

STUDIES ON RAGWEED POLLEN. I. PRELIMINARY FRACTIONATION OF RAGWEED POLLEN

CHARACTERIZATION AND ALLERGENICITY OF THE ISOLATED FRACTIONS

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INTRODUCTION

THE study of the chemical and immunologic properties of ragweed pollen was begun as early as 1831, when Elliotson¹ suggested ragweed as a possible causative agent of hay fever, an assumption that was later fully substantiated by the studies of Blackley.² Since then, numerous attempts have been made to isolate and characterize the active substances present in ragweed pollen, but with little success.

The results of chemical fractionation of ragweed pollen extract by applying different precipitating agents, such as ammonium sulfate,^{6-9, 13, 16, 24, 36} alcohol,^{3-5, 15, 19, 37, 48} acetone,³¹ lead acetate,^{21, 36} acids,^{10, 11, 25, 26} and alkali,^{7, 15, 21} have suggested allergenicity to be due to a carbohydrate or a protein constituent, or to both.

Using Tiselius electrophoresis, Newell³⁸ and Abramson and his associates²³ demonstrated the complex nature of ragweed pollen extract by showing that it can be resolved into a number of electrophoretically distinct components. Abramson and associates²³ using this method, were able to separate one of the faster migrating, pigmented components from the main immobile, colorless fraction. Allergenic activity was elicited by both fractions.

Abramson and associates²³ also determined a sedimentation constant of 1.5 for the major unpigmented, immobile material and calculated a molecular weight of 5,000 for this material. Sanigar⁴⁷ calculated a sedimentation constant of 1.0 for his ragweed extract, and concluded that the molecular weight could not exceed 17,000.

Stevens and his co-workers⁴⁰ determined a sedimentation constant of 1.3 for the nondiffusible fraction of their ragweed extract and calculated a molecular weight of 17,800 for this material. In a second study,³⁹ using free electrophoresis and melting point determinations, they showed that one of the pigments in ragweed pollen was isoquercitrin.

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Loveless and her associates²⁹ in 1951, using standard Visking tubing, were able to obtain a separation between the diffusible and nondiffusible components in ragweed pollen extract. Both fractions exhibited allergenic reactivity, but the nondiffusible material was the more active. Free electrophoresis revealed the presence of an essentially immobile, colorless component in both fractions.

Alexander and his associates^{30, 31} fractionated, by paper chromatography, the dialysate of the ragweed pollen extract into five different components. Four of these stained for peptides and one for carbohydrate. The nondialyzable fraction did not stain for either component; but, after undergoing acid hydrolysis, this latter material stained with ninhydrin. These facts would indicate that the nondialyzable material was proteinous in nature. Allergenic activity was detected in all the fractions.

The results of the fractionation of ragweed pollen by paper chromatography by Bernstein and his co-workers⁴¹ and Perlman⁴² also emphasized the complex composition of ragweed pollen and the presence of numerous allergenically active constituents.

Bookman and Wax,⁴³ using ion exchange resins, demonstrated that the allergenicity of ragweed pollen fractions is totally unrelated to their respective nitrogen contents.

Munoz and Becker,²⁸ applying the Oudin "gel technique,"^{33, 34} clearly established the existence of at least five different antigens in ragweed pollen extracts. Wodehouse³² was able to demonstrate seven or eight precipitin bands by the Ouchterlony "gel technique,"^{34, 35} each band representing a different antigen in the pollen extract.

In 1954 Abramson and his associates⁴⁴ claimed to have prepared a "purified" ragweed extract by treatment of defatted ragweed pollen with alcohol prior to extraction with water. This latter extract, designated by them MLL (methanol leached and lyophilized), exhibited only two peaks on free electrophoresis and was therefore considered to be a "purified" extract.

Since it was our intention to study the immunologic and physicochemical properties of the various "pure" ragweed components, we chose for our initial study the "purified" fraction as prepared by Abramson and associates.⁴⁴ However, as shown in this report, this "purified" fraction proved to be a complex mixture of materials.

METHODS AND MATERIALS

Low ragweed pollen (*Ambrosia artemisiaefolia*) was defatted with diethyl ether in a Soxhlet extraction apparatus for three days. MLL was prepared by the method of Abramson and associates⁴⁴ in the following manner:

Five grams of the defatted pollen was leached with 50 c.c. aliquots of 90 per cent CH₃OH and the extracting medium was changed frequently until it was completely colorless. This alcohol-soluble fraction next was distilled under suction at 50 to 60° C. until alcohol could no longer be detected, and

then it was lyophilized. This fraction is referred to as ROH SOL. The residue from the alcohol extraction was dried and extracted with water for seventy-two hours. This extract was immediately lyophilized and constitutes MLL. A portion of the insoluble residue was then extracted continually with water for twelve days longer, the extracting fluid being replaced every two days, and the former was lyophilized. This preparation is referred to as MLL INSOL. All extractions with alcohol and water were performed in a cold room at 3 to 5° C. The water used in all extractions, except where specified, was distilled water.

To determine the specific effect, if any, of alcohol on the composition and allergenicity of ragweed pollen, MLL was compared with a water extract of ragweed pollen which had not undergone preliminary alcohol leaching. The water extract of pollen was prepared by extracting 10 grams of ether-defatted ragweed in 100 c.c. of water for seventy-two hours. The mixture was then centrifuged at 20,000 r.p.m. for twenty minutes in a Spincro Model L preparative ultracentrifuge. The yellow-colored supernatant was slightly turbid, this opalescence being attributed to some lipoidal material still present in ragweed pollen after the extensive defatting procedure. The solution was then lyophilized, redissolved in a small volume of water (20 c.c.), and recentrifuged at 20,000 r.p.m. for twenty minutes. A white material rose to the surface of the liquid, and the clear, yellow-colored supernatant was drawn off with a syringe provided with a long needle and lyophilized. This material is referred to as WSR (water-soluble ragweed). The insoluble residue left after the seventy-two hours' extraction of the defatted pollen with water was further extracted continually for sixteen days with water, the extracting fluid being changed every two days. The insoluble residue which remained was lyophilized and is referred to as WSR INSOL.

Both WSR and MLL were dialyzed through Visking tubing against water, the ratio of the volume of the solution within the Visking tubing to that outside being 1:5. In each case, the water solution of the fraction was first dialyzed for thirty-six hours, and the dialysate was subsequently lyophilized. This fraction is termed *dialysate*. The residue remaining within the sac was then dialyzed continually for ten days against running tap water to ensure complete removal of dialyzable material. It was then lyophilized and designated as *dialyzed residue*.

