

FRACTIONATION OF POLLEN EXTRACTS BY CHROMATOGRAPHY*†‡

I. PRELIMINARY STUDIES WITH RAGWEED EXTRACT

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DURING the past half century numerous investigations have been conducted in an effort to isolate and define chemically the antigenic substances in ragweed pollen. It was at first generally accepted that the antigenic material was a protein, but later studies indicated the presence of active carbohydrate components.¹⁻⁶ Gradually, evidence has accumulated which suggests that the allergenic activity of ragweed pollen extracts is shared by a number of components^{4, 7-22} Recent investigations indicate that these active components vary in molecular weight from 650 to 5000,^{15, 17} and one investigator has presented evidence to suggest that the antigenic components may be pigment-carbohydrate-polypeptide complexes.^{17, 23} Past studies of the chemical nature of the ragweed pollen components have been extensively reviewed in several recent publications.²⁴⁻²⁷

Paper chromatography provides a method for the isolation and identification of the components of a complex mixture. The extensive literature pertaining to the techniques and previous applications of paper chromatography has been summarized in a number of publications.²⁸⁻³⁹ This study was undertaken in an effort to resolve and identify the components of ragweed pollen extract by the application of paper chromatography.

EXPERIMENTAL PROCEDURES AND RESULTS

Preparation of Ragweed Pollen Extract.—Low ragweed pollen was defatted with ether in a Soxhlet extraction apparatus. The defatted pollen was extracted with Coca's alkaline extracting fluid⁴⁰ modified by the addition of 5 per cent glycerine. The ratio of pollen to extracting fluid was 1:14 and extraction was allowed to progress for 15 minutes with vigorous agitation in a Waring blender. The pollen residue was removed by filtration through Whatman #1 filter paper. The extract was sterilized by Seitz filtration and stored in the refrigerator at 4° C. The total nitrogen content of the extract was 0.9 mg. per cubic centimeter and the phosphotungstic acid precipitable nitrogen was 0.5 mg. per cubic centimeter. This extract will be referred to as whole ragweed extract.

Dialysis of Whole Ragweed Extract.—An aliquot of whole ragweed extract was placed in a 36/32 Visking sac and dialyzed against running tap water at 4° C. for 16 days. The fluid within the sac remained clear and no precipitate formed. After Seitz filtration this fluid had a total nitrogen content of 0.15

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mg. per cubic centimeter and a phosphotungstic acid precipitable nitrogen content of 0.15 mg. per cubic centimeter. This liquid will be referred to as dialyzed residue.

A second aliquot of whole ragweed extract was placed in a 36/32 Visking sac and dialyzed against a seven fold volume of distilled water for 72 hours at 4° C. A stream of air bubbles was used to keep the fluid outside of the sac constantly agitated. At the end of the 72 hour period the fluid outside of the Visking sac was concentrated to $\frac{1}{4}$ of its original volume by rapid evaporation at room temperature. This fluid was labelled dialysate of ragweed extract. Its total nitrogen content was 0.41 mg. per cubic centimeter and phosphotungstic acid precipitable nitrogen was 0.09 mg. per cubic centimeter after Seitz filtration.

Preparation of Two-Dimensional Chromatograms.—Two-dimensional paper chromatograms were prepared by downward migration of the solvents on 18 × 22½ inch Whatman #1 filter paper. With minor variations the procedure was carried out as described by Consden, Gordon and Martin.⁴¹ Near a corner of the paper, 6 cm. from 2 edges was applied 0.025 ml. to 0.050 ml. of the solution to be analyzed. The sample was allowed to dry at room temperature. One edge of the paper was then placed in the trough of the first chamber where water-saturated phenol was allowed to migrate downwards for 18 hours. The atmosphere in this chamber contained ammonia and was saturated in respect to both phenol and water. After removal from the chamber the paper was dried in a strong air current at room temperature for 8 to 12 hours. The paper was then secured in the trough of a second chamber where water-saturated lutidin (2,4 methyl pyridine) was allowed to migrate downward for 30 hours in a direction at right angle to the previous phenol development. The atmosphere in this chamber was saturated in respect to lutidin and water. The chromatogram was then dried and inspected in ordinary and ultraviolet light.* The paper was then sprayed with 0.1 per cent ninhydrin (triketohydrindene-hydrate) in water-saturated butyl alcohol and the colors allowed to develop at room temperature. The ninhydrin reaction is used to detect the positions of amines, amino acids, peptides and related substances on the chromatograms. Other chromatograms were treated with Partridge's aniline hydrogen oxalate reagent²¹ which reacts with aldohexoses and pentoses.

