

Characterization of ragweed pollen extracts prepared by sonication and other methods

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Extracts of ragweed pollen were prepared by stirring for 48 hours, homogenization, or sonication, and by various combinations of these methods. The extracts were compared with respect to protein yield and to degree and stability of skin-sensitizing activity in ragweed-sensitive patients. Compared with the usual method of extraction by stirring, both homogenization and sonication proved to be highly efficient, rapid methods with respect to all criteria evaluated. A decrease in allergenic activity was observed in all extracts during storage over a 20 day period. Following fractionation of extracts on Sephadex G-25 and G-100 columns, most of the skin-sensitizing activity was found in protein fractions with a molecular weight between 5,000 and 70,000, although some activity resided also in a fraction of low molecular weight.

The first detailed description of a technique for preparing allergens can be credited to Dunbar,¹ who in 1905 reported a method of pollen extraction with distilled water followed by rapid freezing and thawing. Following a number of studies by other authors²⁻⁷ on the use of various extraction media for the preparation of allergenic material, Brown⁸ observed in 1932 that highly stable and allergenic extracts of ragweed pollen were obtained by suspending the pollen in a glycerine-saline buffer at 7° C. for one week. He recommended intermittent shaking with glass beads to prevent clumping. Subsequently, Cooke and Stull⁹ reported that in their studies with ragweed pollen the highest yields of allergen were obtained by using 3 to 12 Gm. of pollen per 100 ml. of extraction

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Supported by United States Public Health Training Grant 1-T1-AI-281-01 from the National Institute of Allergy and Infectious Diseases.

Received for publication Nov. 7, 1966.

*Postdoctoral Fellow in Microbiology and Instructor in Medicine (Allergy), supported by the above training grant.

medium. These authors recommended extraction for not more than 18 hours since most of the protein nitrogen and allergenic material were removed during this period of time. Hampton and co-workers,¹⁰ who extracted ragweed and timothy pollen at increasing pollen-to-buffer ratios, studied protein nitrogen yield and the ability of the extracts to neutralize skin-sensitizing antibody. Their extraction method consisted of mixing the suspension in an automatic shaker for one hour and then refrigerating the suspension overnight.

Although since that time no detailed studies have been reported on the preparation of ragweed pollen extracts, there have been a number of publications on the characterization of antigenic and allergenic components of the pollen. Studies by Schon and Richter,¹¹ Goldfarb, Bhattacharya, and Kaplan,¹² Cebra,¹³ and Robbins and co-workers¹⁴ have been concerned with the identification of various proteins and/or polypeptides as potential allergens in ragweed pollen. King and Norman,¹⁵ and King, Norman, and Connell¹⁶ have isolated a protein with a molecular weight of 37,800 using chromatography on DEAE cellulose and Sephadex. This protein was found to represent 6 per cent of the protein extracted from ragweed pollen and was responsible for 90 per cent of the allergenic activity in the whole pollen extract.

In the above studies, protein was generally extracted by mixing the ragweed pollen with buffer for 1 hour to 7 days. Most commercial laboratories that prepare allergenic extracts for physicians use a modification of this mixing technique.

In our study, ragweed pollen extracts were prepared by three basic methods—sonication, homogenization, and stirring—and various combinations of these methods. The extracts were compared with respect to protein yield and skin-sensitizing activity in ragweed-sensitive patients. Allergenic activity was also determined in fractions obtained from preparations subjected to Sephadex gel filtration.

MATERIALS AND METHODS

Ragweed pollen

A 10 per cent suspension (w/v) of defatted dwarf ragweed pollen (*Ambrosia elatior*)* in tris-buffered saline (0.01M tris buffer, pH 8.0, 0.15M NaCl), was used for all extractions.