To determine whether allergenic reactivity was still associated with the MLL INSOL, a portion of this material was extracted with 0.05 N NaOH for seventy-two hours. The insoluble residue was then lyophilized, and is referred to as NaOH INSOL. The yellow-colored supernatant was then acidified to pH 6.0 with 0.1 N HCl, at which point a white, fluffy precipitate separated out. The supernatant was separated from this fine precipitate by centrifugation at 25,000 r.p.m. for twenty minutes, and is referred to as pH 6 SOL. The precipitate is referred to as NaOH SOL. Both extracts were subsequently lyophilized.

Fig. 1 shows a flow sheet of the complete extraction procedure of the various fractions from ragweed pollen.

The extracts were compared by the following four methods:

I. *Free Electrophoresis*.—The three fractions—MLL, WSR, and ROH SOL—were analyzed by free electrophoresis.

The Spinco Tiselius apparatus was used throughout the present study to determine the electrophoretic pattern of each fraction. The solutions for electrophoresis were prepared by dissolving the fractions in the following buffers:

- Veronal buffer, pH 8.6; ionic strength 0.1
- Borate buffer, pH 8.6; ionic strength 0.1
- Phosphate buffer, pH 7.8; total ionic strength 0.17 (contribution of NaCl 0.15)
- Glycine buffer, pH 10.6; total ionic strength 0.17 (contribution of NaCl 0.15)
- Glycine buffer, pH 3.5; total ionic strength 0.17 (contribution of NaCl 0.15)

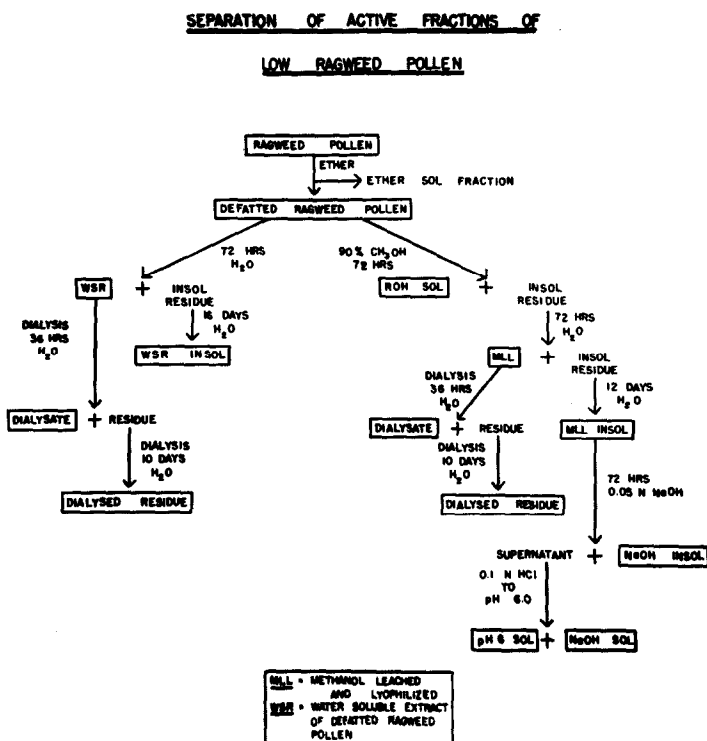


Fig. 1.—Flow sheet of the extractions of fractions from low ragweed pollen.

Each of the fractions was found to contain both diffusible and nondiffusible components on dialysis through Visking tubing. Therefore, when each fraction was analyzed by electrophoresis for all its constituents, initial dial-

ysis against the respective buffer was dispensed with. To minimize anomalies in the electrophoretic experiments, the conductivities of the buffer and of the respective extract-buffer solution were equalized prior to each run. The pH of the extract buffer solution was raised or lowered to that of the buffer by the addition of either 0.1 N NaOH or 0.1 N HCl; the conductivities were then equalized by adding solid NaCl to the buffer. These steps were necessary since, in all cases, the solution of any particular fraction in the buffer used had a higher conductivity than, as well as a different pH from, the corresponding buffer.

II. *Paper Electrophoresis*.—Whatman No. 1 filter paper was used throughout.

The aqueous solution of the material to be separated by paper electrophoresis was applied at the center of the strip of filter paper as a narrow band about 30 to 35 mm. long. The filter paper was previously immersed in the buffer and semidried between sheets of filter paper. The paper was then layered onto a horizontal Plexiglas plate* which was used as the supporting surface. This, in turn, was placed onto the buffer vessels, the two extremities of the paper dipping into the buffer to a depth of about one inch. The buffer vessels had previously been filled to an equal level, which is essential to prevent chromatographic effects from disturbing the separation pattern of the material under observation. For similar reasons, the plate supporting the filter paper strips was leveled both longitudinally and vertically.

Heating, caused by passage of current through the paper strips, results in some evaporation of water from the filter papers saturated with buffer. To minimize evaporation from the paper during electrophoresis, a glass plate supported by a foam rubber frame was placed onto the papers, forming a closed system. A sheet of Whatman No. 3 MM. filter paper was placed between the glass plate and the foam rubber frame. Its function was to absorb any water vapor which might condense on the glass plate during electrophoresis. Application of high voltages (above 170 volts) results in more evaporation from the paper. This, in turn, causes absorption of more buffer into the paper strips with a concomitant chromatographic displacement of the electrophoretically separated bands toward the center of the paper. The effect of absorption of more buffer into the paper results in a further increase of the concentration of buffer salts in the filter papers. This, in turn, results in even more evaporation and in serious disturbances of the electrophoretic separation. These effects were minimized by immersion of the ends of the Whatman No. 3 MM. paper into the buffer vessels. Due to the lower resistance of this buffer-saturated filter paper, as compared to that of the Whatman No. 1 filter paper strips, more heat was dissipated from it. Consequently, the electrophoretic chamber was saturated with vapor emanating mainly from the What-

*The upper surface of the Plexiglas plate was provided with small, closely spaced, pyramidal projections, extending to a height of $\frac{1}{8}$ inch above the surface of the plate. This arrangement prevented the paper from sagging and the formation of a surface liquid film between the paper and the Plexiglas. The dimensions of the Plexiglas plate were 16 inches long by 15 inches wide.

man No. 3 MM. paper. The buffer vessels were then connected by paper wicks with the electrode vessels, filled to equal levels with 20 per cent KCl solution, containing silver-silver chloride electrodes.* These electrodes were used in preference to the conventional platinum or carbon and steel electrodes to ensure constancy of pH during electrophoresis.