Samples of each material were hydrolyzed with 6N HCl at 100° for 18 hours and the hydrolysate applied to sheets for the preparation of chromatograms in the same manner. By comparing these chromatograms with those prepared from unhydrolyzed material it was possible to identify the positions of peptides and to determine their qualitative amino acid composition. Peptides appear as single ninhydrin reactive areas on the two-dimensional chromatograms. After acid hydrolysis the ninhydrin spot characteristic of a peptide disappears and multiple ninhydrin spots appear corresponding to the constituent amino acids of the peptide.

Amino acid identifications were made by establishing the precise superimposition of the unidentified ninhydrin spots with those ninhydrin spots of

*GE-CH4 bulb with double rondel filter Corning #5874 wave length .32-.40 Microns.

known amino acids. This was accomplished by the simultaneous development of 4 chromatograms. The first chromatogram was prepared from a sample of the material being analyzed, the second from an aliquot of this same material after acid hydrolysis, the third from selected known amino acids, and the fourth from a mixture of the hydrolyzed material being analyzed and the selected known amino acids.

Chromatographic Analysis of Dialysate of Ragweed Extract.—Aliquots of the dialysate containing a total of 0.1 mg. of nitrogen resulted in satisfactory chromatograms whereas quantities containing 0.2 mg. of nitrogen resulted in considerable overlapping of spots. Fig. 1 is a composite tracing of several chromatograms of dialysate of ragweed extract. Two areas labelled P and D were yellow before chromatograms were treated with color producing reagents and these areas were fluorescent under the ultraviolet lamp. When chromatograms of dialysate were treated with aniline hydrogen oxalate reagent the area labelled CHO appeared. Chromatograms treated with ninhydrin revealed the areas labelled 1 through 10 which are the positions of leucine, valine, proline, hydroxyproline, alanine, γ -aminobutyric acid, β -alanine, glutamine, asparagine and aspartic acid respectively. An occasional trace of glycine was found to overlap the upper portion of the asparagine spot but because this was inconstant it is omitted from the tracing. Also appearing on chromatograms treated with ninhydrin were the areas labelled A, B, C, and the previously noted area D which became blue after ninhydrin treatment.

Chromatograms of dialysate after acid hydrolysis did not contain the ninhydrin reactive areas A, B, C, and D. Asparagine and glutamine spots were absent, valine, aspartic acid and alanine spots were definitely increased and leucine, proline, hydroxyproline amino butyric acid and β -alanine spots appeared with approximately the same size and intensity noted with unhydrolyzed samples of dialysate. In addition ninhydrin reactive spots in the positions of glutamic acid, glycine, serine and lysine appeared, the serine and lysine spots being quite faint.

These observations strongly suggested that the substances labelled A, B, C, and D were peptides. This interpretation was based on the disappearance of these spots after acid hydrolysis, the concomitant increase of some of the previously noted amino acids, and the appearance of amino acids not present on chromatograms of unhydrolyzed dialysate. Inspection of two-dimensional chromatograms (Fig. 1) revealed that areas A, B, C, D, and CHO were well separated by the lutidin development. Accordingly, this solvent was used to fractionate the dialysate so that these substances might be analyzed independently.

Chromatographic Fractionation of Dialysate of Ragweed Extract.—Aliquots of dialysate, each containing a total of 0.5 mg. of nitrogen, were applied to sheets of filter paper as 17 inch streaks 3 inches from one edge of the paper. The papers were then developed with lutidin so that the components were separated as parallel bands across the paper. After 30 hours of development the chromatograms were dried and exposed to a strong air current for 12 hours to remove the lutidin. Strips were then cut from the sides of each chromatogram

and these were treated with aniline hydrogen oxalate and ninhydrin. Using these as reference strips the untreated center portion of the chromatograms could be cut into sections containing the fractions CHO, A, B, C, and D respectively (Fig. 2). The sections of the chromatograms interspaced between these areas were retained for further study.

The fractions were obtained from the cut sections of the chromatograms by elution with distilled water. For this purpose a Petri dish containing distilled water was placed on a ringstand and one end of a paper strip to be eluted was secured below the water level in the dish by a glass weight. The remainder of the strip was allowed to hang over the edge of the Petri dish with the lower end of the paper inserted in the mouth of a test tube. The apparatus was covered

