

Qualitative and quantitative variations in dwarf ragweed pollens

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With the use of two different fractionation procedures, it is shown that differences exist among dwarf ragweed pollens harvested in different years. Quantitative chromatographic differences frequently occur which are accompanied by differences of yields in each fraction. Many fractions also showed differences in chemical analysis, gel diffusion patterns, and skin reactivity. The results are related to the yearly variations of potency of ragweed extracts. It is suggested that the differences between the products containing Antigen E isolated separately by King and by Robbins may be related to these differences.

The fractionation of dwarf ragweed pollen extracts has been under investigation in this laboratory for some time. Earlier methods of fractionation were developed which were reasonably reproducible with batches of varying sizes (50 to 500 Gm.) of the same pollen.^{1, 2} However, we have found that marked differences in both a qualitative and quantitative nature have appeared in pollens which were collected in different years. In developing a later method we have found similar differences. Since such variations may be of clinical significance, we are reporting the results of these experiments at this time.

MATERIALS AND METHODS

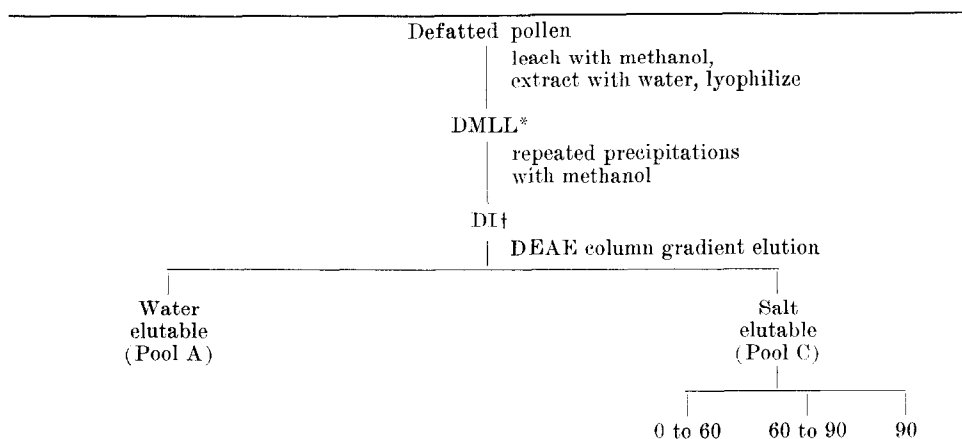
The pollens were obtained from two sources. The 1959 and 1961 pollens were obtained from commercial sources in a defatted condition. The others were supplied by the Institute of Allergy and Infectious Diseases of the National Institutes of Health. All pollens were at least 98 per cent pure dwarf form by microscopic analysis.

Diethylaminoethyl cellulose (DEAE) was supplied by Carl Schleicher and Schull Co. and was further purified by the method of Peterson and Sober.³

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Supported by United States Public Health Grant AI-05386.

Received for publication June 30, 1967.

Table I. Outline of Method A for fractionation of pollen extracts

*DMLL stands for the extract of dwarf pollen which has been methanol leached and lyophilized.

†DI is the water soluble fractions resulting from repeated methanol precipitations of DMLL.

The treated DEAE was washed free of Na^+ and Cl^- with distilled water and equilibrated with either 0.005M phosphate buffer (pH = 7.4) or 0.005M Tris borate buffer (pH = 7.8).

Two kinds of rabbit anti-whole—ragweed serum (AWRW) were used for the immunodiffusion studies. Serum No. 90* was prepared with a 1960 dwarf ragweed pollen extract. The second AWRW was prepared by us in the usual manner with a 1963 pollen extract emulsified with complete Freund's adjuvant.