Preliminary experiments demonstrated that optimal separation of ragweed pollen extracts was obtained by applying a potential of 110 volts, the current varying from 20 to 40 milliamperes, depending on the buffer used. Electrophoresis was carried out at room temperature. The duration of a single electrophoretic experiment never exceeded fourteen hours, but varied with the fraction being analyzed.

Upon completion of the run, the papers were dried quickly at 120° C. and then stained for carbohydrate, protein, amino acids, and peptides.

The following staining reagents were used:

- (a) 1 per cent ninhydrin solution in 95 per cent ethyl alcohol to detect amino acids and peptides⁵⁰
- (b) The Lemieux-Bauer reagent to detect carbohydrates⁴⁹
- (c) Amido-black (10 B) to stain for proteins⁴⁶

For the sake of brevity, all bands revealed by ninhydrin staining are referred to as peptide bands.

Buffers used in this study were:

- (a) Veronal buffer, pH 8.6; ionic strength 0.10
- (b) Borate buffer, pH 8.6; ionic strength 0.10
- (c) Phosphate buffer, pH 7.8; ionic strength 0.17 (contribution of NaCl 0.15)
- (d) Citrate buffer, pH 4.56; ionic strength 0.10

Preliminary experiments indicated that borate buffer provided the resolution of a maximum number of components of WSR and MLL. Therefore, results obtained with this buffer are the only ones reported here.

III. *Ultracentrifugation.*—The Spinco Model E optical ultracentrifuge was used to determine the sedimentation constants of the various fractions. The experiments also served to disclose any heterogeneity of the materials with respect to molecular weights.†

The synthetic boundary cell, as devised by Pickels and his co-workers⁴⁵ was used instead of the standard cell. This cell allows for the formation of a sharp initial boundary between the solution under observation and the solvent at the start of the experiment. Formation of initial boundaries is essential in determining the sedimentation rate of small molecular weight materials, which otherwise would diffuse at such a rapid rate as to prevent

*The silver-silver chloride electrodes were prepared from silver strips $\frac{1}{4}$ inch wide and $\frac{1}{32}$ inch thick.

†Assuming that diffusion coefficients and partial specific volumes are equal for all fractions.

the formation of a sedimenting boundary. The average rotor temperature was 20.5° C. The sedimentation constants were not recalculated for standard conditions.

All the fractions, with the exception of ROH SOL, were dissolved in 0.9 per cent NaCl. The ROH SOL fraction was dissolved in glycine buffer at pH 10.6 due to its low solubility in 0.9 per cent NaCl and in solutions of lower alkalinity.

IV. *Cutaneous Reactivity*.—All the fractions were made up to 1,000 units per cubic centimeter (1 unit = 10^{-5} mg. nitrogen). Nitrogen values were determined by a modification of the method of Lanni, Dillon, and Beard.⁵¹ All the fractions were tested on ragweed-sensitive persons. The activity was determined by scratch test and reactions were read after twenty minutes. Reactions were tabulated as 0, 1+, 2+, 3+, and 4+, depending on the size and extent of the wheal and surrounding erythema.

RESULTS

Initial extraction with alcohol, followed by extraction with water, resulted in a lower yield of extractable material from defatted pollen as compared with the yield of material extracted with water only, as shown in Table I. The data in this table were arrived at by assuming that the lyophilized fractions were completely dehydrated.

TABLE I

FRACTION	PERCENTAGE OF WHOLE DEFATTED RAGWEED
WSR 16-day extraction	30
ROH soluble ragweed	12
MLL	6
pH 6 soluble ragweed	20
NaOH soluble ragweed	8
NaOH insoluble ragweed	52

Free Electrophoresis.*—The three fractions examined by free electrophoresis (WSR, MLL, and ROH SOL), using different buffer systems, were all found to be electrophoretically heterogeneous. An essentially immobile peak was present in all the electrophoretic patterns of the fraction. At least three distinct peaks were identified in each pattern. The most striking electrophoretic separation occurred with WSR in glycine buffer at pH 3.5. At least five well-characterized, small peaks were seen migrating to the negative pole at a rapid rate. The results are illustrated in Figs. 2, 3, 4, and 5.

Due to the low solubility of ROH SOL in buffers of pH lower than 9, it was analyzed by free electrophoresis only in glycine buffer at pH 10.6 (Fig. 5).

Paper Electrophoresis.*—By paper electrophoresis in borate buffer at pH 8.6, WSR and MLL were resolved into visible pigment bands, in addition to distinct bands revealed by staining with ninhydrin, amido-black, and the carbo-

*In Figs. 2 through 11 the arrows represent the initial boundaries and zones of application in experiments using free electrophoresis and paper electrophoresis, respectively.

hydrate reagents. It is evident from Figs. 6 and 7 that WSR and MLL are distinct materials, containing different numbers of electrophoretically separable fractions. WSR is comprised of four pigment bands, one protein band, one carbohydrate band, and one peptide band which migrated independently

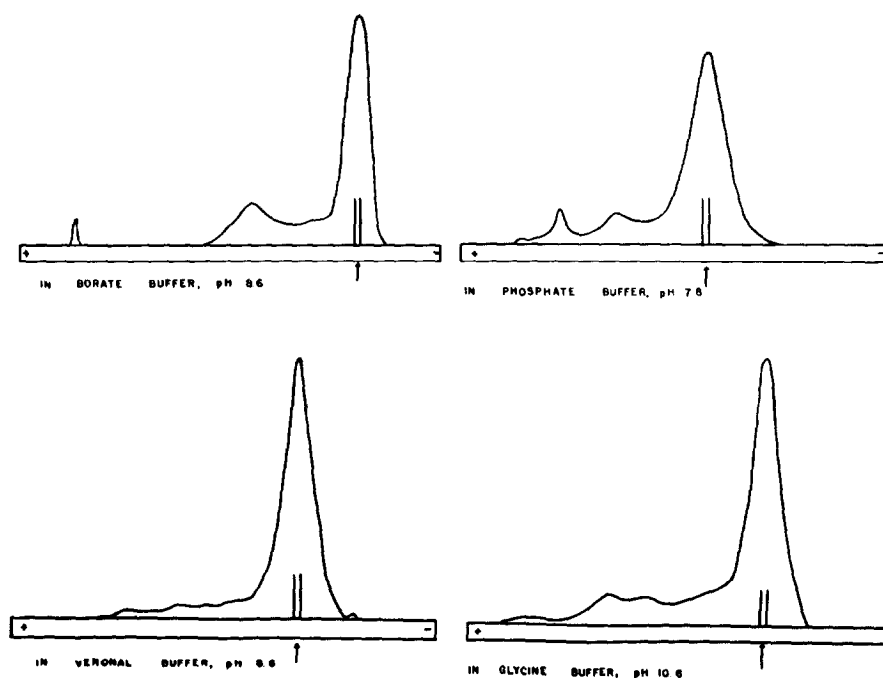


Fig. 2.—Free electrophoresis of the water extract of defatted low ragweed pollen (WSR).