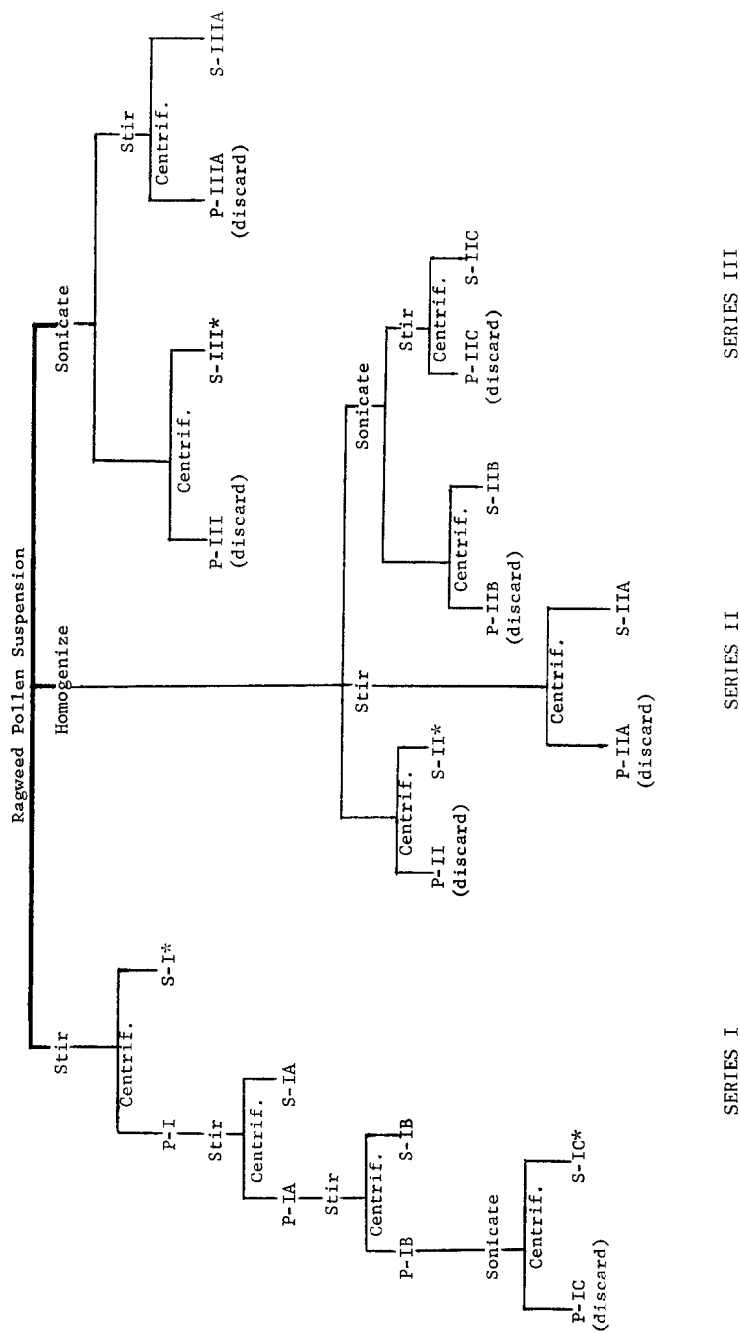
Preparation of extracts

The general protocol for the preparation of pollen extracts by various methods is shown in Fig. 1. Extracts were obtained by three basic techniques—stirring, homogenization, and sonication—as well as by various combinations of these methods.

Stirring. Ten-milliliter aliquots of the pollen suspension were mixed in a 30 ml. beaker by slow magnetic stirring for 48 hours at 5° C.

Homogenization. Ten-milliliter aliquots of the suspension were homogenized in a motor-driven tissue grinder with a 40 ml. glass tube and Teflon pestle. The suspension was ground by 3 down-up excursions of the pestle at 200 r.p.m.

*The dry, defatted pollen was donated by Center Laboratories, Port Washington, N. Y.

**Fig. 1**

General protocol for the preparation of ragweed pollen extracts by stirring, homogenization, sonication, and various combinations of these techniques. Extracts selected for skin testing are identified by an asterisk. For procedural details, see Materials and Methods section.

