

CHARACTERIZATION OF THE DIALYZABLE CONSTITUENTS OF THE AQUEOUS EXTRACT OF RAGWEED POLLEN

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IN a recent review on the allergens of ragweed pollen,¹ it was concluded that there was no agreement as to whether or not the dialyzable constituents (dialysate) of aqueous ragweed pollen extracts had skin activity. These conflicting observations seemed to be partially reconciled by the findings of Richter and associates,² who reported that a dialysate fraction collected during the first 24 hours of dialysis reacted in passive transfer tests with most (86 per cent) of the sera† of ragweed-allergic individuals who had received injection therapy with the whole aqueous extract (WSR). However, with some exceptions (17 per cent), the dialysate fraction did not react with the sera‡ of those who had not received such treatment. From these findings it was inferred that during injection treatment with WSR new reagin(s) is formed which is not produced "spontaneously" by nontreated allergic individuals. The present study was undertaken to investigate more fully the nature of the dialyzable skin-active materials, which had been thought to induce the production of new reagins.

The results to be reported demonstrate that all the dialysate fractions prepared in the present study reacted with sera from both treated and nontreated ragweed-sensitive individuals. Furthermore, the presence of new reagins in treated sera could not be detected by cross-neutralization studies in which the dialysate fractions were compared with the nondialyzable components (DWSR‡) of the whole aqueous ragweed pollen extract.

MATERIALS AND METHODS

Ragweed Fractions.—WSR was prepared from defatted short ragweed pollen§ and was lyophilized.^{4, 5} The lyophilized material (3 Gm.) was dissolved in distilled water (50 ml.) and dialyzed at 4° C. in a Visking cellophane tube

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†The sera of nontreated and treated allergic individuals will be referred to hereafter as nontreated and treated sera, respectively.

‡The preparation of DWSR is described elsewhere.³

§This pollen was purchased from Mr. Fred J. Hodgson, "Fine Pollens," Verona, N. J., in 1958.

against distilled water (300 ml.). The water was changed after 1, 2, 6, and 10 days, the 4 solutions were lyophilized, and the corresponding dialysate fractions were designated as D₁, D₂, D₆, and D₁₀, respectively. The yields of these 4 fractions were 1.07, 0.20, 0.17, and 0.09 Gm., respectively, and were reproducible within the limits of experimental error. The materials were hygroscopic and were stored in airtight containers at 4° C. until used. (Fraction D₁ was essentially equivalent to the 24 hour dialysate fraction of Richter and associates.²)

Acid hydrolysis of the fractions and identification* of the sugars present were performed as described previously.³ The amino acid composition of the fractions, before and after acid hydrolysis, was determined by two dimensional paper chromatography using Whatman No. 1 paper and the solvent systems ethanol : n-butanol : water : propionic acid (volumes 10:10:5:2) and acetone : n-butanol : water : dicyclohexylamine (volumes 10:10:5:2).⁶ The chromatograms were stained with a solution of 0.25 Gm. of ninhydrin in 100 ml. of acetone acidified with acetic acid (7 per cent by volume). Nitrogen determinations were performed on 2 to 4 mg. quantities of material by the method of McKenzie and Wallace.⁵

Immunologic Procedures.—Antisera were prepared by immunizing rabbits with WSR and with DWSR according to the schedule described earlier.⁸ For the demonstration of precipitating antibodies the micro-Ouchterlony technique described by Perelmutter and co-workers⁸ was used. In addition to the whole rabbit antisera, an antibody-enriched γ -globulin preparation (R γ G), obtained by precipitation with ethanol from an anti-WSR rabbit serum, was used at a concentration of 0.8 per cent in saline. The dialysate fractions and DWSR were employed at concentrations of 100 and 1 mg. per milliliter, respectively.

Human sera were obtained from nontreated ragweed-allergic individuals and from allergic individuals immunized with WSR and were sterilized by Seitz filtration. Solutions of the various ragweed fractions were sterilized either by filtration through Seitz filters† or by filtration through "Oxoid" cellulose acetate membranes,‡ according to the experimental requirements, using Swinny† filter adapters.

