γ-glutamyl dipeptides are formed from glutathione and an amino acid in the presence of a γ-glutamyl transpeptidase (Thompson *et al.*, in press).

The lengthening list of amino substances which are found in the form of γ-glutamyl compounds in diverse places in the plant kindgom suggests that they may play an interesting role in the nitrogen metabolism of plants.

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Isolation Studies of Allergens from Ragweed Pollen*

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A highly allergenic protein preparation apparently of 32,000 molecular weight has been isolated from the ragweed pollen extract by ammonium sulfate precipitation followed by chromatography on DEAE cellulose and on Sephadex. A second component of about 10,000 molecular weight was found to be also highly reactive in four out of thirty-two untreated hay fever patients. A cause for the gradual loss of the allergenic activity of ragweed pollen extract on storage is suggested.

One of the most common seasonal hay fevers is caused by ragweed pollen. Extensive studies designed for the isolation and characterization of the active allergens have been under way in many different laboratories ever since the recognition that this pollen is the causative agent. These studies have been reviewed by several different authors (Newell, 1941; Sherman, 1959; Richter and Sehon, 1960).

In spite of all this careful work, the objective has not thus far been realized and the real chemical nature of the allergen is not known. The results from most laboratories indicate the activity to be associated with protein fractions, but others indicate it to be present in peptide fractions. Obviously the problem is one of considerable difficulty and one which presents a challenge to any laboratory interested in the isolation and characterization of active principles. It has been taken up with the hope that certain techniques being developed in this laboratory may prove of value.

One major difficulty in the isolation of the ragweed allergens is the lack of methods capable of providing quantitative estimations of the allergenic activity. At present estimations are usually made by direct skin tests on sensitized individuals or by indirect skin tests on normal individuals passively sensitized with sera from ragweed patients (Sherman, 1959). Both tests are subject to at least tenfold variations. Because of such variations, it is helpful to have an independent *in vitro* assay method. One such possible method is agar gel diffusion analysis of antigens with precipitating antibodies from rabbits immunized with ragweed pollen extracts. In the present work, the Ouchterlony analysis of antigens was used for rapid identification and comparison of different ragweed antigens. Since the substances which are antigenic in rabbits are not necessarily the allergens in man, direct skin tests were carried out later on the isolated fractions to identify the allergens.

This paper will describe the isolation of a highly allergenic protein preparation of 32,000 molecular

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weight from the ragweed pollen extract by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose and on Sephadex. A second component of about 10,000 molecular weight was also highly active in about 10% of untreated hay fever patients.

Experimental

Materials.—The low ragweed (Ambrosia elatior) pollens of 1956 and 1960 crops were stored at deep-freeze temperature. The 1960 crop was furnished by the Division of Biologics Standards, U. S. Department of Health, Education and Welfare. Comparison of the extracts from the two crops by the Ouchterlony technique showed no discernible difference in antigen content.

Rabbit anti-ragweed sera were prepared by the procedure of Cebra (Cebra, 1956). Two of the sera used were generous gifts from him.

The proteins used for the standard chromatogram in Figure 4B were as follows: bovine plasma albumin (Armour Co., Lot No. T68204), bovine β-lactoglobulin (Pentex Co., Lot No. F23), and bovine pancreatic ribonuclease (Armour Co., Lot No. 647-213).

Sephadex G-75 and G-25 (Pharmacia Co., Uppsala, Sweden) were dry sieved through an 80-mesh screen, and only particles larger than 80-mesh size were used. DEAE-cellulose (Carl Schleicher and Schuell Co., Lot No. 1126) had an exchange capacity of 0.9 meq/g.

Carboxypeptidase A was a Sigma Chemical Co. preparation (Lot No. C-50-B-82). *p*-Nitrophenylacetate, *p*-nitrophenylphosphate, and bis-*p*-nitrophenylphosphate were purchased from Mann Research Laboratories.

Isolation of the Allergens from Pollen.—Low ragweed pollen (200 g) was defatted five times with 1-liter portions of ethyl ether by decantation. After removal of the residual ether under reduced pressure, the pollen was extracted in 1400 ml of water at 25° for 5 hours with stirring. The solution was filtered through a Buchner funnel and the pollen residue was washed with 300 ml of water. The combined filtrates (1500 ml, *p*H 5.9) were adjusted to *p*H 7 with 4.5 ml of 3 N NH_4OH, then 900 g of ammonium sulfate was added with stirring. After 3 hours at 4°, the precipitate was collected by centrifugation. The dark brown gummy precipitate (ca. 20 ml) was dissolved in 65 ml of 0.05 M Tris hydrochloride buffer (*p*H 7.9). A small amount of insoluble material was removed by centrifugation. The solution was then passed through a Sephadex G-25 column (50 × 4 cm) with water as the eluant at a flow rate of 300 ml per hour, and the effluent was collected in 20 ml fractions. The ammonium sulfate peak was located by ninhydrin analyses (Moore *et al.*, 1954). Fraction A, which contained all the antigens, was quite turbid and was clarified by centrifugation before lyophilization. To insure the removal of ammonium sulfate, the lyophilized fraction A was passed through the G-25 column a second time.