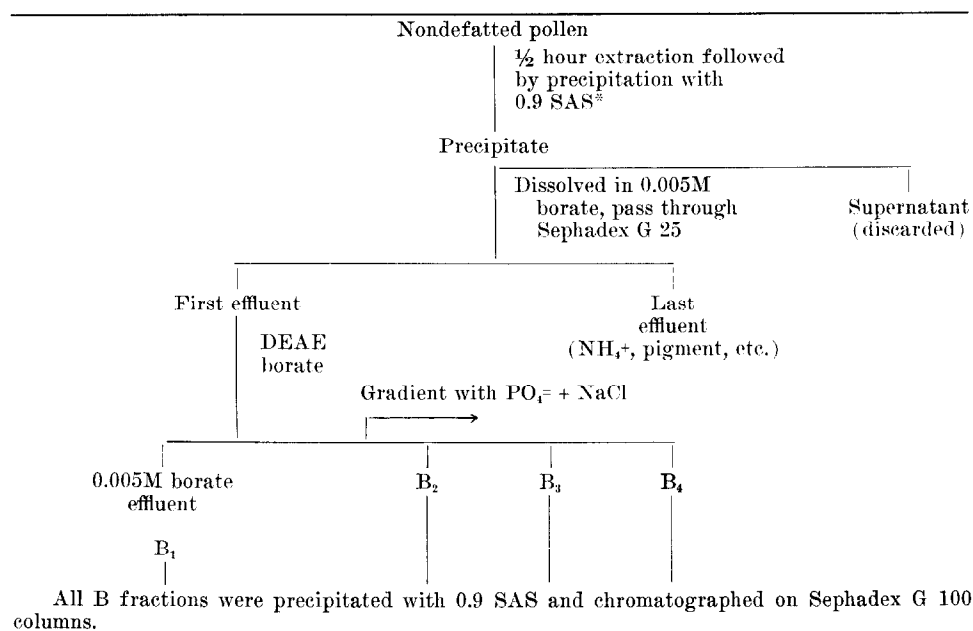
The rabbit anti-IV C (A.E.) was prepared for the Committee on Standardization of the National Institute of Allergy and Infectious Diseases by Dr. Merrill W. Chase. The antigen used for injection was King's IV C.⁴ The procedure consisted of a single injection of IV C on alumina and a second intravenous administration of a further amount of IV C. The serum was a pool of the bleedings from four of the five animals used. It was reported to give a major single line, which King associated with antigen E, and several weak lines in immunodiffusion studies at an antigen concentration of 0.5 mg. per 0.1 milliliter.

Immunodiffusion and immunoelectrophoresis studies were carried out by methods which have already been described.^{1, 2} The concentration of the various fractions was 10 mg. per milliliter. Pool 90 serum was used to study the fractions isolated by Method A and AWRW was used on the products isolated by Method B.

The preparation of Pool C (Method A with defatted pollen) has been described in detail² and an outline of the method is found in Table I. The

*Supplied by Dr. R. T. Wodehouse and C. Gussoni of the Lederle Laboratories, Pearl River, N. Y.

Table II. Outline of Method B for fractionation of pollen extracts



*Nine-tenths SAS is 90 per cent saturated ammonium sulfate.

method of preparation of the BG fractions (Method B with nondefatted pollen) is being reported in detail elsewhere and is summarized in Table II.

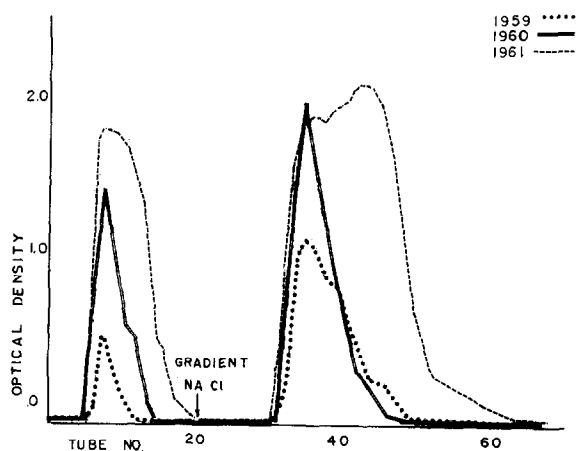
Comparative skin tests were performed on treated patients at the Allergy Clinic of the Mt. Sinai Hospital in Chicago.

Absorption spectrum measurements were performed on a Beckman DU spectrophotometer. The fractions were dissolved in 0.005M phosphate buffer at a pH of 7.3 to 7.5.