Passive transfer (P-K) tests were performed on the backs of normal, nonallergic volunteers. To determine the limits of sensitivity of the allergenic preparations, sites were sensitized intracutaneously with 0.05 ml. portions of a suitably diluted serum and were challenged 24 hours later with 0.025 ml. of the allergenic solutions in tenfold serial dilutions. The allergenic solutions were freshly prepared before each test. The P-K titers of the sera were determined by sensitizing sites with 0.05 ml. of twofold saline dilutions of the sera. Twenty-four hours later the sites were challenged with 0.025 ml. of a saline solution of WSR (50 μ g per milliliter). In all these experiments the reactions were graded as described previously.⁹

To establish whether or not new additional reagins were indeed produced

*These analyses were performed by Dr. T. E. Timell at the Pulp and Paper Research Institute, McGill University, Montreal.

†Commercially available from Beckton, Dickinson and Co., Rutherford, N. J.

‡Purchased from British Drug Houses, Toronto (Ltd.).

by hyposensitization treatment with WSR, the 4 dialysate fractions and DWSR were compared by cross-neutralization tests using treated sera. In one series of experiments, each serum was mixed with a solution of DWSR to give a fourfold dilution of the serum and a final concentration of 75 μg per milliliter of DWSR. Multiple sites were sensitized with 0.05 ml. portions of this mixture and challenged after 24 hours with 0.025 ml. of DWSR (100 μg per milliliter) and the reactions were recorded. Twenty-four hours later control sites were rechallenged with DWSR; the absence of reactions on this occasion was taken as evidence that all sites had been neutralized with respect to the skin-active components of DWSR. The experimental sites (i.e., the sites not retested with DWSR) were then challenged with the various dialysate fractions (100 μg per milliliter) and the reactions recorded in the usual manner.

In a second series of experiments, 3 treated sera and 3 nontreated sera were diluted twentyfold with saline solutions of D_{10} to give final concentrations with respect to D_{10} of 380, 570, 760, and 1900 μg per milliliter. Sites were sensitized with 0.05 ml. of these solutions, challenged 24 hours later with 0.025 ml. of DWSR (50 μg per milliliter), and the reactions were recorded in the usual manner.

RESULTS AND DISCUSSION

Physicochemical Characterization of Dialysate Fractions.—The amino acid analyses of the dialysate fractions are given in Table I. It is evident that many free amino acids dialyzed during the first and the second day, thus accounting for the higher nitrogen contents of fractions D_1 (8.11 per cent N) and D_2 (7.95 per cent N), as compared with D_6 (3.86 per cent N) and D_{10} (3.58 per cent N). It can also be seen that the nitrogen contents of fractions D_6 and D_{10} were due mainly to the unidentified peptide material, which moved chromatographically with the front of the second solvent (most of the yellow pigments were observed in the same regions of the chromatogram). In spite of the improved conditions of hydrolysis used,³ the basic amino acids arginine and lysine seemed to be partially destroyed. The amino acids of fraction D_{10} were the same as those already reported for the nondialyzable electrophoretic fraction δ .³ The detection of glycine and threonine only in the hydrolysates but not in free form indicated that these amino acids were present solely as peptide material. It is worth pointing out that the analyses of Dankner and co-workers¹⁰ for a 72 hour dialysate of ragweed pollen extract agree reasonably well with the findings presented for D_1 and D_2 in Table I.

Arabinose and galactose were found in the acid hydrolysates of all 4 fractions. Glucuronic and/or galacturonic acids were found only in the hydrolysates of fractions D_6 and D_{10} . It was not possible to distinguish between these 2 acids with the solvent systems used. All of these sugars had also been detected in the electrophoretic fractions of DWSR.⁵ Glucose was found in the hydrolysates of fractions D_1 and D_2 and probably occurred as the glucoside of the flavanoid pigment.¹¹

By use of a Spinco Model E analytical ultracentrifuge with a synthetic boundary cell, the apparent sedimentation coefficients of D_1 , D_2 , D_6 , and D_{10}

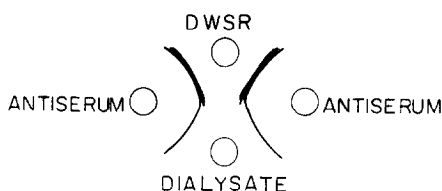


Fig. 1.—Antigenic relationship between DWSR and any of the dialysate fractions D_2 , D_6 , or D_{10} .

at a concentration of 12 mg. per milliliter in water at 25° C. were found to be 0.4 S, 0.4 S, 0.4 S, and 0.8 S, respectively. By contrast, the sedimentation coefficients of the nondialyzable components of ragweed pollen extract (i.e., DWSR) was of the order of 1.8 S and that of the electrophoretic fraction δ of DWSR was 3.6 S.³ All ultracentrifugal patterns consisted of single broad peaks, confirming the heterogeneity of these preparations. The sedimentation coefficients, therefore, were not corrected for standard conditions.