A DEAE-cellulose column was prepared from 30 g of dry DEAE-cellulose which previously had been passed through acid and base cycles. The column (18 × 4 cm) was equilibrated with 0.025 M Tris hydrochloride buffer (*p*H 7.9). Fraction A (5.3 g) was dissolved in 40 ml of the Tris hydrochloride buffer, and the *p*H of the solution was adjusted to 7.9 with the addition of 3 N NH_4OH. The solution was then passed through the DEAE-cellulose column with 0.025 M Tris hydrochloride buffer as eluant at a flow rate of 300 ml per hour. After the collection of 30–35 fractions (25 ml each fraction), the eluant was changed to 0.05 M Tris hydrochloride buffer containing 0.20 M NaCl (*p*H 7.9). There was also a significant amount of material adsorbed on the column which could be eluted only with strong alkali. For recovery, 60 g of solid ammonium sulfate was added to each 100 ml of the pooled fraction. After 3 hours at 4°, the precipitate was collected by centrifugation, dissolved in 15–20 ml of water, dialyzed against several changes of distilled water at 25° for 6 hours, and lyophilized. The dialysis casings used were Visking 23/32 grade previously washed with water and 2 × 10^{-3} N disodium versenate.

Fraction D (1.0 g) was then chromatographed on Sephadex G-75 as described in the Methods section. For recovery of the fractions, 53 g of solid ammonium sulfate was added to each 100 ml of solution. After 3 hours at 4°, the precipitate was collected, dissolved in 10–15 ml of 1 × 10^{-3} M NH_4HCO_3, and dialyzed against several changes of 1 × 10^{-3} M NH_4HCO_3 at 25° for 6 hours. The use of NH_4HCO_3 was found to reduce the precipitation which took place with fractions III_1, IV_1, and V_1. After dialysis, the fractions were lyophilized. The fractions were rechromatographed on Sephadex G-75, and the following amounts were used: 147 mg (fraction II_1), 212 mg (fraction III_1), 302 mg (fraction IV_1), 150 mg (fraction V_1), and 140 mg (fraction VI_1).

Methods

A. Chromatography on Sephadex G-75.—The column (600 × 2.5 cm) was constructed in three 200-cm sections, mounted at the same level and connected in series with polyethylene tubing (1.0–1.4 mm I.D.). Before packing, the Sephadex gels were washed with 50% acetic acid and water, then equilibrated with 0.5 N ammonium acetate. Air pressure of 5–6 cm Hg was applied to drive the buffer through the columns and to reduce the degassing of buffer solution on the top of the second and third columns. The flow rate was adjusted to 60–65 ml per hour by the attachment of a fine-glass capillary tube to the exit polyethylene tubing. Chromatography was carried out at 25°. Samples (up to 2 g in 15 ml of the buffer) were used and the effluent was collected in 15-ml fractions. The positions of the peaks seldom varied by more than two fractions. The eluant, 0.5 N ammonium acetate, was chosen because it serves the dual purpose of buffering the solution at neutrality and preventing the clogging

of the column. When a salt-free concentrated protein solution passes through the Sephadex G-75 gels, there is always a shrinkage of the gel and the column becomes clogged. This can be avoided by using an eluant of high ionic strength.

B. CHEMICAL ANALYSES.—Phosphates were determined colorimetrically (Lowry and Lopez, 1946). For the determination of covalently linked phosphate, the sample was previously hydrolyzed in 3 N HCl for 2 hours at 100°.

Hexoses and pentoses were analyzed by the orcinol-sulfuric acid method (Tsugita and Akabori, 1959) and the orcinol–ferric chloride method Volkin and Cohn, 1954). With the first method, both hexoses and pentoses gave the same color yield; with the second method, arabinose gave fourteen times more absorption at 660 mμ than glucose and twice that of glucuronic acid. No hydrolysis of the sample was required with either method.

Hexuronic acid was determined by the carbazole method (Dische, 1950) without prior hydrolysis. A standard curve was constructed with glucuronic acid.