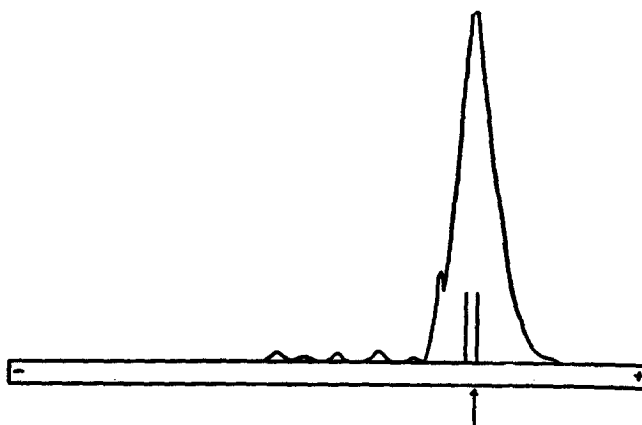


Fig. 3.—Free electrophoresis of the water extract of defatted low ragweed pollen (WSR) in glycine buffer, pH 3.5.

to the positive pole; three peptide bands which migrated to the negative pole; and one diffuse peptide band which maintained itself at the center of the paper, but extended into both the cathodic and anodic regions of the paper. Since it extended more into the cathodic region, this slow migration

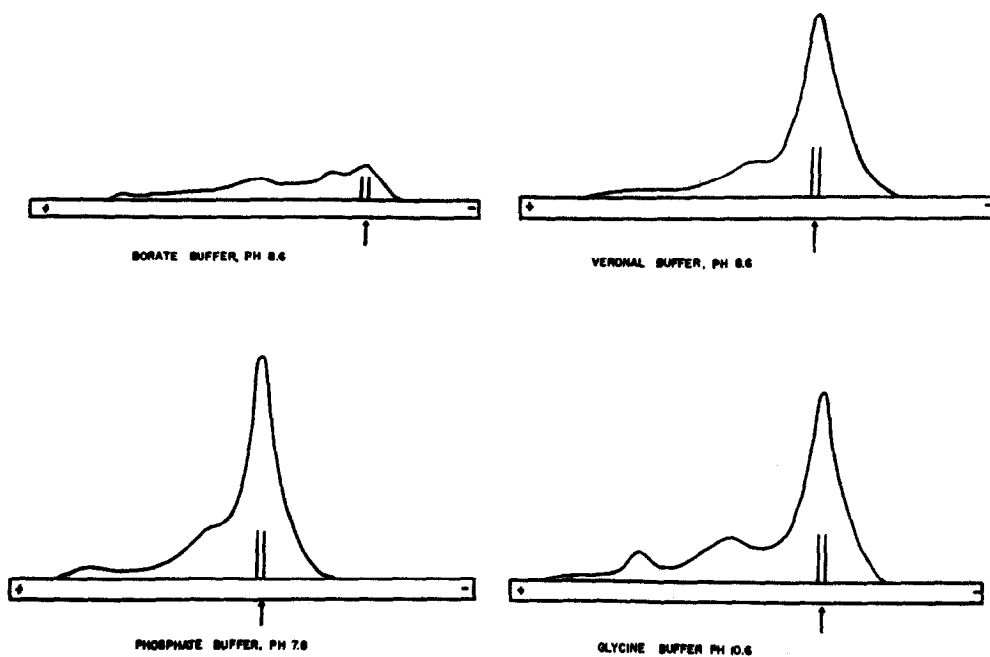


Fig. 4.—Free electrophoresis of the water extract of defatted low ragweed pollen after methanol extraction (MLL).

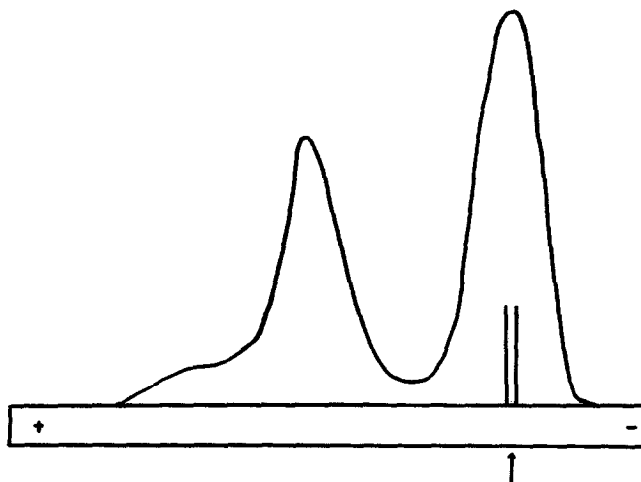


Fig. 5.—Free electrophoresis of the methanol extract of the defatted low ragweed pollen (ROH SOL) in glycine buffer, pH 10.6.

is considered to be due to electrophoresis rather than to diffusion. It ought to be mentioned that all the five peptide bands were detected only in the early stages (six hours) of electrophoresis of WSR in borate buffer at pH 8.6 (Fig. 6). In experiments of longer duration (twelve to fourteen hours), however, the two rapidly migrating cathodic bands were permitted to run off the paper in order to obtain an optimal resolution of the other components.

On the other hand, MLL in borate buffer at pH 8.6 (Fig. 7) separated into one diffuse pigment, one protein, one carbohydrate, and three peptide bands.

ROH SOL in borate buffer gave rise to three pigment bands (Fig. 8). No carbohydrate-stainable material could be detected in this fraction, while a faint ninhydrin band was noted at the cathodic side of the point of application.

The pH 6 SOL could not be resolved into more than one component by paper electrophoresis using Veronal and borate buffers. Electrophoretic separations with these two buffers at pH 8.6 resulted in identical patterns (Fig. 9).

