, 1941.
- King TP, Norman PS: Isolation studies of allergens from ragweed pollen. *Biochemistry* **1**:709, 1962.
- King TP, Norman PS, Connell JT: Isolation and characterization of allergens from ragweed pollen. II. *Biochemistry* **3**:458, 1964.
- Lichtenstein LM, King TP, Osler AG: In vitro assay of allergenic properties of ragweed pollen antigens. *J ALLERGY* **38**:174, 1966.
- Norman PS, Lichtenstein LM: Capacity of purified antigens and whole pollen extracts to release histamine from leukocytes of hay fever patients. *J ALLERGY* **52**:94, 1973.
- Baer H, Godfrey H, Maloney CJ, Norman PS, Lichtenstein LM: The potency and antigen E content of commercially prepared ragweed extracts. *J ALLERGY* **45**:347, 1970.
- Underdown BJ, Goodfriend L: Isolation and characterization of an allergen from short ragweed pollen. *Biochemistry* **8**:980, 1969.
- Lapkoff CB, Goodfriend L: Isolation of a low molecular weight ragweed pollen allergen: Ra5. *Int Arch Allergy* **46**:215, 1974.
- Campbell DH, Garvey JS, Cremer NE, Sussdorf SH: Methods of immunology. New York, 1970, W. A. Benjamin Inc., p. 247.
- Baer H, Maloney CJ, Norman PS, Marsh DG: The potency and group I antigen content of commercial preparations of six species of grass pollen extracts. *J ALLERGY CLIN IMMUNOL* **54**:157, 1974.
- Axelsson NH, Kroll J, Weeke B: A manual of quantitative immunoelectrophoresis. Oslo, 1973, Universitetsforlaget.
- Ishizaka K, Tomioka H, Ishizaka T: Mechanisms of passive sensitization. I. Presence of IgE and IgG molecules on human leukocytes. *J Immunol* **105**:1459, 1970.
- Craig LC, King TP: Dialysis. *Methods Biochem Anal* **10**:175, 1962.
- Bruce CA, Rosenthal RR, Lichtenstein LM, Norman PS: Quantitative inhalation bronchial challenge in ragweed hay fever patients: A comparison with ragweed allergic asthmatics. *J ALLERGY CLIN IMMUNOL* **53**:230, 1974.
- Norman PS, Winkenwerder WL, Lichtenstein LM: Trials of alum-precipitated pollen extracts in the treatment of hay fever. *J ALLERGY CLIN IMMUNOL* **50**:31, 1972.
- Norman PS, in Rose NR, Friedman H, editors: Manual of clinical immunology. Washington, D.C., 1976, American Society for Microbiology, p. 585.
- Belin L, Norman PS: Diagnostic tests in the skin and serum of workers sensitized to *Bacillus subtilis* enzymes. *Clin Allergy* **7**:55, 1977.
- Lichtenstein LM, Osler AG: Studies on the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leukocytes by ragweed pollen antigen. *J Exp Med* **120**:507, 1964.
- May CD, Lyman M, Alberto R, Cheng J: Procedures for immunochemical study of histamine release from leukocytes with small volumes of blood. *J ALLERGY* **46**:12, 1970.
- Laurell CB: Electroimmunoassay. *Scand J Clin Lab Invest* **29**:21, 1972.
- Roebber MG: Isolation, structure and activity of ragweed pollen antigens Ra3, Ra4 and Ra5. Montreal, 1975, McGill University.
- Belin L, Rowley JR: Demonstration of birch pollen allergen from isolated pollen grains using immunofluorescence and a single radial immunodiffusion technique. *Int Arch Allergy* **40**:754, 1971.
- Belin L: Immunological analysis of birch pollen antigens with special reference to the allergenic components. *Int Arch Allergy* **42**:300, 1972.
- Brown HM, Thantrey N: Rapid extraction of grass pollen allergens and separation of their active fractions. *Acta Allergol* **31**:22, 1976.

27. Knox RB, Heslop-Harrison J: Pollen-wall proteins: Localization of antigenic and allergenic proteins in the pollen grain walls of *Ambrosia* spp. (ragweeds). *Cytobios* **4**:49, 1971.
28. Howlett BJ, Knox RB, Heslop-Harrison J: Pollen-wall proteins: release of the allergen antigen E from intine and exine sites in pollen grains of ragweed and *Cosmos*. *J Cell Sci* **13**:603, 1973.
29. Marsh DG, Bias WB, Santilli J, Schacter B, Goodfriend L: Ragweed allergen Ra5: A new tool in understanding the genetics and immunochemistry of immune response in man. *Immunology* **12**:539, 1975.
30. Goldfarb AR: Separation of new skin reactive antigens from dwarf ragweed pollens. *J Immunol* **100**:902, 1968.
31. Lowenstein H, King TP, Goodfriend L, Hussain R, Roebber M, Marsh DG: Antigens of *Ambrosia elatior* (short ragweed) pollen. II. Immunochemical identification of known antigens by quantitative immunoelectrophoresis. (Submitted to *J Immunol*.)
32. Norman PS, Windenwerder WL, Lichtenstein LM: Immunotherapy of hay fever with ragweed antigen E: Comparisons with whole pollen extract and placebos. *J ALLERGY* **42**:93, 1968.
33. Reisman RE, Wicher K, Arbesman CE: Immunotherapy with antigen E. *J ALLERGY* **44**:82, 1969.
34. Norman PS, Lichtenstein LM: Capacity of purified antigens and whole pollen extracts to release histamine from leukocytes of hay fever patients. *J ALLERGY* **52**:94, 1973.