Hexosamines were determined by the *p*-dimethylaminobenzaldehyde method (Elson and Morgan, 1933). The samples were hydrolyzed in 3 N HCl at 100° for 2.5 hours, then neutralized with NaOH for analysis. A standard curve was prepared with galactosamine. The values for hexuronic acid and hexosamines were not corrected for decomposition.

For identification of the neutral carbohydrate present in fraction A, samples (3 mg) were hydrolyzed in 1 N HCl for 4 hours at 110°. After passage through a mixed bed ion-exchange column, the hydrolysate was used for paper chromatography in a solvent system of isopropanol, *n*-butanol, and water (140:20:40). The chromatograms were developed with *p*-anisidine spray. For isolation of the carbohydrate, a larger sample (200 mg) was hydrolyzed. The carbohydrate was recrystallized from a mixture of ethyl acetate, ethanol, and water.

Sulfhydryl group was determined by the amperometric titration method (Benesch *et al.*, 1955). The titration medium contained 0.15 μmole of protein in 5 ml of H_2O and 1.5 ml of 1 M Tris nitrate buffer (*p*H 7.4), and the titrant, 2×10^{-3} N $AgNO_3$, was added in 5-μl aliquots.

Amino acid analysis was carried out on a Spinco automatic amino acid analyzer (Moore *et al.*, 1958). The samples (3–4 mg) were hydrolyzed in 6 N HCl in sealed evacuated tubes for 22 hours at 108°. The hydrolysates were taken to dryness on a rotatory evaporator, then dissolved in 5 ml of buffer for analysis (Crestfield, unpublished observation). Tryptophan was determined spectrophotometrically (Goodwin and Morton, 1946).

C. IMMUNOLOGIC ANALYSES.—Agar gel diffusion of antigens (Ouchterlony, 1958) was carried out as follows: microscope slides (3 × 2 in) were coated with 5 ml of warm 0.8% Difco Noble agar in Tris hydrochloride buffer (*p*H 7.9, Γ/2 0.11) containing 0.01% merthiolate. After gelling, wells (4 mm diameter) were made by gentle suction with a fine glass tubing. Three parallel rows of six wells each were made, and the wells were spaced with centers at 1-cm intervals. Antisera were in the middle row and antigen solutions in the first and third rows; solutions were introduced in 10-μl aliquots. A second addition to the wells after 24 hours sharpened the precipitin lines even when antigen or antibody were in excess. The stated amount of antigen and antiserum was added in two equal portions. After 48 to 72 hours, the precipitin lines were photographed. Near the midzone between wells, the precipitin line position varies with the logarithm of the antigen concentration, and from this line position it is possible to compare concentrations of an antigen in different fractions. However, it is not valid to compare concentrations of different antigens, as precipitin line positions depend on the concentration of the specific antibody and the rate of diffusion of the particular antigen. These are different in each antigen-antibody system.

Direct skin testing on untreated ragweed-sensitive subjects was carried out by intracutaneous injection of 0.05 ml of test solution. Test solutions were made up in isotonic saline buffered at *p*H 7.4 with Tris hydrochloride. Each fraction was tested at serial tenfold dilutions until a negative reaction was observed. The reactions were graded after 15 minutes as follows:

Grade	Erythema	Wheal
0	<5 mm	<5 mm
±	5–10	5–10
1+	10–20	5–10
2+	20–30	5–10
3+	30–40	10–15 or with pseudopods
4+	>40	>15 or with many pseudopods

Scratch tests were performed by making a 1-cm scratch on the skin with a vaccination needle. One drop of test solution was placed on the scratch and after 15 minutes reactions were graded 1+ to 4+ by the size of the wheal.

D. SEDIMENTATION ANALYSES.—Sedimentation analyses were carried out in a Spinco model E ultracentrifuge with double-channel cells at 50,740 rpm with 1% protein solutions in a buffer of 0.10 M NaCl + 0.004 M KH_2PO_4 + 0.035 M K_2HPO_4 (*p*H 7.7). Synthetic boundary cell runs at 12,590 rpm gave the total protein concentration. The rapidly sedimenting material referred to that fraction of the sample absent in the high-speed runs. Diffusion coefficients were calculated from the synthetic boundary cell runs by the height-area method. Partial specific volume was calculated from the determined amino acid composition (Cohn and Edsall, 1943).

E. ELECTROPHORESIS.—Zone electrophoresis in starch was carried out in a half cylindrical

trough (50 × 3 × 1.5 cm) with 4 mg of the samples; a potential drop of 2.3 volts per cm at 4° for 41 hours was used. The block was equilibrated with a buffer of 0.05 M sodium barbital +0.01 M barbital +0.05 M NaCl (*p*H 8.55). After the block was sectioned into 1-cm segments, each segment was extracted with 4 ml of water, and the protein concentration was determined colorimetrically on 1-ml aliquots of the extract (Lowry *et al.*, 1951).