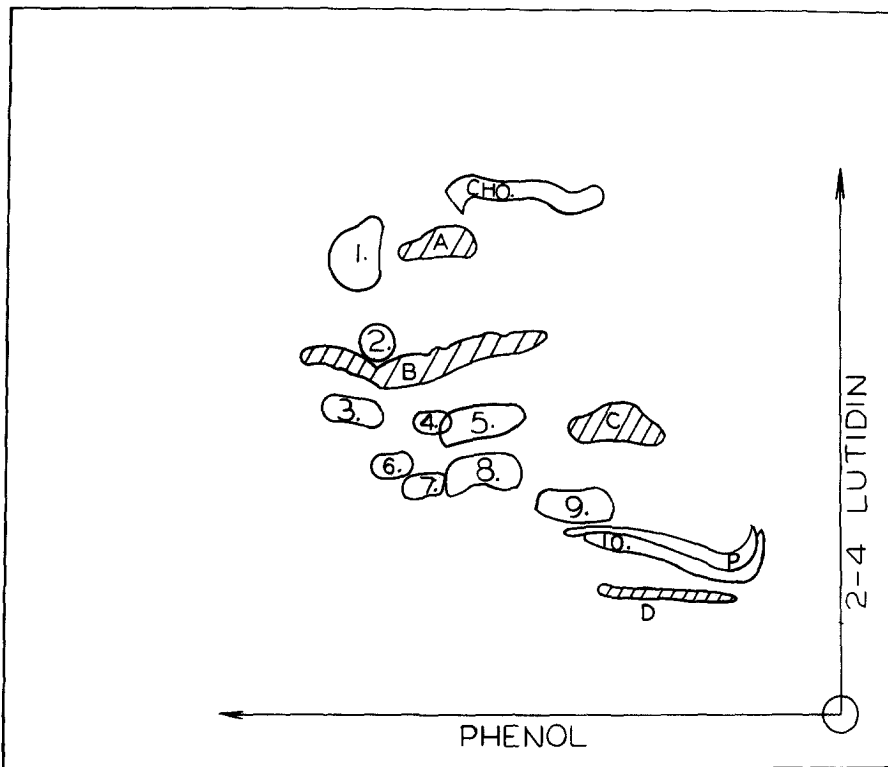


Fig. 1.—A composite tracing from chromatograms of the dialysate of ragweed extract. 1-leucine, 2-valine, 3-proline, 4-hydroxyproline, 5-alanine, 6- γ -aminobutyric acid, 7- β -alanine, 8-glutamine, 9-asparagine, 10-aspartic acid; P-pigment; CHO-carbohydrate; A, B, C, and D-peptides (see text).

with a bell jar to prevent evaporation during the downward migration of the water through the paper strip. Treatment of a few of the strips with ninhydrin or aniline hydrogen oxalate, after elution with varying quantities of water, revealed that most of the reactive material was contained in the first few milliliters entering the collecting test tube.

The eluates from 250 strips were evaporated to dryness in watch glasses at room temperature under the air current of an electric fan. The material in

each watch glass was weighed and then redissolved in distilled water for further chromatographic studies or redissolved in physiologic saline with the addition of Merthiolate 1:10,000 for immunologic studies.

Analysis of Dialysate Fractions.—

Fraction A: Two-dimensional chromatograms of 1 mg. of fraction A treated with ninhydrin revealed the presence of leucine and the spot previously labelled A. After acid hydrolysis of 1 mg. of this fraction the leucine spot appeared unchanged but the spot denoting the position of A substance was no longer present. Ninhydrin reactive areas in the positions of aspartic acid, glutamic acid, glycine and alanine appeared. Fig. 3 is a composite tracing of these chromatograms.

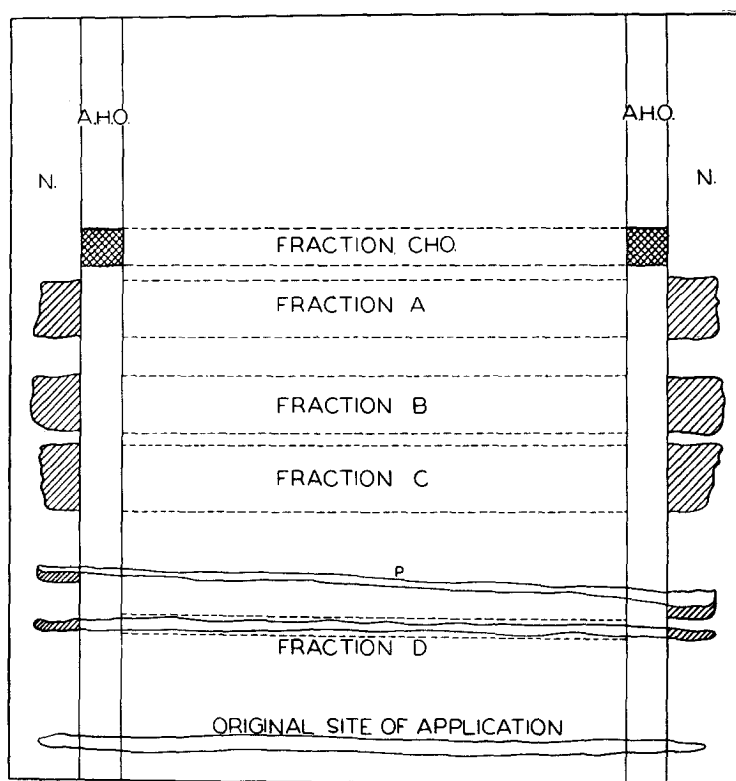


Fig. 2.—Chromatographic fractionation of dialysate of ragweed extract. A.H.O.—reference strips treated with aniline hydrogen oxalate. N—reference strips treated with ninhydrin. The broken lines indicate how the center untreated portion of the chromatogram was cut into strips containing fractions CHO, A, B, C and D respectively.