RESULTS

Fractionation of pollens from 1959, 1960, and 1961 (Method A)

The yields obtained from 100 Gm. of pollen for each of the batches are given in Table III. The amounts of DMLL from the 1959 and 1961 pollens were comparable to, but somewhat less than, that from the 1960 batch. On the other hand, while the 1959 and 1960 pollens gave somewhat similar yields of DI, the 1961 pollen gave almost twice these. With respect to the chromatography of DI and DEAE cellulose columns, the 1959 and 1960 material gave similar patterns but that from 1961 gave a double Pool C peak (Fig. 1). The relative total yields of the Pool C fractions differed considerably, e.g., the combined 1961 ($C_1 + C_2$) contained twice as much material as the 1960 pollen, which in turn gave almost twice as much of the Pool C product as the 1959 pollen did. Even with fractionation with ammonium sulfate there were differences in the distribution between the amounts obtained in the fraction precipitated in 60

**Fig. 1**

Chromatography of DI on DEAE.

Table III. Yield data of fractions from the 1959, 1960, and 1961 crops of dwarf ragweed pollen (100 Gm.)

Year of crop	DMLL (Gm.)	DI (Gm.)	Pool C (mg.)	
			0 to 60*	60 to 90*
1959	7.0	1.54	37	55
1960	19.6	1.70	102	62
1961 C ₁			110	80
1961 C ₂	7.0	3.33	103	168

*The ranges shown indicate the per cent saturation with ammonium sulfate in which the products were precipitated.

per cent saturated ammonium sulfate (SAS) and in the fraction precipitated in the 60 to 90 per cent SAS range.

Chemical analyses and specific absorbances for the various fractions are recorded in Table IV. Fractions of Pool C (0 to 60 and 60 to 90) prepared at different times from the same pollen gave analytical figures which were within 10 per cent of each other. On this basis, the 0 to 60 fractions of 1959 and 1960 pollen gave identical nitrogen and arabinose values. Neither C₁ nor C₂ of the 1961 pollen, however, gave a 0 to 60 fraction which had analyses that corresponded with the analyses of the products obtained from the other crops. The 60 to 90 fractions from the three pollen batches were completely different in all respects. Differences in specific absorbance occurred between those products which had shown differences of nitrogen and arabinose values.

Table V contains the comparative skin test data on treated ragweed-sensitive patients. Since it was recognized that skin tests varied from time to time for a given subject and from individual to individual, it was felt desirable to have a reference standard for any given test. We arbitrarily chose Pool C, 1960, 0 to 60,

Table IV. *Chemical analyses of fractions*

<i>Fraction</i>	<i>Nitrogen (%)</i>	<i>Arabinose (%)</i>	<i>d₂₇₆* (1 mg./ml.)</i>
<i>Pool C, 0 to 60</i>			
1959	11.9	11.5	1.178
1960	12.7	11.7	1.178
1961 C ₁	11.30	11.3	0.781
1961 C ₂	11.50	15.4	1.095
<i>Pool C, 60 to 90</i>			
1959	12.4	15.4	1.178
1960	13.7	18.1	0.4 - 0.6
1961 C ₁	11.6	11.5	0.170
1961 C ₂	10.2	12.5	0.905

*d₂₇₆ (1 mg. per milliliter) is the absorbance at 276 mm. for a solution containing 1 mg. of solids per milliliter.

Table V. *Comparative skin test data on treated ragweed-sensitive patients*

<i>Relative activity</i>	<i>P</i>	<i>T</i>	<i>C</i>	<i>W</i>	<i>B</i>	<i>F</i>
<i>Pool C, 0 to 60</i>						
1959	-2	-1	0	-1		
1960	0* (10 ⁻⁵)†	0* (10 ⁻⁵)†	0* (10 ⁻⁵)†	0* (10 ⁻⁷)†	0* (10 ⁻⁵)†	0* (10 ⁻⁵)†
1961 C ₁		0		0	-2	-2
1961 C ₂				+2	-1	-1
<i>Pool C, 60 to 90</i>						
1959		-1	+1	-1		
1960	0	-1	-1	0	-1	-2
1961 C ₁				+1	-1	-1
1961 C ₂				+3	0	0

*The numbers indicate the relative number of tubes (factors of ten) by which each fraction varies. The plus sign indicates greater activity and the minus sign indicates smaller activity.