Sonication. With the use of a Branson Sonifier (Model S-75) equipped with a titanium probe and water-jacketed steel chamber, 3 ml. aliquots of the pollen suspension and 0.5 ml. of small glass beads were sonicated for 15 minutes at 0 to 5° C. (power source set at 8). The relationship between time of sonication and yield of extracted protein is shown in Fig. 2. It is apparent that sonication for more than 15 minutes added only a small amount of protein to the yield.

Combined methods. As shown in Fig. 1, certain pollen preparations were treated sequentially by more than one basic technique. If such treatment followed a centrifugation step (as in Series I), the pollen residue was resuspended in 10 ml. buffer and then subjected to further treatment. In the absence of centrifugation, further treatment was done on the whole suspension.

All centrifugations were performed at $1,500 \times g$, at 5° C., for 60 minutes.

Protein determinations

Protein concentrations were determined by the biuret method,¹⁷ with the use of a solution of bovine serum albumin as standard.

Skin testing

The extracts selected for skin testing are identified by an asterisk in Fig. 1. Preparations *S-I*, *S-II*, and *S-III* were obtained by stirring, homogenization, and sonication, respectively. Preparation *S-IC* was material removable by sonication after the pollen had been subjected previously to extensive extraction by stirring. Certain fractions eluted from Sephadex columns were also used for skin testing. These fractions are identified in Figs. 4 to 6.

Each of the 4 extracts tested was a pool of material prepared on three sep-

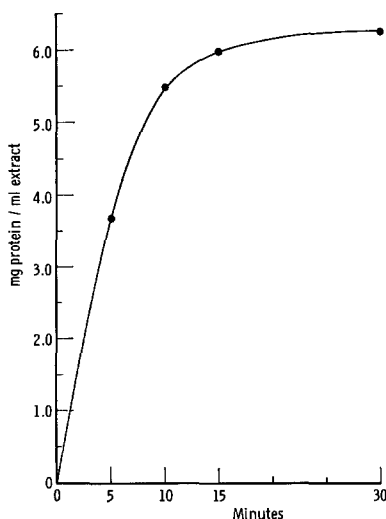


Fig. 2

Relationship between time of sonication of ragweed pollen and amount of protein released. Three milliliter aliquots of a 10 per cent suspension of pollen were sonicated for the periods shown.

arate occasions by the same technique. Standard stock preparations of the extracts were obtained by dilution in phosphate-buffered saline (0.15M NaCl, 0.01M phosphate buffer, pH 7.9) to a concentration of 0.4 to 0.5 mg. protein per milliliter. The Sephadex fractions used for testing were diluted in the same buffer to the same concentration. All stock preparations were sterilized by Millipore filtration, and sterility of the filtered solutions was checked by culture on blood-deoxycholate agar.

For skin testing, fivefold serial dilutions in sterile phosphate-buffered saline were made from the stock preparations. The ability of these dilutions to elicit a positive skin test was determined on the day they were prepared (Day 0). After the initial testing, the dilutions were frozen at -15°C ., retested on Day 5, refrozen, and tested again on Day 20.

The tests were performed by injecting 0.05 ml. of the dilutions intracutaneously into 3 ragweed-sensitive volunteers, one of whom was undergoing hyposensitization. The tests were graded according to the criteria of King and Norman.¹⁵ With the use of increasing concentrations of extracted protein, that dilution was found which gave a reaction of 2+ or greater. The skin test titer was expressed as the reciprocal of this dilution.

Sephadex fractionation

All extracts that were used for skin testing were also subjected to gel filtration by use of Sephadex G-25 and G-100 columns. Equilibration of the 1×25 cm. column and elution were done with 0.15M NaCl at 25°C . Two-milliliter aliquots of the extracts were passed through the column. The protein contents of these aliquots are noted in Figs. 4 and 6. The flow rates of the G-25 and G-100 columns were 30 and 10 ml. per hour, respectively, and 1.5 ml. fractions were collected. Normal human serum was passed through the G-100 column under the same conditions.

RESULTS

The extraction of ragweed pollen as outlined in Fig. 1 was performed three times. Individual and average protein yields, expressed in milligrams of protein extracted per 100 mg. pollen, are listed in Table I.