F. Determination of the Carboxyl-Terminal and Amino-Terminal Amino Acids of Fraction IV_2.—Carboxypeptidase A digestions were carried out under the following conditions: ca. 12–15 mg of the sample (ca. 0.35–0.44 μmole) was digested with 0.02 μmole of diisopropylfluorophosphate-treated carboxypeptidase A in 2.0 ml of 0.05 N NH_4HCO_3 at 25°. Carboxypeptidase A concentration was estimated spectrophotometrically (Neurath, 1955). At zero time, the entire mixture was transferred into a thin film dialysis apparatus (Craig *et al.*, 1957). The sac used was Visking 23/32 cellophane membrane, which is impermeable to proteins but permeable to free amino acids. The apparatus was designed so as to provide ca. 50 cm^2 of dialyzing area, and the volume of the outside solution was 25 ml of 0.05 N NH_4HCO_3. At intervals, the outside solution was changed and set aside. The collected solutions, after lyophilization to remove water and NH_4HCO_3, were dissolved in *p*H 2.2 citrate buffer and the liberated amino acids were then determined on the amino acid analyzer.

For the detection of amino-terminal amino acid, ca. 12 mg of the protein sample was converted to its dinitrophenyl derivative by treatment with 50 mg of 2,4-fluorodinitrobenzene in 3 ml of 50% ethanol containing 0.24 mmole of $NaHCO_3$ for 2 hours at 25°. The derivative precipitated out on acidification and was triturated with ether. After drying, the sample was hydrolyzed in 1 ml of 6 N HCl in an evacuated tube at 108° for 13 hours. After dilution with water, the hydrolysate was extracted with ether. The ether extract and the aqueous phase were examined by paper chromatography (Levy, 1955).

G. Enzyme Assays.—Proteolytic activity was determined by the method of Kunitz (Northrup *et al.*, 1948). The assay was carried out at *p*H 7.6, 5.3, and 4.0. Casein was used as the substrate at *p*H 7.6. and bovine plasma albumin was used at *p*H 5.3 and 4.0.

Esterase activity was determined with *p*-nitrophenylacetate as substrate. The assay system contained 1 μmole of substrate and *x* μg of the pollen fraction in 3.25 ml of 0.05 M potassium phosphate buffer (*p*H 5.9). The release of *p*-nitrophenol at 25° was followed spectrophotometrically at 400 or 440 mμ for 10 minutes. In the region of 0–60% hydrolysis, the rate of release was directly proportional to the amount of enzyme used. A unit of activity was defined as the release of 0.01 μmole of *p*-nitrophenol per minute, and the specific activity was defined as the number of units present per mg of the fraction tested.

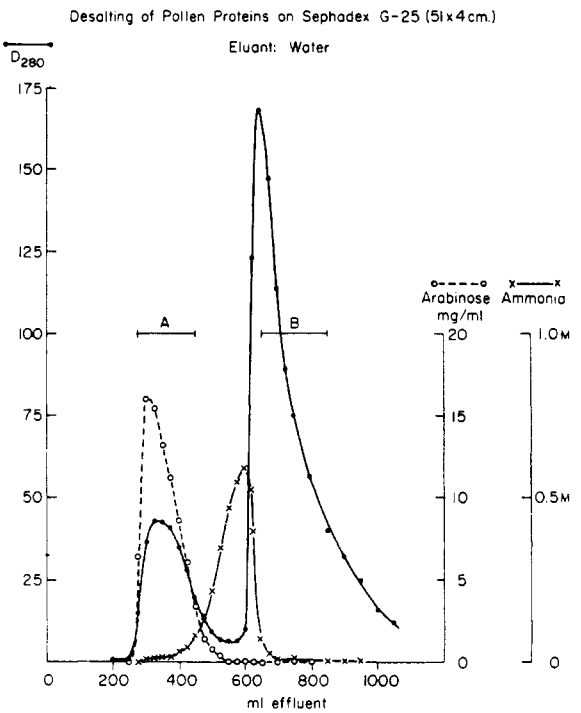

Fig. 1.—Chromatography of the ammonium sulfate precipitate from pollen extract on Sephadex G-25.

Phosphodiesterase and phosphomonoesterase activities were measured with bis-*p*-nitrophenylphosphate and *p*-nitrophenylphosphate as substrates, respectively. The assay system contained 1 μmole of substrate and *x* μg of the pollen fraction in 3.25 ml of 0.05 M sodium maleate buffer (*p*H 6.1). Hydrolysis at 25° was followed by measuring the liberated *p*-nitrophenol spectrophotometrically. In the region of 0–30% hydrolysis, the rates were proportional to the amount of enzyme used. The unit of activity and the specific activity are defined as above.