†The numbers in parentheses are the range of limiting concentrations milligram per milliliter of the solid material which elicit a positive skin test for the given subject.

as the reference standard, and each patient was tested with serial dilutions of this standard along with the other test solutions. The lowest concentration that gave a distinctly discernible skin test was used as an estimate of the skin activity of the material. For most patients and fractions, Pool C 0 to 60 for 1960 had stronger skin-reactive properties than the other 0 to 60 fractions. Patient W, however, was clearly more sensitive to the 1961 C₂ 0 to 60 fraction. The same results were found for the 60 to 90 fractions, again with the exception of Patient W and the 1961 C₁ and C₂ fractions.

In general the immunodiffusion patterns (Figs. 2 and 3) from different years for both fractions 0 to 60 and 60 to 90 showed common lines. However, there were many cases where lines present in one fraction did not appear to be present in the other parallel fractions. In Fig. 3 a comparison of the 0 to 60 fractions from 1959 (Well A), 1960 (Well B), and 1961 C₂ (Well C) showed

Fig. 2

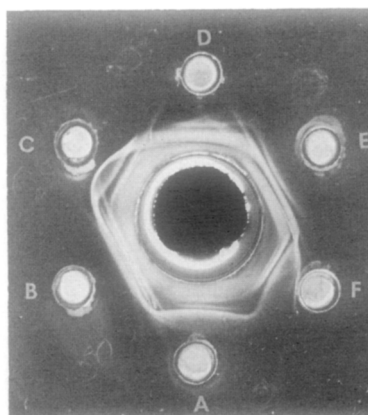


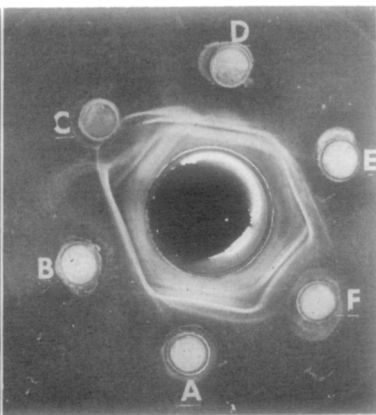
Fig. 2

Ouchterlony patterns: *A*, Pool C, 0 to 60, 1959; *B*, Pool C, 0 to 60, 1960; *C*, Pool C₂, 0 to 60, 1961; *D*, Pool C, 60 to 90, 1959; *E*, Pool C, 60 to 90, 1960; *F*, Pool C₂, 60 to 90, 1961. The antiserum was Pool 90.

Fig. 3

Ouchterlony patterns: *A*, Pool C, 0 to 60, 1960; *B*, Pool C₁, 0 to 60, 1961; *C*, Pool C₂, 0 to 60, 1961; *D*, Pool C, 60 to 90, 1960; *E*, Pool C₁, 60 to 90, 1961; *F*, Pool C₂, 60 to 90, 1961. The antiserum was Pool 90.

Fig. 3



that the 1960 fractions gave three distinct lines which differed from the 1959 and 1961 fractions and that a line common to the 1959 and 1961 C₂ fractions was only present in small amounts in the 1960 fractions. The 1960 60 to 90 fraction had at least two components which differed from the other corresponding fractions. On the other hand, the 1959 and 1961 60 to 90 fractions had two components which differed from the other corresponding fractions. The 1959 and 1961 60 to 90 fractions gave two components which differed from the 1961 C₂ fraction.

Fractionation of pollens of 1961 and 1963 (Method B)

Table VI is a comparison of the yields of each product obtained under identical conditions from 1961 and 1963 pollens. In general, the yields of the fractions obtained from the 1963 pollen, with some few exceptions, greatly exceeded the yields of the corresponding fractions from 1961 pollens. Only in the case of fraction B₄G₁ did the 1961 pollen give a larger yield. In some cases the same yields were obtained from the different pollens, e.g., B₂G₁, B₂G₂, B₃G₁, B₃G₂, and B₄G₃.