Immunologic Characterization.—In the agar gel diffusion studies, no precipitin band was formed between the rabbit antisera and fraction D_1 , even at the high concentration of 100 mg. per milliliter of D_1 used. However, a single weak band of identity was observed between the antisera and fractions D_2 , D_6 , and D_{10} , showing that all the antigenic constituents present in these fractions were identical. In another series of experiments, each of the fractions D_2 , D_6 , and D_{10} was compared with DWSR. A typical band pattern obtained with a dialysate fraction is given in Fig. 1. A single precipitin band formed between the dialysate fraction and the antisera and merged with one of the bands obtained with DWSR. Identical patterns were obtained with D_2 , D_6 , or D_{10} , showing that all the antigenic constituents of dialysate were present in DWSR. Furthermore, the extra bands obtained with DWSR indicated that there were antigenic constituents in DWSR which could not be detected in the dialysate fractions.

Passive Transfer Tests.—Lea and associates⁹ reported that erratic results were obtained in P-K experiments with ragweed pollen extracts which had been sterilized by filtration through Seitz pads. Furthermore, these Seitz-filtered solutions were appreciably less active than the solutions sterilized by filtration through cellulose acetate membranes; this effect was attributed to the removal of active materials from the pollen extracts by the Seitz filters, as had also been reported previously by Stull and co-workers.¹² It was clear, therefore, that the conclusions of Richter and associates² were questionable, since in their P-K experiments only Seitz-filtered ragweed solutions had been used; moreover, their 24 hour dialysate and WSR solutions had been used after filtration at the low concentration of 62.5 μ g per milliliter.

To decide whether or not the Seitz filters were responsible for the removal of the skin-active constituents, the allergenic activity of Seitz-filtered dialysate solutions was compared with that of the same solutions which had been membrane-filtered. Solutions of each of fractions D_1 , D_2 , D_6 , and D_{10} were prepared at a concentration of 100 μ g per milliliter and divided into 2 equal portions. One portion was Seitz-filtered and the other was membrane-filtered. These

solutions were then serially diluted and used in P-K tests. Five fortyfold diluted nontreated sera gave no reaction with the Seitz-filtered dialysate solutions, whereas the corresponding membrane-filtered solutions showed considerable allergenic activity. From these results it may be concluded that Seitz filtration had led to a reduction in the concentration of skin-active material from the dialysate solutions below the threshold level required to give skin activity. It was just conceivable, however, that all skin-active components, excepting those responsible for producing new reagins during hyposensitization with WSR, had been preferentially adsorbed on the Seitz filters during sterilization of the dialysate solutions. The filtered solutions would then, understandably, have reacted with treated sera but not with nontreated sera. On the other hand, if on Seitz filtration a general reduction of all the skin-active components had occurred, the filtrates after concentration would have been expected to react with sera of both nontreated and treated patients.

To settle this point unequivocally, 5 ml. portions of 100 ml. of an aqueous solution of fraction D₁ at a concentration of 75 μ g per milliliter were each passed through a Swinny Seitz filter and the 20 filtrates were pooled. Sterile sodium chloride solution (9 per cent) was added to a small portion of the pooled filtrate to make it isotonic (solution A). The remainder of the filtrate was lyophilized, and weighed amounts of the resulting pale yellow powder were used to prepare saline solutions at concentrations of 100, 1000, and 2000 μ g per milliliter (solutions B); these latter solutions were sterilized by membrane filtration before use. The skin activity of solutions A and B was then compared by P-K tests with 10 nontreated sera and 10 treated sera; all these sera were diluted only twofold so as not to decrease the concentration of any of the reagins below the level of detection. The results obtained are given in Table II and may be summarized as follows: (1) solution A did not react with any of the nontreated sera but showed threshold activity with 3 of the 10 treated sera, and (2) all of the solutions B, even at the lowest concentration of 100 μ g per milliliter, gave marked reactions with all of the 20 sera tested. The results obtained with solution A are in accordance with the observations made in the previous study.² However, the positive reactions obtained with solutions B indicated that Seitz filtration did not preferentially remove any of the allergenic components of D₁. The two sets of results, taken together, demonstrate, therefore, that Seitz filtration of solutions of fraction D₁ leads to a reduction in the concentration of all skin-active components in this fraction rather than to a selective removal of some of the allergens. Furthermore, it is proposed that the threshold reactions obtained with some of the treated sera may simply reflect the increase in reaginic content of these sera on hyposensitization treatment.¹³