Results

The allergens were isolated from defatted pollen by water extraction and subsequently precipitated with ammonium sulfate at 0.9 saturation. The precipitate was desalted by chromatography on Sephadex G-25 (Fig. 1). Adsorbed yellow pigments and other low-molecular-weight substances were removed together with the ammonium sulfate in the process. In the extract five antigens were detected with the antiserum used. These antigens were designated as A to E according to their precipitin line positions (Fig. 2, upper row). The first peak, fraction A, was found to contain all the antigens present in the extract except that there was a decrease in antigen A.

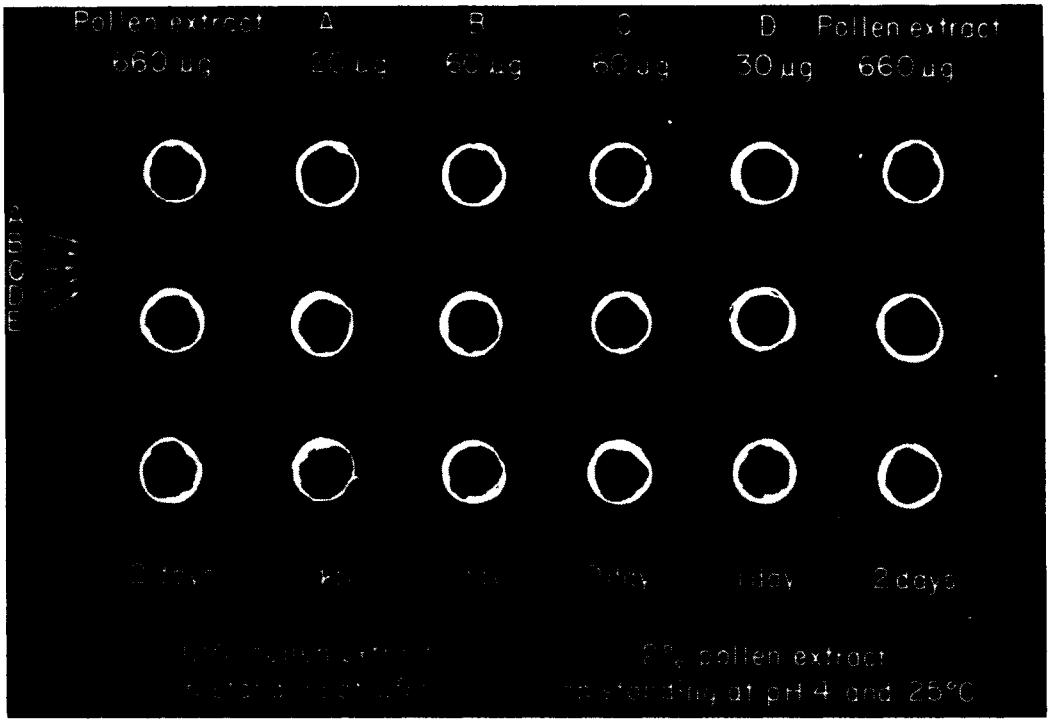

FIG. 2.—Agar gel diffusion analyses of ragweed pollen antigens (see text for designations).

The pigment-containing peak, fraction B, was devoid of antigens.

Direct skin tests showed that the allergenic activity was concentrated in fraction A, as fraction B had diminished activity (Table I). The tests were carried out on ten different patients, and only one representative case is given in Table I. The supernatant from the ammonium sulfate precipitation was not tested, since previous work (Stull and Sherman, 1939) has shown it to be inactive.

Fraction A was found to contain 10% nitrogen, < 0.1% phosphorus, 37% L-arabinose, and traces of hexosamine and hexuronic acid. The phosphorus present was found to be due to absorption of inorganic phosphate, since the same values were obtained before and after acid hydrolysis. The neutral carbohydrate was identified as L-arabinose by paper chromatography and by actual isolation. The isolated sugar had an identical melting point and optical rotation with an authentic sample. The arabinose was shown to be present in the form of a polymer by the observation that it dialyzed through a cellophane membrane Visking 23/32 grade at a rate much too slow for the monomer. The material that did dialyze probably represented enzymic degradation products, since a boiled solution of fraction A showed a decreased amount of dialyzable arabinose. Removal of 50% of the arabinose in fraction A did not cause any detectable change in its antigen composition as judged by the precipitin line positions.