Fraction B: One milligram samples of fraction B were analysed by two-dimensional chromatography before and after acid hydrolysis. Prior to hydrolysis only the ninhydrin reactive spot previously labelled as denoting substance B was observed on the chromatograms. This spot was absent after the material was hydrolyzed and the chromatograms then contained ninhydrin reactive areas in the positions of valine, alanine, glycine, serine, glutamic acid and aspartic acid. Fig. 4 is a composite tracing of these chromatograms.

Fraction C: Chromatograms of fraction C were found to contain, in addition to the substance labelled C, ninhydrin reactive spots in the positions of proline, hydroxyproline, alanine, asparagine, β -alanine, γ -aminobutyric acid, and glutamine. After hydrolysis of the fraction there was a definite increase in alanine, an appearance of at least 5 new ninhydrin reactive spots, and the disappearance of the spot labelled C. A further analysis of fraction C is in progress.

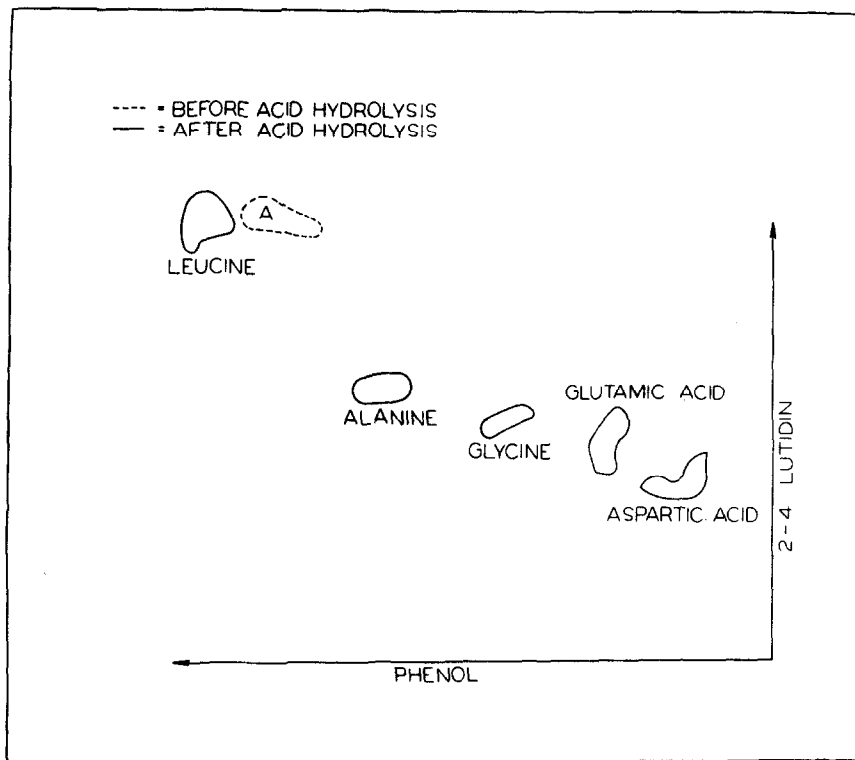


Fig. 3.—A composite tracing of the ninhydrin reactive areas on the chromatograms of fraction A before and after acid hydrolysis. The area labelled A was present before acid hydrolysis and absent after acid hydrolysis. Ninhydrin reactive areas in the positions of aspartic acid, glutamic acid, glycine and alanine were present only after acid hydrolysis. A spot in the leucine position was present in approximately the same size and intensity both before and after acid hydrolysis. These chromatograms indicate that the area labelled A is a peptide and that fraction A contains free leucine as well as "peptide A."

Fraction D: One milligram samples of fraction D were analysed by phenol-lutidin chromatography before and after acid hydrolysis. The chromatograms of the unhydrolyzed material revealed ninhydrin reactive areas in the positions of substance D and aspartic acid. Chromatograms of the hydrolyzed material revealed an absence of the substance D spot, an increase in the aspartic acid spot, and the appearance of spots in the positions of alanine, lysine, glycine, serine, and glutamic acid. Tracings of these chromatograms are presented in Fig. 5.

Fraction CHO: Fraction CHO reduced Benedict's solution with heat and also gave a strong Molisch reaction. Further analysis of this carbohydrate fraction is in progress.