A comparison of the electrophoretic patterns of the dialysate, dialyzed residue, and whole extract for WSR and MLL, respectively, as represented in Figs. 10 and 11, suggests that both WSR and MLL can be partially fractionated by dialysis through Visking membrane against water. The dialysate of WSR contained four pigment, three peptide, and one carbohydrate bands.

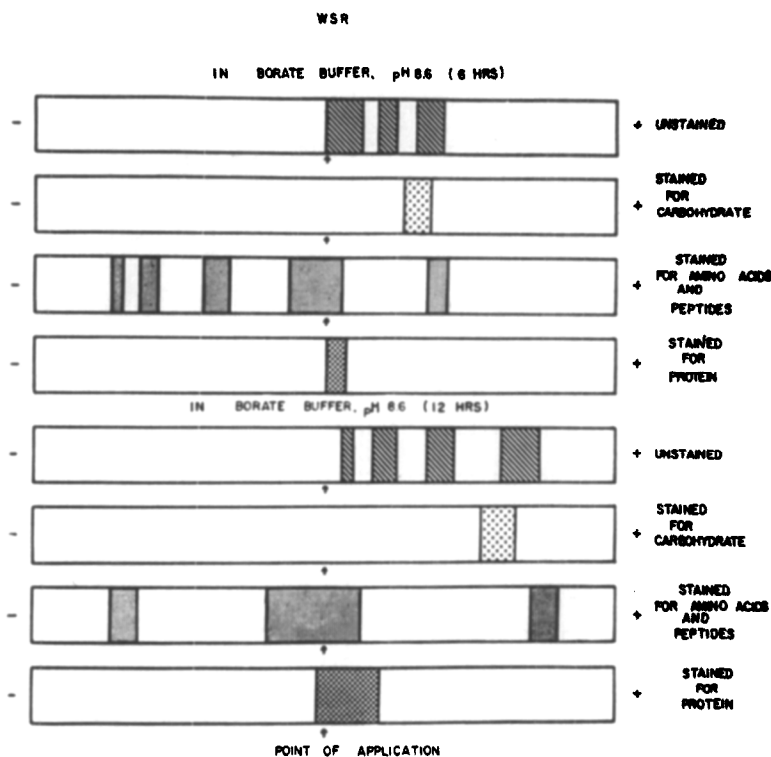


Fig. 6.—Paper electrophoresis of the water-soluble extract of defatted low ragweed pollen (WSR). The upper four diagrams represent the resolution of the constituents after six hours of electrophoresis. The lower four diagrams represent the separation of the components after twelve hours of electrophoresis.

The dialyzed residue consisted mainly of a protein-stainable material which migrated slowly to the anode. A slightly visible pigment band and a barely stainable carbohydrate band were seen migrating with it. In addition, a faint peptide band was detected which migrated slowly to the cathode.

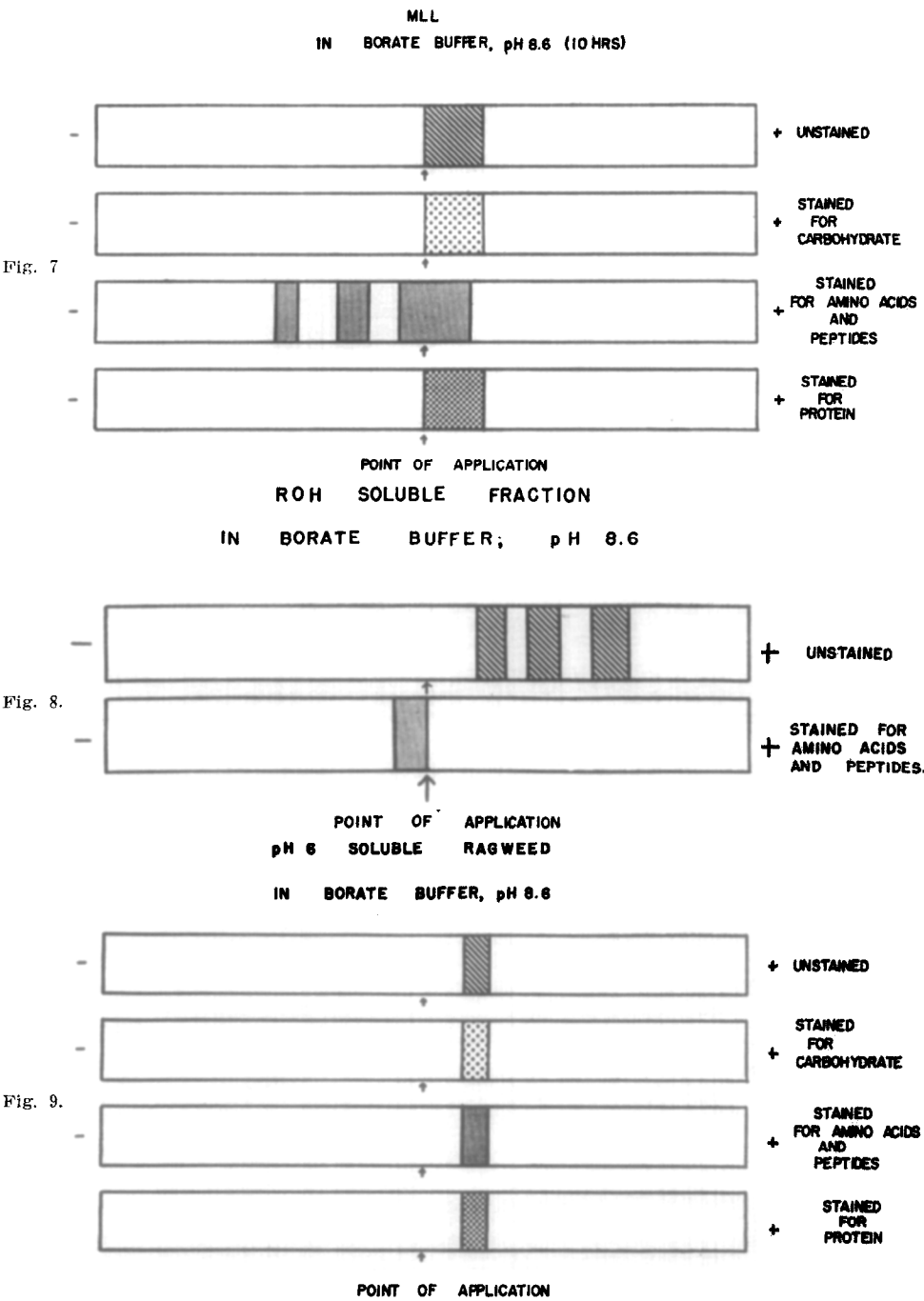


Fig. 7.—Paper electrophoresis of the water extract of defatted low ragweed pollen after methanol extraction (MLL).