TABLE I
COMPARATIVE SKIN TESTS OF FRACTIONS A, B, C, AND D ON A SINGLE RAGWEED-SENSITIVE PATIENT

Gram Used	Pollen Extract	Fraction A	Fraction B	Fraction C	Fraction D
10^{-6}			2+		
10^{-7}					
10^{-8}			±		
10^{-9}	4+	3+		1+	
10^{-10}	1+	1+			2+
10^{-11}	0	±		0	±

The symbols ± to 4+ refer to the grade of the skin reaction on intracutaneous injection of the stated amount of the material (see text for designations).

To gain a preliminary insight into the properties of fraction A, zone electrophoresis on starch and thin film dialysis through cellophane membrane, Visking 20/32 grade (Craig *et al.*, 1957), were carried out. At *p*H 8.5, fraction A migrated as a negatively charged broad band with a mobility of two thirds of that of plasma albumin. At *p*H 3.7, it migrated mainly as cationic substances. Therefore, the isoelectric points of the pollen proteins are in the *p*H range of 4–8. The dialysis experiment indicated a wide distribution of molecular sizes in fraction A, up to 50–60% in the molecular weight range of several thousand to twenty thousand.

Fraction A was readily separated into two fractions, C and D, by chromatography on DEAE-cellulose with a stepwise increase of the ionic strength of the eluant (Fig. 3). Only antigen C was present in fraction C (Fig. 2). All the other

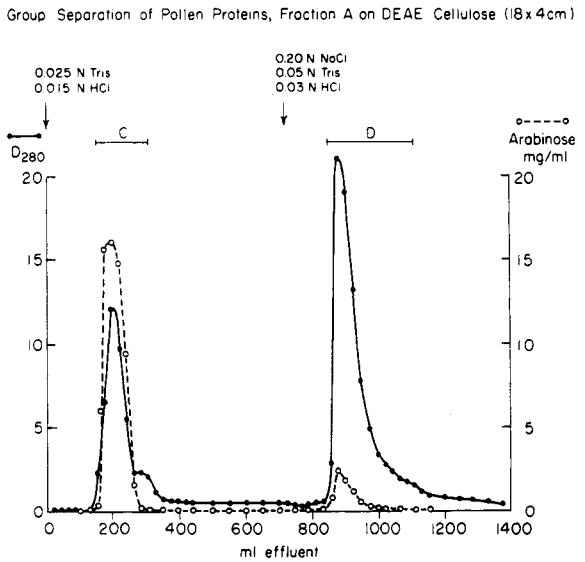

FIG. 3.—Chromatography of fraction A on DEAE-cellulose.

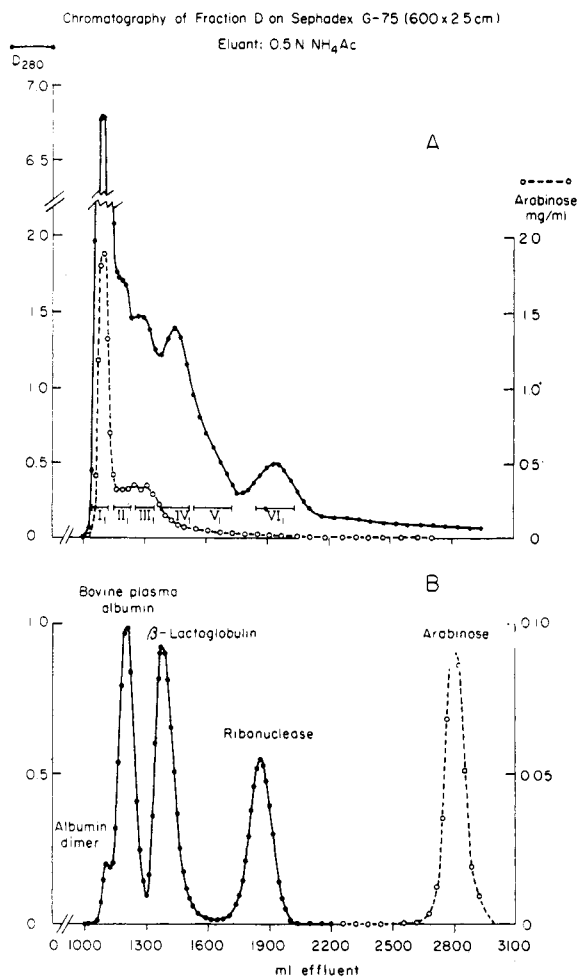

FIG. 4.—Chromatography of fraction D (A) and an admixture of known proteins and arabinose (B) on Sephadex G-75.