Chromatographic Analysis of Dialyzed Residue.—Quantities of the dialyzed residue containing 0.2 mg. of nitrogen were used for the preparation of chromatograms developed with phenol and lutidin. No "spots" appeared when these chromatograms were treated with ninhydrin or alinine hydrogen oxalate. The dialyzed residue also failed to react with these reagents when the same quantities were merely applied to small spots on filter paper which was not developed with solvents. Chromatograms of 0.2 mg. of acid hydrolyzed dialyzed residue revealed the presence of 15 ninhydrin reactive spots. These have been identified as occupying the positions of tyrosine, leucine, valine, proline, γ -aminobutyric acid, hydroxyproline, alanine, threonine, arginine, glycine, serine, cystine, lysine, glutamic acid, and aspartic acid (Fig. 6).

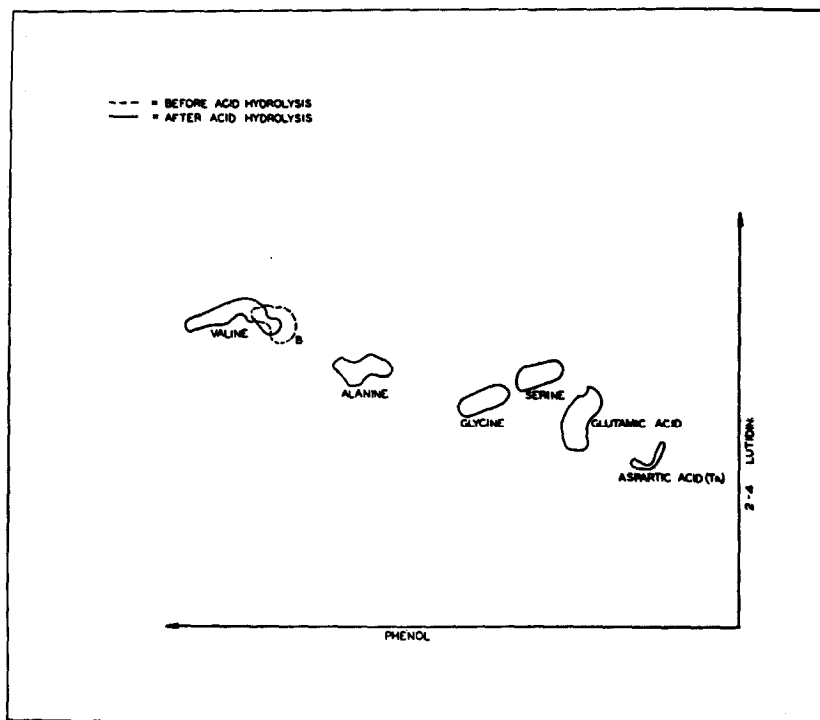


Fig. 4.—A composite tracing of the ninhydrin reactive areas on the chromatograms of fraction B before and after acid hydrolysis. A single ninhydrin reactive area was present on chromatograms of the unhydrolyzed fraction and this area is labelled B. Chromatograms of fraction B after acid hydrolysis revealed the presence of valine, alanine, glycine, serine, glutamic acid and a trace of aspartic acid but the area labelled B was absent. The chromatograms indicate that the area labelled B is representative of a peptide. Since this "peptide B" partially overlaps the position of valine, traces of which are in the dialysate, fraction B probably contains a small quantity of free valine in addition to "peptide B."

Chromatographic Analysis of Whole Ragweed Extract.—Chromatograms of aliquots of whole ragweed extract containing 0.2 mg. of nitrogen appeared identical to those observed with the dialysate. Chromatograms of this material after acid hydrolysis, however, differed from the posthydrolysis chromatograms of dialysate in that there was an increase in proline and hydroxyproline and an appearance of the other ninhydrin reactive spots observed on the chromatograms of hydrolyzed dialyzed residue.

Immunologic Reactions with Fractions.—Each of 16 control subjects was tested intradermally with whole ragweed extract and dialyzed residue in concentrations of 125 protein nitrogen units (P.N.U.), and with dialysate fractions A, B, C, D, and CHO in concentrations of 2 mg. per milliliter. There were no positive reactions in this control group. Using the same test concentrations in 17 ragweed sensitive subjects, all reacted positively to the whole extract and to the dialyzed residue, and 15 of the 17 reacted positively to one or more of the dialysate fractions. These reactions are recorded in Table I. Material eluted from the areas of the chromatograms interspaced between the ninhydrin reactive fractions was found to be inactive.