These conclusions were confirmed by further experiments in which fraction D₁ was Seitz-filtered as above at a concentration of 50 and 100 μ g per milliliter; the corresponding solution A of the weaker solution did not react with any of the treated or nontreated sera, while solution A, corresponding to the stronger solution, reacted with 4 of the 10 nontreated sera and with

TABLE I. AMINO ACID ANALYSIS BY PAPER CHROMATOGRAPHY OF THE DIALYSATE FRACTIONS BEFORE AND AFTER ACID HYDROLYSIS

AMINO ACIDS	BEFORE HYDROLYSIS				AFTER HYDROLYSIS			
	D ₁	D ₂	D ₆	D ₁₀	D ₁	D ₂	D ₆	D ₁₀
Alanine	+	+	-	-	+	+	+	+
Arginine	+	+	-	-	-	-	-	?
Aspartic acid	+	+	-	-	+	+	+	+
Glutamic acid	+	+	-	-	+	+	+	+
Glycine	-	-	-	-	+	+	+	+
Histidine	?	?	-	-	-	-	+	+
Hydroxyproline	+	+	-	-	?	+	+	+
Leucine(s)	+	+	-	-	+	+	+	+
Lysine	+	+	-	-	-	-	-	+
Phenylalanine	-	-	-	-	-	-	-	?
Proline	+	+	+	-	+	+	+	+
Serine	?	-	-	-	?	+	+	+
Threonine	-	-	-	-	+	+	+	+
Tryptophane	?	-	-	-	-	-	-	-
Tyrosine	?	?	-	-	-	-	-	?
Valine	+	+	-	-	+	+	+	+
Unidentified	+	+	+	+	-	+	+	+

TABLE II. SKIN ACTIVITIES OF SOLUTIONS A AND B PREPARED FROM A SEITZ-FILTERED SOLUTION OF FRACTION D₁ AT A CONCENTRATION OF 75 µg PER MILLILITER

P-K TITERS OF NON- TREATED SERA	SOLU- TION A	SOLUTIONS B			P-K TITERS OF TREATED SERA	SOLU- TION A	SOLUTIONS B		
		100 µg/ml.	1000 µg/ml.	2000 µg/ml.			100 µg/ml.	1000 µg/ml.	2000 µg/ml.
>> 1200	-	++	+++	+++	> 1200	±	++	+++	+++
> 1200	-	++	+++	++	> 1200	±	++	++	+++
> 1200	-	+	++	++	1200	+	++	+++	+++
1200	-	++	++	+	800	-	++	+++	++
1200	-	+	++	++	800	-	+++	+++	+++
800	-	++	+++	++	400	-	+++	+++	+++
400	-	+	+	+	400	-	++	++	+++
200	-	++	++	++	200	-	++	++	+++
200	-	++	++	++	200	-	++	++	++
< 100	-	+	++	++	200	-	++	+++	+++

TABLE III. SKIN ACTIVITY OF DIALYSATE FRACTIONS D₂, D₆, AND D₁₀ AFTER SEITZ FILTRATION

P-K TITERS OF NONTREATED SERA	SITES SENSITIZED WITH SERA DILUTED TWOFOLD; CHALLENGED WITH DIALYSATE FRACTION (500 µg/ml.)			SITES SENSITIZED WITH SERA DILUTED TENFOLD; CHALLENGED WITH DIALYSATE FRACTION (500 µg/ml.)		
	D ₂	D ₆	D ₁₀	D ₂	D ₆	D ₁₀
>> 1200	++	+++	+++	++	++	+++
> 1200	++	+++	+++	++	++	++
1200	±	++	+++	-	++	±
1200	+	+++	+++	-	+++	+++
800	±	++	++	-	+	±
400	++	+++	+++	±	+++	+++
200	+	+++	+++	++	+++	+++
200	-	+	+	-	++	-
100	-	-	±	-	+	+
< 100	-	++	++	-	++	++

7 of the 10 treated sera. Two solutions B at 100 and 1000 μg per milliliter were prepared from fraction D_1 filtered at 50 μg per milliliter; solution B at 100 μg per milliliter did not react with any of the treated or nontreated sera, whereas solution B at 1000 μg per milliliter reacted with 2 of the 10 treated sera. On the other hand solution B at 300 μg per milliliter, which was prepared from fraction D_1 filtered at 100 μg per milliliter, gave marked reactions with all treated and nontreated sera. It follows from these experiments that the skin activity of all solutions prepared by Seitz filtration depends on their initial concentrations.