The three basic extraction methods, stirring for 48 hours, homogenization, and sonication, produced very similar mean protein yields, ranging between 4.9 and 5.6 mg. protein per 100 mg. pollen. Of the combined methods studied, sonication followed by stirring (Extract *IIIA*) consistently gave the highest protein yield. The efficiency of extraction by stirring alone decreased with increasing periods of stirring. Thus, during three successive 48 hour periods of stirring, approximately 50 per cent of the extractable protein was released during the first 48 hours.

The results of skin testing are shown in Fig. 3. On Day 0, all four extracts studied were about equally allergenic in the three volunteers tested. On Day 5, the activity in all extracts had decreased, except for Extract *I* in DS. This decrease was most marked in Extract *IC*, in which skin reactivity was lowered by 6 dilutions. After Day 5, the allergenicity of all extracts remained constant ex-

Table I. Protein extracted from ragweed pollen by various techniques
(for details, see *Materials and Methods*)

Extract No.	Method	Experiment No.			Mean
		1	2	3	
I	Stirring	5.6*	5.2	4.7	5.2
IA	Stirring of Pollen I	2.9	1.3	3.8	2.7
IB	Stirring of Pollen IA	1.7	0.6	2.0	1.4
IC	Sonication of Pollen IB	1.2	0.4	0.7	0.8
II	Homogenization	4.8	5.6	4.3	4.9
IIA	Homogenization + stirring	6.4	6.2	5.8	6.1
IIB	Homogenization + sonication	6.1	6.4	4.4	5.6
IIC	Homogenization + sonication + stirring	6.9	6.6	6.4	6.6
III	Sonication	6.0	5.9	5.0	5.6
IIIA	Sonication + stirring	7.6	7.2	7.2	7.3

*Milligrams of protein extracted per 100 mg. pollen.

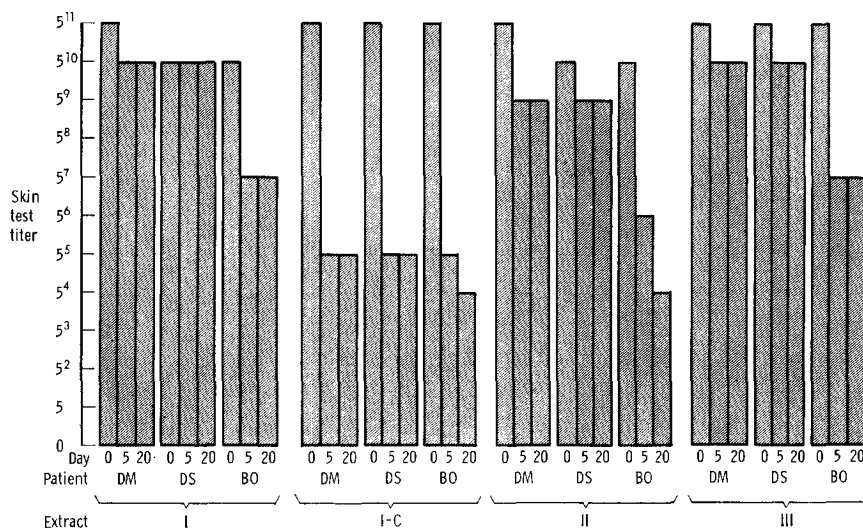


Fig. 3

Skin-test titers in 3 ragweed-sensitive patients tested on 3 occasions with extracts prepared by various methods. Titters are expressed as the reciprocal of the highest dilution of standardized extract giving a positive reaction.

Extract I, prepared by stirring for 48 hrs.