Fig. 8.—Paper electrophoresis of the methanol extract of defatted low ragweed pollen (ROH SOL).

Fig. 9.—Paper electrophoresis of pH 6 soluble fraction. (For definition, see text.)

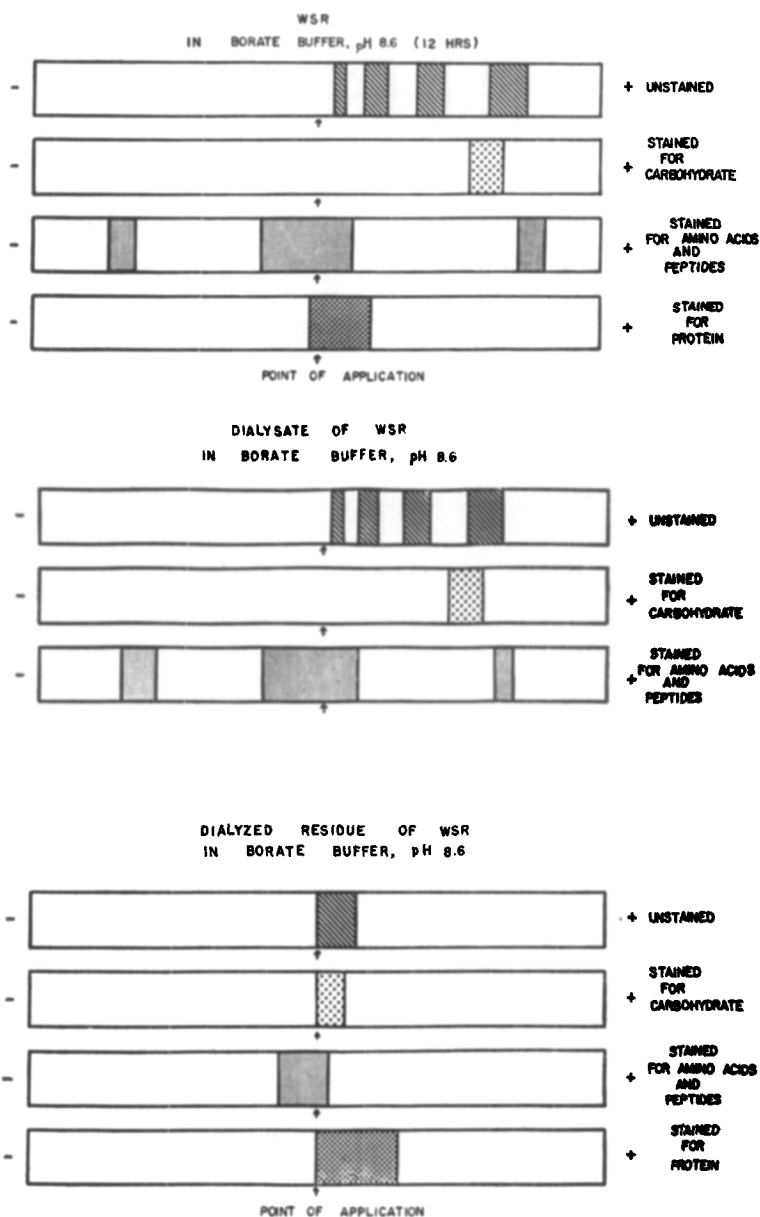


Fig. 10.—The upper four diagrams represent the electrophoretic separation of the components of WSR on paper. The middle three diagrams represent the separation of the constituents of the dialysate of WSR. The lower four diagrams represent the electrophoretic separation of the components of the dialyzed residue of WSR. Time for electrophoretic separation was twelve hours.

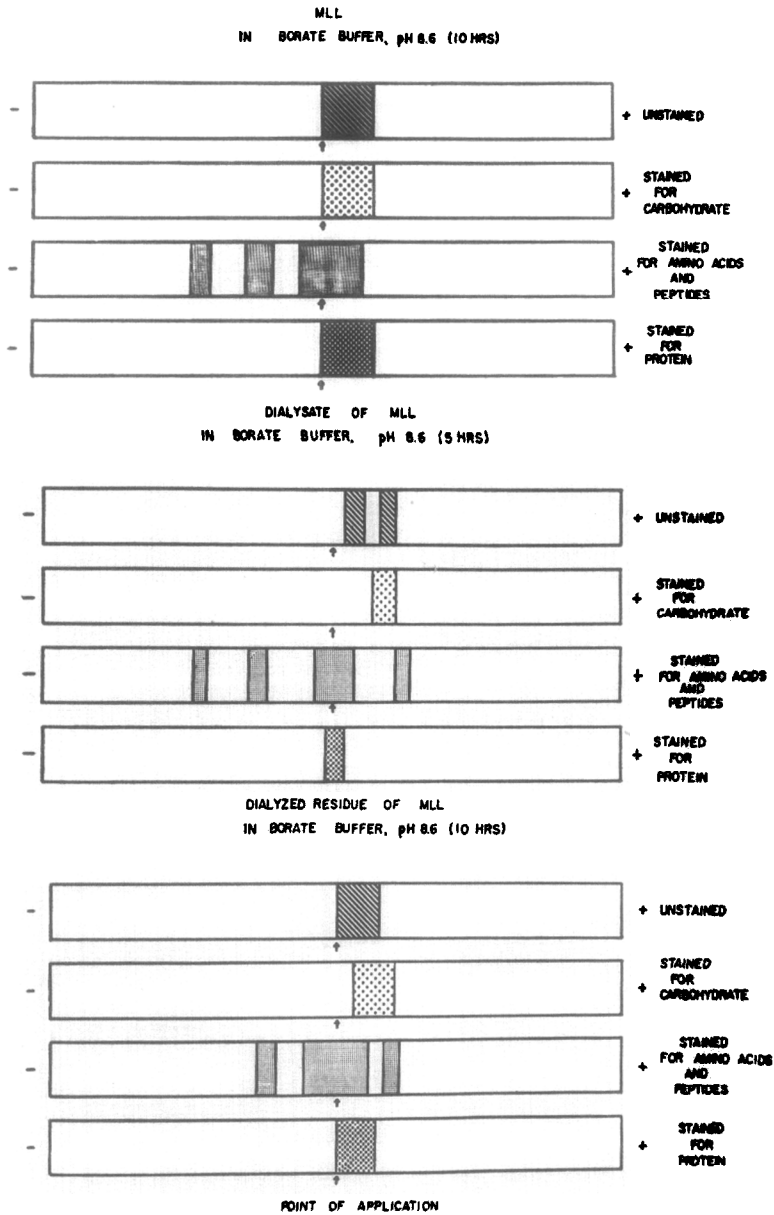


Fig. 11.—The upper four diagrams represent the electrophoretic separation of the components of MLL on paper after ten hours. The middle four diagrams represent the separation of the constituents of the dialysate of MLL after five hours. The lower four diagrams represent the components of the dialyzed residue of MLL resolved after ten hours.