A comparison of the immunodiffusion reactions of the products with AWRW and AE showed good agreement for the most part. However, several fractions, which were isolated in the same chromatographic position, were found to give different immunodiffusion patterns when derived from the 1961 or 1963 pollens (Table VII). All of the products listed were skin reactive at concentrations of 10⁻⁵ mg. per milliliter or less. There were fractions which differed only in respect

Table VI. Yields isolated from dwarf pollen

Fractions	Ve/V_o^*									
	G_1 1-1.1* (mg.)		G_2 1.5* (mg.)		G_3 1.8-2.0* (mg.)		G_4 2.2-2.3* (mg.)		G_5 2.7* (mg.)	
	61	63	61	63	61	63	61	63	61	63
B ₁	120	177	0	171	14	142	9	137	0	89
B ₂	16	27	14	19	0	115	tr†	40	tr†	9
B ₃	5	6	34	40	0	0	10	42	0	0
B ₄	25	6	0	0	49	50	0	4	0	0

*This is the range of values of Ve/V_o where V_o is the effluent volume of the Sephadex G 100 column for complete exclusion as determined with Blue Dextran and Ve is the volume at the peak for the fraction. This ratio is specific for the eluted fraction.

†Only trace solids recovered.

Table VII. Immunological properties of fractions*

Sample	anti-WRW		anti-IV C	
	61	63	61	63
B ₁ G ₃	++†	++	neg.	+wk.
B ₁ G ₄	++	neg.	neg.	+wk.
B ₂ G ₁	++wk.	++	neg.	+
B ₄ G ₁	+	+wk.	neg.	neg.
B ₄ G ₃	++	++	+	++

*Positive skin tests at 10^{-6} mg. per milliliter at least. The most active skin reacting product was B₁C₃.

†Abbreviations: ++ = multiple distinct lines, by immunoelectrophoresis; + = single distinct line by immunoelectrophoresis; +wk = single line but comparatively weak; neg = no lines.

to the presence or absence of IV C antigens (B₂G₁) or in the reaction with AWRW (B₁G₄). Other qualitative differences also appeared, e.g., the reaction with AE to give either one or several lines (B₄G₃).

DISCUSSION

From the evidence presented above it appears that there were major differences among the dwarf ragweed pollens harvested in the 4 years under study. If the evidence for this conclusion had been based on a single method of fractionation, e.g., the preparation of Pool C (Method A), it might have been surmised that the differences were due to experimental anomalies peculiar to the method of fractionation. However, a second and distinctly different method of fractionation also gave rise to major differences.

Although the pollens have been labeled by the year of harvest, it does not necessarily follow that the time was the major factor. It is quite possible that the geographical origin of the pollens were different. We have no information on this matter. All that can be stated definitely is that the pollens had been examined microscopically and were found to contain more than 95 per cent of the dwarf variety. It does not seem reasonable to expect that a 5 per cent impurity would be sufficient to account for the major differences observed.

The data describe quantitative and qualitative differences among corresponding fractions isolated according to the prescribed procedures; these differences were known to be reproducible with the same batch of pollen. The differences extended to the relative skin reactivities of the corresponding fractions of different batches and this should have a clinical significance. The same difference in potency has been briefly mentioned some time ago by Feinberg.⁵ On the other hand, the physicochemical differences between the active fractions, e.g., Pool C 0 to 60 for 1959 and 1960 versus Pool C₁ or C₂ for 1961, create problems for investigators who attempt to reproduce results with different batches of pollens. This is emphasized by the report of Robbins and associates⁶ in which two products (IVRBB and IVRCC) were isolated from a mixture of 1960, 1961, and 1962 pollens and which contained 100 per cent of antigen E but differed significantly from the comparable product of King, Norman, and Connell with respect to amino acid analysis and specific absorbances. It is not to be presumed from our conclusions that we can or do infer any large change in the nature of the allergen or allergenic sites.

The observation which we have not emphasized in this report (that there are skin-reactive materials present in whole ragweed which do not react with A.E.) is a separate observation which is being reported elsewhere.

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