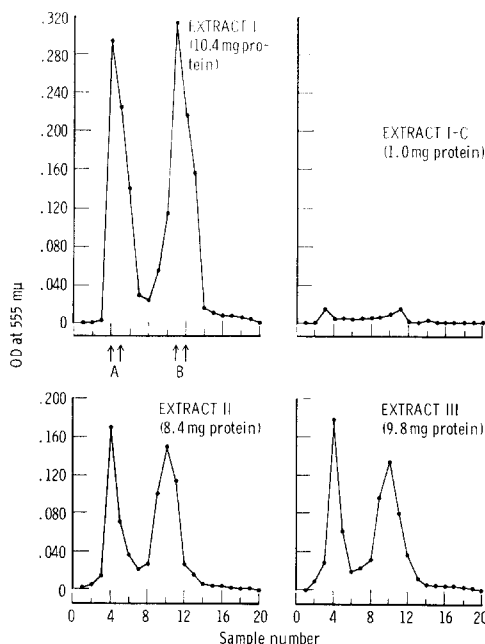
Extract IC, prepared by sonication of pollen following extensive previous extraction.

Extract II, prepared by homogenization.

Extract III, prepared by sonication.

cept in patient BO, who differed from the other volunteers in that she was receiving hyposensitization to ragweed at the time of the study.

Fractionation on Sephadex G-25 resulted in the same bimodal distribution of proteins in all four extracts, as shown in Fig. 4. Peak A, falling within the void volume, represented proteins with a molecular weight greater than 5,000 and peak B material with a molecular weight less than 5,000. Fractions from

**Fig. 4**

Fractionation on a Sephadex G-25 column of pollen extracts prepared by various methods. The *protein* content of each sample is given (see Fig. 3 for identification of extracts fractionated). *Protein* concentration in the column fractions is represented by the *OD at 555 mμ* of the biuret-assayed effluents. Fractions used for skin testing are identified by arrows.

both peaks were tested for skin-sensitizing activity. While both peaks contained active material, the activity of fraction A was higher by 6 dilutions in all three volunteers than that of fraction B (see Fig. 6).

When fractionation was done on Sephadex G-100, most of the protein in Extracts *I*, *II*, and *III* (representing the three basic methods), appeared in samples collected beyond the void volume, indicating a molecular weight of less than 100,000. The profiles obtained consisted of a rather broad, low peak followed by a sharp rise and fall in protein concentration (peaks *X* and *Y*, respectively, in Fig. 5). (Because of the small amount of protein in sample *IC*, its G-100 profile was difficult to evaluate.)

Skin-testing of fractions *X* and *Y* of Extract *I* revealed a greater activity by 4 to 5 dilutions in fraction *X* than in fraction *Y* (Fig. 6). Fraction *Y* was recycled through the G-100 column, and skin testing of material in the single peak obtained revealed a decrease in allergenicity by 3 to 4 dilutions as compared with fraction *Y* (see fraction *Y'* in Fig. 6).

Normal human serum was passed through the G-100 column for calibration purposes. The single peak obtained was located in front of peaks *X* and *Y* of Extract *I*, indicating a molecular weight of less than 70,000 for these fractions.

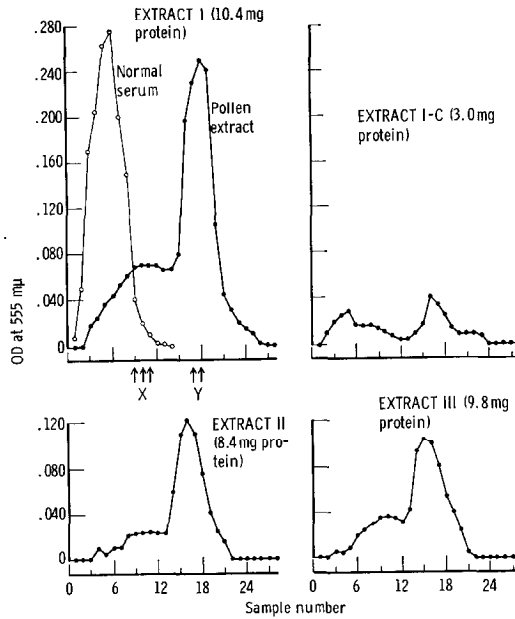


Fig. 5

Fractionation on a Sephadex G-100 column of pollen extracts prepared by various methods. See legend for Fig. 4 for additional information.