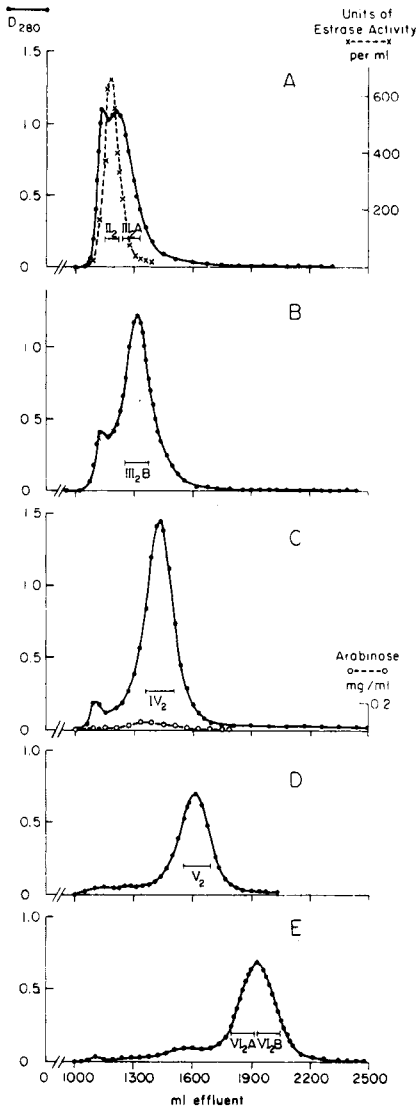

FIG. 5.—Rechromatography of the fractions from Figure 4A on Sephadex G-75.

antigens were in fraction D. The precipitin lines corresponding to antigens A and B split into new components, and these were designated as antigens A_1, A_2, B_1, and B_2 (see also Fig. 6). The allergenic activity was found to be concentrated in fraction D (Table I). Attempts to resolve fraction D with linear gradients of increasing ionic strength were unsuccessful owing to excessive trailing of the peaks.

Further resolution of the antigens in fraction D was obtained by chromatography on Sephadex G-75 (Porath, 1960). The indicated fractions (Fig. 4A) were recovered, and antigenic analyses indicated resolution of several of the antigens. The fractions II_1 through VI_1 were therefore rechromatographed (Fig. 5). Fraction I_1 was not rechromatographed, as it gave a weak diffuse antigen pattern and showed low allergenic activity. The yields of the various fractions from

TABLE II
SUMMARY OF YIELDS FROM VARIOUS FRACTIONS AND THEIR ESTERASE ACTIVITIES

Fraction[a]	Yield[b] (g)	Esterase		Phosphodiesterase	Phosphomonoesterase
		Specific Activity	Total Units	Specific Activity	Specific Activity
Extract	50.500	1.9	95,000	0.25	0.43
A	5.600	14	78,000	2.5	4.6
C	2.100	0.6	1,300	0.4	0.4
D	1.100	54	59,000	5.5	9.4
I_1	0.240	62	15,000	1.4	12
II_1	0.110	260	29,000	12	22
III_1	0.130	34	4,400	3.2	1.6
IV_1	0.170	—	—	0	—
V_1	0.080	—	—	—	—
VI_1	0.050	—	—	—	—
II_2	0.038	600	23,000	—	—
III_2A	0.024	82	2,000	—	—
III_2B	0.056	40	2,200	—	—
IV_2	0.087	32	2,800	—	—
V_2	0.029	2	60	—	—
VI_2A	0.011	—	—	—	—
VI_2B	0.014	—	—	—	—

[a] Fraction A is isolated from G-25 chromatography of the ammonium sulfate precipitate of pollen extract (Fig. 1). Fractions C and D are from DEAE-cellulose chromatography of fraction A (Fig. 3). Fractions I_1 to VI_1 are from G-75 chromatography of fraction D (Fig. 4A). Fractions II_2 to VI_2 are from rechromatographies of fractions II_1 to VI_1 (Fig. 5). [b] Yield from 200 g of pollen.