In Table III are recorded the skin activities of fractions D_2 , D_6 , and D_{10} , Seitz-filtered at a concentration of 500 μg per milliliter using 10 nontreated sera. These sera were diluted only twofold and tenfold so as not to decrease the concentration of any of the reagins below the level of detection. First, it is evident that these Seitz-filtered solutions had considerable skin activity. Furthermore, fractions D_6 and D_{10} were the most active and had almost comparable skin activity, and fraction D_2 was less active. It is worth noting that there is no evident relationship between the P-K titer of the serum used and the size of the skin reaction observed, suggesting that the over-all P-K titer is due to multiple reagins¹⁴ occurring in different ratios in different sera.

As has been indicated above, all the 4 membrane-filtered dialysate fractions D_1 , D_2 , D_6 , and D_{10} were skin active. The relative skin activities of these fractions are recorded in Table IV with respect to 5 nontreated sera, diluted fortyfold. Fraction D_1 was found to be very much less active than D_2 , D_6 , and D_{10} . The latter 2 fractions had comparable activity and were slightly more active than D_2 . These results are in agreement with the gradation of activity of the dialysate fractions observed in Table III. As previously pointed out, the corresponding Seitz-filtered solutions of the 4 fractions did not display skin activity with fortyfold diluted sera even at an initial concentration of 100 μg per milliliter, whereas the membrane-filtered solutions of D_2 , D_6 , and D_{10} still retained skin activity after dilution to 1 μg per milliliter. Therefore, it cannot be too strongly emphasized that the *choice of filters* used for sterilization of dilute allergenic preparations is important if valid conclusions are to be drawn from P-K experiments.

It was still possible that new reagins directed specifically against dialyzable components were being produced in allergic individuals during hyposensitization with WSR. To test this hypothesis, cross-neutralization experiments with DWSR and the 4 dialysate fractions were carried out using 6 treated sera. In these experiments only membrane-filtered ragweed solutions were used. The final challenge with the 4 dialysate fractions of the sites previously desensitized with DWSR gave no skin reactions, indicating that these sera did not contain any new reagin directed solely against dialysate and that no such reagin had been produced by the hyposensitization treatment. Furthermore, the results of these experiments demonstrated that DWSR contained all the determinant groups responsible for the skin activity of WSR.

In other experiments, attempts to neutralize sera from either treated or nontreated individuals with fraction D_{10} , which was the most active of the 4

TABLE IV. SKIN ACTIVITY OF DIALYSATE FRACTIONS AFTER MEMBRANE-FILTRATION, OBTAINED WITH 5 SERA (DILUTED FORTYFOLD) FROM NONTREATED PATIENTS

CONCENTRA- TION/ML.	D ₁					D ₂					D ₅					D ₁₀				
100 μ g	+	-	+	±	+	++	++	++	++	+++	++	+	+	++	++	++	+	+	++	++
10 μ g	-	-	-	-	-	+	++	±	+	+	++	+	+	++	++	++	+	+	++	++
1 μ g	-	-	-	-	-	-	-	±	±	±	+	+	±	±	±	+	±	-	±	±
0.1 μ g											-	-	-	-	-	-	-	-	±	-

dialysate fractions, failed even when the sera were only twentyfold diluted with a solution of D₁₀ at the high concentration of 2000 μ g per milliliter. Thus, sites sensitized with these serum-dialysate solutions always gave large positive reactions on challenge with a solution of DWSR at a concentration of 50 μ g per milliliter. More concentrated solutions of D₁₀ were not used in these experiments, since the results might have been obscured by nonspecific irritation. The alternative, namely dilution of the serum with larger volumes of D₁₀ solution, was not attempted either, since this procedure would have rendered equivocal the detection of reaginic component(s) present in small concentration(s) and not neutralized by D₁₀.

Because of these limitations of the P-K test, it still remains undecided whether or not dialysate contains all or only some of the skin-active determinants found in WSR. Nevertheless, it is clear that during hyposensitization treatment new reagins directed exclusively against the allergenic components of dialysate are not produced, as had been concluded earlier.² However, none of these experiments rules out the possibility that new reagins directed to allergenic component(s) present in DWSR may be formed on treatment. This possibility cannot be tested until all the allergens of ragweed pollen are isolated in a pure form.