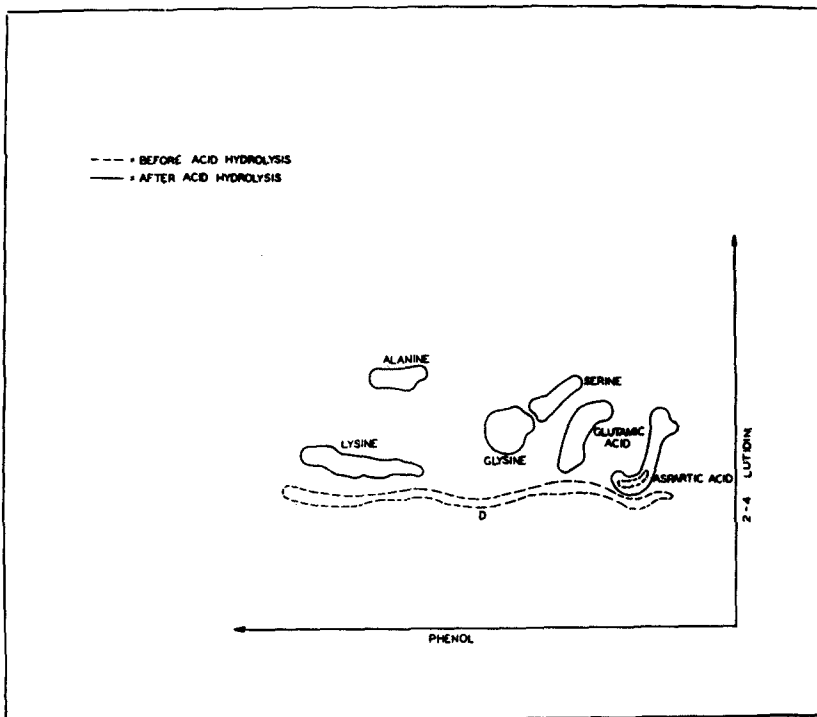


Fig. 5.—A composite tracing of the ninhydrin reactive areas on chromatograms of fraction D before and after acid hydrolysis. The area labelled D and a trace of aspartic acid were identified on chromatograms of the unhydrolyzed fraction. After acid hydrolysis the area labelled D was absent, the aspartic acid area was markedly increased, and ninhydrin reactive areas appeared in the positions of alanine, lysine, glycine, serine and glutamic acid. These chromatograms indicate that the area labelled D is a peptide and that fraction D contains a trace of free aspartic acid in addition to "peptide D."

Twenty-five sera known to contain reagins to whole ragweed extract were used to determine the reactivity of fractions by passive transfer technique. These sera were available from previous studies in this laboratory and were not obtained from the patients tested intradermally. The sera were used undiluted and challenged 48 hours later with fractions A, B, C, D, and CHO in concentrations of 10 mg. per milliliter. and with dialyzed residue and whole extract in a concentration of 1,000 P.N.U. per milliliter. Positive passive transfer reactions

TABLE I. DIRECT INTRADERMAL REACTIONS*

PATIENT	DIALYSATE FRACTIONS†					DIALYZED‡ RESIDUE	WHOLE‡ EXTRACT
	A	B	C	D	CHO		
1	+	+	0	0	+	+++	+++
2	+++	+	++	+		+++	++++
3	0	++	0	+++	++	++	+
4	0	++	0	0	0	++	++
5	0	0	+	+		++++	+++
6	+	0	0	+	+	++++	++++
7	++	+++	++	++++	+	++++	++++
8	0	0	0	+	0	+++	+++
9	++	++	+	++++	+	++	++++
10	0	+	0	++		++++	++++
11	++++	+	++	++++		++++	++++
12	++	+	+	+	0	++++	++++
13	0	+	0	++		++	++
14	+	+	0	+		+	+
15	0	0	0	0		+	+
16	+	+	+	+		+	+
17	0	0	0	0		+	++
Fraction reacting	9/17	12/17	7/17	13/17	5/8	17/17	17/17

*0.01 to 0.02 ml. of each test fraction injected

†2.0 mg./ml. test concentration

‡125 P.N.U. test concentration

Wheal size: + 8 to 25 sq. mm.

++ 26 to 50 sq. mm.

+++ 51 to 65 sq. mm.

++++ >65 sq. mm.