The dialysate of MLL resolved itself into four distinct peptide bands, one carbohydrate band, and two pigment bands. In addition, a faint protein band was also detected. The four peptide bands represent bands migrating at a faster rate than those present in MLL. The dialyzed residue of MLL consisted of one protein, one pigment, one carbohydrate, and three peptide bands. The total number of distinguishable, electrophoretically separated bands in the dialysate and dialyzed residue was greater than the number detected in undialyzed MLL.

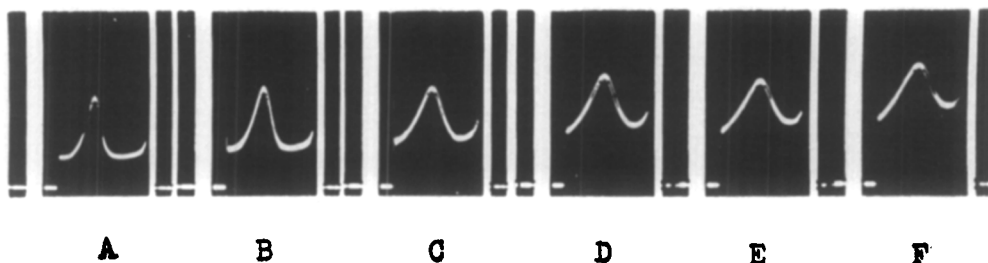


Fig. 12.—Sedimentation photographs of whole ragweed extract (WSR) taken at 59,780 r.p.m. Frame A represents the initial boundary in the synthetic boundary cell; frames B, C, D, E, and F represent photographs taken subsequently after twelve, eighteen, thirty-two, forty, and forty-eight minutes.

Ultracentrifugation.—Each extract gave rise to only one broad peak in the analytical ultracentrifuge. Fig. 12 is a photograph of a typical experiment. Sedimentation constants were determined for the majority of the fractions (Table II). The sedimentation constants were all rather low, varying from 0.45 to 2.54 Svedberg units. The WSR had a sedimentation constant of 0.92, while its dialysate and dialyzed residue had sedimentation constants of 0.61 and 2.54, respectively. The ROH SOL had the lowest sedimentation constant of 0.45, while that of the MLL was 1.34. A sedimentation constant of 1.17 was calculated for the pH 6 SOL.

TABLE II. ULTRACENTRIFUGAL ANALYSIS OF ACTIVE FRACTIONS SEPARATED FROM RAGWEED POLLEN*

FRACTION	SEDIMENTATION CONSTANT†
WSR	0.92
WSR—dialysate	0.61
WSR—dialyzed residue	2.54
ROH SOL ragweed‡	0.45
MLL	1.34
pH 6 SOL ragweed	1.17

*Performed in 0.9 per cent NaCl, except where indicated otherwise.

†Expressed in Svedberg units.

‡In glycine buffer pH 10.6.

Cutaneous Reactivity.—The results of cutaneous tests are shown in Table III. The WSR and ROH SOL fractions appeared to exhibit comparable skin reactivity, and the MLL and pH 6 SOL fractions only slightly less. The activity of the ROH SOL fraction diminished rapidly on standing, however, and completely disappeared after four weeks of storage in aqueous solution at

3 to 5° C. The activity of the other fractions also decreased with time, on storage in solution, but at a much more gradual rate. Loss of activity in other fractions could be demonstrated only after two to three months of storage in aqueous solution at 3 to 5° C.

In the present study, the ether-soluble fraction of ragweed pollen was not tested for skin reactivity. However, it was shown by Moore and his associates¹⁴ to be active.

TABLE III. REACTIVITY OF FRACTIONS BY SCRATCH TEST

FRACTION*	SUBJECT TESTED								
	L. K.	M. G.	S. T.	A. M.	M. L.	D. K.	B. L.	B. K.	M. B.
pH 6 SOL	2+	±	0	4+	2+	±	±	3+	3+
NaOH SOL	±	0	0	0	0	0	0	0	0
NaOH INSOL	0	0	0	0	0	±	0	0	0
WSR	4+	2+	4+	4+	2+	2+	±	4+	-
WSR INSOL extracted with water—5 days	3+	0	±	4+	1+	±	-	3+	-
WSR INSOL extracted with water—14 days	-	-	-	-	2+	-	±	3+	4+
ROH SOL	4+	0	4+	0	0	±	-	0	0
MLL	4+	-	-	4+	3+	2+	-	4+	4+
MLL INSOL	±	0	0	0	1+	±	-	0	-

*Each made up of 1,000 units per cubic centimeter (1 unit = 10⁻⁵ mg. nitrogen).

The WSR INSOL, even after sixteen days of continuous extraction with water, still manifested positive cutaneous reactivity. MLL INSOL was not active when tested by scratch. However, the pH 6 SOL extracted from this seemingly inactive ragweed pollen residue was quite active. The NaOH INSOL, as well as the NaOH SOL, displayed essentially no whealing activity.

DISCUSSION

Free electrophoresis demonstrated that none of the preparations was homogeneous. A stationary component was distinguished in each of the electrophoretic experiments of the MLL, WSR, and ROH SOL fractions. One possible interpretation of these results is that ragweed pollen extract might contain a component having no ionizable groups, for it does not migrate in either acid or alkaline buffers. One type of substance which might behave in this manner could be a closed-ring polypeptide devoid of free amino or carboxyl groups. The large, diffuse stationary peak may also represent the contribution of salt boundaries.