The dialysate solution used by Richter and co-workers² was nearest to being equivalent to a solution of fraction D₁, and, as shown in the present study, Seitz filtration of this fraction resulted in lowering its skin activity to a minimal level. Therefore, it is not surprising that most of the nontreated sera tested with the 24 hour Seitz-filtered dialysate solution² had not displayed any activity. On the basis of the findings of the present study, it is suggested that the previous results² can be reinterpreted as demonstrating that during treatment an increase occurs in the concentration of skin-sensitizing antibodies¹³ already being produced by the allergic individual.

SUMMARY

Four fractions of the dialyzable components of the aqueous extract of ragweed pollen were prepared and their physicochemical and immunologic properties were examined. All these fractions were skin active with respect to sera from both treated and nontreated individuals. All the antigenic and allergenic determinant groups of the dialysate fractions were found to be present in the nondialyzable components of the whole aqueous extract. Contrary to previous

conclusions, it was demonstrated by cross-neutralization studies that new reagin(s) directed exclusively against components of the dialysate is not produced by hyposensitization treatment with the whole aqueous extract. Rather, any increase in reagin concentration caused by hyposensitization is considered to be the result of a rise in the concentration of reagin(s) already being produced by the allergic individual.

REFERENCES

1. Richter, M., and Schon, A. H.: Studies on the Allergens of Ragweed Pollen, *J. ALLERGY* 31: 111, 1960.
2. Richter, M., Schon, A. H., Gordon, J., Grégoire, C., and Rose, B.: Demonstration of Reagin of a New Specificity in Sera of Treated Ragweed-Sensitive Persons, *J. ALLERGY* 29: 287, 1958.
3. Lea, D. J., and Schon, A. H.: The Chemical Characterization of Nondialyzable Constituents of Ragweed Pollen, *Int. Arch. Allergy* 20: 203, 1962.
4. Schon, A. H., Richter, M., Harter, J. G., and Rose, B.: Studies on Ragweed Pollen. I. Preliminary Fractionation of Ragweed Pollen, *J. ALLERGY* 27: 236, 1956.
5. Schon, A. H., and Richter, M.: Studies on Ragweed Pollen. IV. Isolation and Characterization of an Allergenic Fraction From Ragweed Pollen, *J. Immunol.* 82: 190, 1959.
6. Hardy, T. L., Holland, D. O., and Nayler, J. H. C.: One-Phase Solvent Mixtures for the Separation of Amino Acids, *Anal. Chem.* 27: 971, 1955.
7. McKenzie, H. A., and Wallace, H. S.: The Kjeldahl Determination of Nitrogen. A Critical Study of Digestion Conditions, Temperature, Catalyst, and Oxidizing Agent, *Australian J. Chem.* 7: 55, 1954.
8. Perelmutter, L., Lea, D. J., Freedman, S. O., and Schon, A. H.: Demonstration of Precipitating Antibodies in Sera of Ragweed-Allergic Individuals by the Agar Gel Technique, *Int. Arch. Allergy* 20: 355, 1962.
9. Lea, D. J., Freedman, S. O., and Schon, A. H.: The Skin Activity of Nondialyzable Constituents of Ragweed Pollen, *Int. Arch. Allergy* 20: 220, 1962.
10. Dankner, A., Bukantz, S. C., Johnson, M. C., and Alexander, H. L.: Fractionation of Pollen Extracts by Chromatography. I. Preliminary Studies With Ragweed Extract, *J. ALLERGY* 22: 437, 1951.
11. Heyl, F. W.: The Yellow Coloring Substances of Ragweed Pollen, *J. Am. Chem. Soc.* 41: 1285, 1919.
12. Stull, A., Cooke, R. A., and Tennant, J.: The Allergen Content of Pollen Extracts. Its Determination and Deterioration, *J. ALLERGY* 4: 455, 1933.
13. Sherman, W. B., Stull, A., and Cooke, R. A.: Serological Changes in Hay Fever Cases Treated Over a Period of Years, *J. ALLERGY* 11: 225, 1940.
14. Perelmutter, L., Freedman, S. O., and Schon, A. H.: Demonstration of Multiple Hemagglutinating Factors in Sera of Ragweed-Allergic Individuals With the Help of Immunosorbents, *J. Immunol.* 89: 623, 1962.