TABLE II. PASSIVE TRANSFER REACTIONS*

SERUM†	DIALYSATE FRACTIONS‡					DIALYZED§ RESIDUE	WHOLE§ EXTRACT
	A	B	C	D	CHO		
1	++	++	+++	++++	0	++++	++++
2	+++	+++	+++	+++	+++	++++	++++
3	0	++	+	0	0	+++	+++
4	0	+++	++	++	++	++++	++++
5	+	+++	++	+	+	++++	++++
6	++	++++	+++	+++	+++	++++	++++
7	+	++++	+	0	0	++++	++++
8	+++	+++	+++	+++	+++	++++	++++
9	0	0	0	0	0	++	++
10	0	+++	0	0	0	++++	++++
11	0	++	0	0	0	+	+++
12	++	+	++	++	+	++++	++++
13	+++	+++	++	++	+	++++	++++
14	++	0	0	+++	+	++++	++++
15	+++	++	+	++	0	++++	++++
16	0	0	0	+++	0	++++	++++
17	0	0	0	0	0	++++	++++
18	+++	++	++	0	+++	++++	++++
19	0	0	0	0	0	++++	++
20	++	0	0	0	++	++++	++++
21	++	0	0	+	0	++++	++++
22	0	++	0	0	0	++++	++++
23	+++	+	0	++	+++	++++	++++
24	0	+++	0	0	0	++	++
25	++	0	+	+	0	++++	++++
Fraction reacting	15/25	17/25	13/25	14/25	11/25	25/25	25/25

*0.02 ml. of each test fraction injected

†0.05 ml. undiluted serum injected, challenged 48 hours later

‡10.0 mg./ml. test concentration

§1000 P.N.U. test concentration

Wheal size: + 20 to 39 sq. mm.

++ 39 to 85 sq. mm.

+++ 85 to 180 sq. mm.

++++ >180 sq. mm.

occurred with each sera challenged with the dialyzed residue and 22 of the 25 sera reacted to one or more of the dialysate fractions. This data is presented in detail in Table II.

By the usual neutralization technique^{42, 43} homologous neutralization was observed with the dialyzed residue and with each of the 5 dialysate fractions. These observations are presented in Table III.

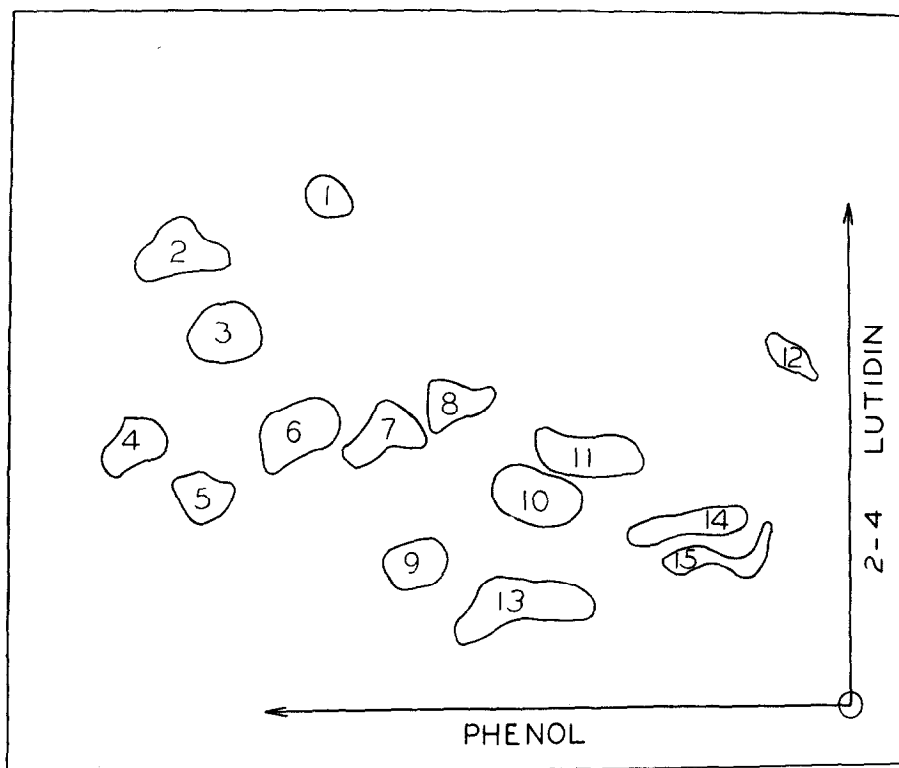


Fig. 6.—A tracing of a chromatogram of dialyzed residue after acid hydrolysis. The ninhydrin reactive areas were identified as 1-tyrosine, 2-leucine, 3-valine, 4-proline, 5- γ -aminobutyric acid, 6-hydroxyproline, 7-alanine, 8-threonine, 9-arginine, 10-glycine, 11-serine, 12-cystine, 13-lysine, 14-glutamic acid and 15-aspartic acid. There were no ninhydrin reactive areas on the chromatograms of unhydrolyzed dialyzed residue. The dialyzed residue therefore contains one or more proteins or large polypeptides.

SUMMARY

A method has been presented whereby a minimum of 6 different active components were obtained from ragweed pollen extract. Five of these components readily diffuse through Visking membrane and are therefore of relatively small molecular size. It has been demonstrated that 4 of the diffusible components are peptides and the major amino acid composition of 3 of these peptides has been defined. The fifth diffusible active component is a carbohydrate.