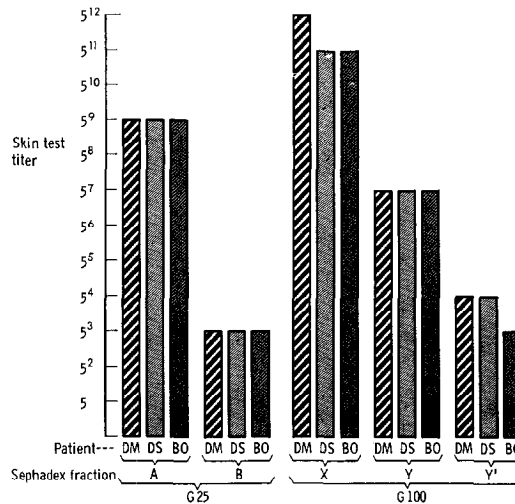


Fig. 6

Skin-test titers in 3 ragweed-sensitive patients tested with *Sephadex G-25* and *G-100* fractions of Extract I. See Figs. 4 and 5 for identification of fractions tested. Fraction Y' was obtained by recycling fraction Y through the *G-100* column. Titers are expressed as the reciprocal of the highest dilution of standardized effluent giving a positive reaction.

DISCUSSION

Over the past 20 years there have been many studies identifying proteins, polypeptides, and carbohydrate-protein complexes as some of the compounds responsible for the immunologic properties of ragweed pollen. There is general agreement that one of the major constituents of the pollen responsible for its allergenicity is a protein. Freedman¹⁸ has shown a correlation between the activity of ragweed pollen extract and the presence of nitrogen precipitable by trichloroacetic acid.

Our studies were concerned with methods of extracting protein from ragweed pollen and with the evaluation of the protein yields, as well as of the allergenicity of extracts prepared by these methods. Our data indicate that stirring, homogenization, and sonication are equally effective in terms of protein yield. Based on the protein content in ragweed pollen determined by Heyl,¹⁹ about 25 per cent of the pollen protein was solubilized by these three methods.

The extracts that were most similar with respect to allergenicity and stability were those produced by stirring for 48 hours and sonication for 15 minutes (Fig. 3). This similarity and the fact that sonication yielded at least as much protein as did other basic techniques indicate that sonication is an effective, rapid method for preparing ragweed extracts. Homogenization yielded about 10 per cent less protein and material of somewhat lower allergenic stability than did sonication.

The loss of allergenicity of pollen extracts on storage has been known for many years. Batchelder and Gondor²⁰ observed a progressive loss of allergenic activity when the pH of pollen extracts was dropped below 5.0. According to King and Norman,¹⁵ a lowering of pH during storage of extracts may be caused by the release of hydrogen ions following the action of esterases on some unknown substrate (probably of low molecular weight). Our data, too, clearly demonstrate the reduction in allergenicity in stored pollen extracts, the most pronounced loss having occurred in material obtainable after extensive, previous extraction of pollen (see Extract *IC* in Fig. 3). It is apparent that whenever skin-test titers are to be determined in several individuals with respect to the same preparation of allergen, the tests should be performed on the same day.

Similar protein profiles were obtained when extracts prepared by the 3 basic methods were compared by fractionation on Sephadex gel columns. Skin-test data obtained with Sephadex G-25 fractions confirm the findings of King and Norman¹⁵ that most of the allergenic activity resides in material with a molecular weight greater than 5,000. This finding, together with the results obtained following fractionation of Extract *I* and normal human serum on Sephadex G-100, suggests a molecular weight between 5,000 and 70,000 for most of the allergenic protein in ragweed pollen. Although there was a difference of at least 4 dilutions between fractions X and Y with respect to skin reactivity, the incomplete separation of the two corresponding peaks on the relatively short G-100 column suggested that much of the activity of peak Y was due to the overlap of protein from peak X. For this reason, fraction Y was recycled. Skin-testing of the resulting single protein peak (fraction Y') indicated that the allergenicity of fraction Y was due to material distinct from fraction X, as well as contaminating

fraction X material. The localization of peaks B and Y', and the similarity between the skin-test titers of these two fractions suggest that they may contain the same low molecular weight allergen.

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