Paper electrophoresis permits the fractionation of a complex solution of materials without preliminary adjustment of its pH and conductivity to those of the buffer. It proved to be a far more effective tool than free electrophoresis in the study of ragweed pollen. The technique yielded more distinct and reproducible patterns and allowed for simultaneous detection and identification of pigment, carbohydrate, peptide, and protein bands by visual observation and selective staining.

WSR resolved itself into eleven components in borate buffer, whereas MLL resolved itself into a lesser number of components. Regardless of the buffer used, MLL was always resolved into a smaller number of components than WSR.

Alcohol extraction, prior to water extraction, appeared to free the ragweed of much of its water-soluble pigments, which is in accordance with the findings of Abramson and associates.⁴⁴ The three faster migrating pigments of WSR were absent in MLL, but were present in ROH SOL ragweed.

The dialysate of WSR apparently contained all the components present in whole WSR, with the exception of the protein constituent. The dialyzed residue still contained a pigment which migrated with the diffuse protein band; it also stained slightly with ninhydrin. Thus, all the components identified in WSR can be accounted for in the dialysate plus the dialyzed residue. Since no new component was detected in the dialysate and dialyzed residue of WSR, it is highly unlikely that ragweed pollen constituents are constantly breaking down during dialysis, as suggested by Loveless and co-workers.²⁹

There is a suggestion, however, of some alteration occurring in WSR on dialysis. The dialyzed residue stained for carbohydrate, although very weakly, in the area of the protein band, whereas the original WSR extract contained no carbohydrate-stainable material associated with the protein constituent. There are at least two explanations to account for these observations:

- (1) Some configurational change might have taken place within the protein moiety of WSR during the extensive dialysis liberating carbohydrate stainable groups.

- (2) The fact that carbohydrate-stainable material was detected in the dialyzed residue of WSR and not in the WSR itself may be due to a concentration effect. WSR may not contain a sufficient amount of the component necessary to be revealed by the carbohydrate reagent. On the other hand, this component might be concentrated to a relatively much higher level on dialysis.

The presence of detectable pigment migrating with the protein in the dialyzed residue and its apparent absence in the whole WSR could be explained in the same manner.

On the other hand, the total number of electrophoretically distinct components present in the dialysate and dialyzed residue of MLL exceeded the number found in undialyzed MLL. This finding suggests that degradation of larger into smaller molecules did occur in the MLL on dialysis. As in the case with the dialyzed residue of WSR, pigment was still present in the dialyzed residue of MLL which also migrated with the protein. These data imply that a pigment either is specifically bound to the protein or that it has similar electrophoretic properties.

The pH 6 SOL could not be resolved into more than one component by paper electrophoresis. One could interpret this finding in terms of a one-component system, the protein, carbohydrate, peptide, and pigment all being part of one and the same molecule which is resistant to alkaline hydrolysis.

On the basis of the sedimentation constants found for the various extracts, molecular weights conceivably could be calculated, provided that the diffusion

coefficient and partial specific volume were known for each fraction. However, diffusion coefficients and partial specific volumes are significant only when calculated for one-component systems. Since none of our fractions, except possibly the pH 6 SOL, complied with this requirement, molecular weight determinations were dispensed with. Due to the high concentration of NaCl in this latter extract and our inability to equilibrate it against the buffer by dialysis without loss of low molecular weight components, free electrophoresis on this material was not performed.

There is no doubt that a shift of small-molecular-weight material occurred through the Visking tubing on dialysis of WSR against water. This was reflected by the great discrepancy in the sedimentation constants of the dialysate and dialyzed residue of WSR. This latter finding served as evidence for the presence of some large molecular-weight material in ragweed pollen extract, which might have been masked by the relatively greater concentration of small molecular-weight material, when the whole extract was examined in the ultracentrifuge. This assumption seems reasonable in view of our experimental findings that 90 per cent by weight of the WSR dialyzed through Visking tubing.

The sedimentation constant of MLL was considerably greater than that of WSR, suggesting that some low molecular-weight material had been removed by the preliminary alcohol extraction. The low sedimentation constant of the ROH SOL supports this interpretation.

It is worth noting that ragweed pollen powder displayed cutaneous activity even after sixteen days of unabated extraction with water,* whereas the extracting fluid at the end of the sixteen days' extraction displayed no such activity. This observation suggested that allergenically active groups were still incorporated in the WSR INSOL residue. Since the WSR INSOL was active when in contact with tissue fluids during scratch test, the possibility of extracting this material with media similar in composition to tissue fluids was investigated. On incubating this material with normal human serum at 3 to 5° C., allergenically active material was detected in solution. Similar results were obtained on extraction of WSR INSOL with physiologic saline and phosphate buffers. Since, in the present study, we were mainly interested in the properties of WSR, the investigation of the properties of the WSR INSOL was discontinued for the time being.

As mentioned previously, the MLL INSOL ragweed, which exhibited no reactivity by scratch test, nevertheless was able to liberate active soluble material when extracted with 0.05 N NaOH for seventy-two hours. As it is highly inconceivable that sodium hydroxide would convert an inert material into an allergenically active one, a possible explanation for these findings would be that there exist residual active groups in the MLL INSOL fraction which are shielded in some way from direct contact with water and alcohol during extraction.

*As mentioned previously, the extracting fluid was replaced every two days.

In testing the various fractions for allergenicity on sensitive persons, scratch tests were performed. In spite of the fact that the intracutaneous test is far more sensitive, the scratch test was adopted in view of the greater danger of inducing constitutional reactions by the intracutaneous injection. For this same reason, no sensitive person was tested with more than seven to eight fractions at any one sitting. All tests were performed in duplicate. At no time were all the fractions tested on a single person at any one sitting. Since each sensitive person displayed his own pattern of reactivity to different fractions, it was impossible to evaluate on an absolute basis the activity displayed by each fraction.

To circumvent this difficulty, in subsequent studies on ragweed pollen extracts the passive transfer technique was used in preference to the scratch test.

SUMMARY

1. Defatted ragweed pollen was extracted with a number of solvents and the various fractions were analyzed by free and paper electrophoresis and ultracentrifugation. All fractions were compared for their allergenic reactivity by skin tests.

2. Extraction of defatted ragweed pollen with different solvents yielded fractions of different composition.

3. All the extracts soluble in the different solvents displayed allergenic reactivity, while the water-insoluble ragweed was the only one of the insoluble residues to display reactivity consistently.

4. The results of this study suggest that zone electrophoresis is suitable for the further fractionation of the various ragweed pollen fractions.

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