In addition to the diffusible active components there is a highly active fraction of ragweed extract which is retained by Visking membrane after 16 days of dialysis against running water. It has been demonstrated that this dialyzed residue contains at least one large polypeptide or protein. The observation that this material is not readily demonstrable by the ninhydrin reaction on paper

suggests that the polypeptide or protein contains relatively few free amino groups. The major amino acid composition of the protein or polypeptide components of the dialyzed residue has been determined.

TABLE III. HOMOLOGOUS NEUTRALIZATION OF FRACTIONS

	MIXTURE* FINAL SERUM DILUTION	FINAL FRACTION CONTENT	REINJECTION† REACTION
A Fraction	1:2	0	+++
	1:2	2 mg./ml.	+
	1:2	10 mg./ml.	0
	1:20	0	+
	1:20	2 mg./ml.	0
	1:20	10 mg./ml.	0
B Fraction	1:2	0	++++
	1:2	2 mg./ml.	++
	1:2	10 mg./ml.	+
	1:20	0	++++
	1:20	2 mg./ml.	++
	1:20	10 mg./ml.	0
C Fraction	1:2	0	++++
	1:2	2 mg./ml.	++++
	1:2	10 mg./ml.	+
	1:20	0	++++
	1:20	2 mg./ml.	++
	1:20	10 mg./ml.	0
D Fraction	1:2	0	++++
	1:2	2 mg./ml.	++++
	1:2	10 mg./ml.	+++
	1:20	0	++++
	1:20	2 mg./ml.	++
	1:20	10 mg./ml.	+
CHO Fraction	1:2	0	++++
	1:2	2 mg./ml.	+++
	1:2	10 mg./ml.	++
	1:20	0	+++
	1:20	2 mg./ml.	+
	1:20	10 mg./ml.	0
Dialyzed residue fraction	1:2	0	++++
	1:2	25 P.N.U./ml.	+++
	1:2	100 P.N.U./ml.	+
	1:20	0	++++
	1:20	25 P.N.U./ml.	++
	1:20	100 P.N.U./ml.	0

*Each fraction was dissolved in normal saline and mixed with serum to yield the final concentrations of fraction and serum indicated. The recipients received intradermal injections of 0.05 ml. of each mixture.

†The recipient sites were reinjected after 48 hours with 0.02 ml. of the homologous fraction. The concentration used for retesting was 10 mg. per milliliter for fractions A, B, C, D, and CHO; and 1000 P.N.U. per milliliter for the dialyzed residue.

Wheal size: + 20 to 39 sq. mm.
 ++ 39 to 85 sq. mm.
 +++ 85 to 180 sq. mm.
 ++++ >180 sq. mm.

The activity of each of the fractions described has been demonstrated by direct intradermal test in ragweed-sensitive subjects and by passive transfer and neutralization techniques. Each ragweed-sensitive subject reacted to the dialyzed residue and a positive passive transfer reaction was obtained with each reaginic serum challenged with this material. Fifteen of 17 ragweed-sensitive subjects and 22 of 25 reaginic sera reacted to one or more of the diffusible components (CHO, A, B, C, and D).

The observations presented (Tables I and II) suggest that each ragweed sensitive subject has an individual pattern of reactivity to the smaller molecular-size components: the data suggesting that most subjects are reactive to certain of these components and not to others, some are reactive to all, and a few to none; however, because of a scarcity of material, observations were made with only a single concentration of the dialysate fractions and, therefore, quantitative variations in reactivity have not been fully evaluated.

Regarding the origin of the fractions of dialysate, it is possible that the process of dialysis or the action of lutidin on the extract results in a separation of active fragments from a larger complex antigen. Since the active diffusible components have been identified on chromatograms of whole ragweed extract, the process of dialysis does not seem to be responsible for the breakdown of a large complex. Since electrophoretic and ultracentrifuge studies have indicated the presence of a number of active components of small molecular weight¹⁵ it seems further probable that the active diffusible fractions obtained from chromatograms are not the result of any solvent action on a complex but rather that they are native to ragweed extracts.

This study demonstrates that paper chromatography is a valuable adjunct in the investigation of a crude antigenic material and the methods employed might profitably be applied to the study of other crude allergenic extracts. Ragweed extract has been fractionated heretofore by electrophoretic techniques and by more classical chemical procedures. The fractions obtained in those studies have been difficult to evaluate and correlate since their chemical definition has in most studies been based on general qualitative chemical reactions. The methods employed in this study provide not only for a separation of the active components of ragweed extract but also provide extensive information concerning the composition of these components.

A continuation of the present study of ragweed extract by paper chromatography, immunologic, and classical chemical methods is in progress.

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