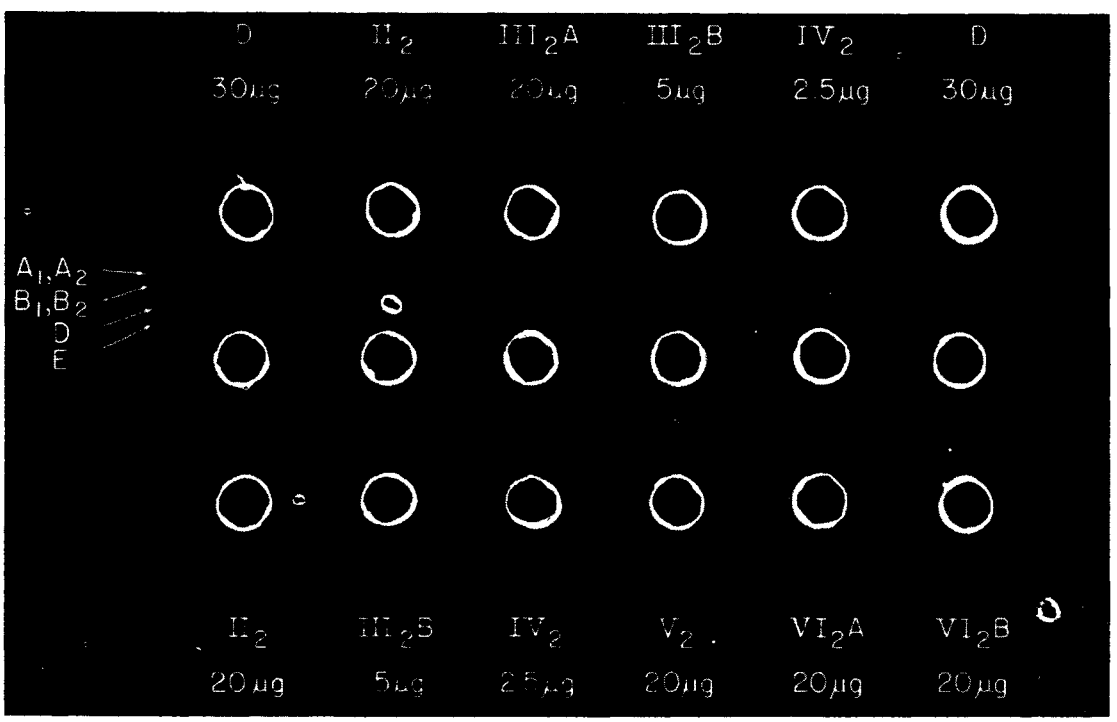

FIG. 6.—Agar gel diffusion analysis of antigens in the fractions from Figure 5.

200 g of pollen are listed in Table II. In Figure 4B is shown the Sephadex G-75 chromatogram of a mixture of bovine plasma albumin, β-lactoglobulin, ribonuclease, and arabinose. The relative molecular sizes of the various fractions can be estimated by a comparison of their positions of emergence from the column with those of the standard substances.

Analyses of antigen contents of the rechromatographed fractions are shown in Figure 6. Fraction IV_2 contained antigen E, as the precipitin line can be traced to that in fraction D. Fraction III_2B contained mainly antigen B_1 and traces of antigens B_2 and E, and the identity of antigen B_1 can be followed by tracing the precipitin line through fractions III_2A and II_2 to that in fraction

TABLE III

COMPARATIVE SKIN TESTS OF THE FRACTIONS DERIVED FROM FRACTION D IN FIGURES 4A AND 5 ON A SINGLE RAGWEED-SENSITIVE PATIENT

Gram Used	Pollen Extract	Fraction							
		I_1	II_2	III_2A	III_2B	IV_2	V_2	VI_2A	VI_2B
5×10^{-8}	3+								
5×10^{-9}	1+								2+
5×10^{-10}	0				3+		3+		0
5×10^{-11}		2+	1+	2+	1+		2+		
5×10^{-12}		±	0	±	0	3+	±	3+	
5×10^{-13}		0		0		1+	0	1+	
5×10^{-14}						±		0	

The symbols ± to 3+ refer to the grade of the skin reaction on intracutaneous injection of the stated amount of the material (see text for designations).

D. Fraction III_2A contained antigens B_1, B_2, A_1, and A_2. Fraction II_2 contained antigens B_1 and B_2. Fractions II_2 and III_2A also contained a component corresponding to E. It is not certain whether this component is a dimer or polymer form of antigen E or another large protein with an antigenic determinant group similar to that of E. Fractions VI_2A and VI_2B contained antigen D. Fraction V contained antigens D and E and an unidentified component.

Comparisons of the relative allergenic activities of these fractions were carried out on seven hay fever patients. The entire test on one of the patients is given in detail (Table III) to serve as an example. The others are summarized in graphic form (Fig. 7); only the smallest quantities required to give positive reactions are given. The scattering of data in Figure 7 is caused in part by the inadequacies of the skin tests, but is also due to varying degrees of sensitivity in patients. The results in general suggest that fraction IV_2 was about ten times more reactive than the other fractions. Identical results were also obtained with six other patients in a preliminary experiment when fraction A was chromatographed on the Sephadex G-75 column to give a similar set of fractions.

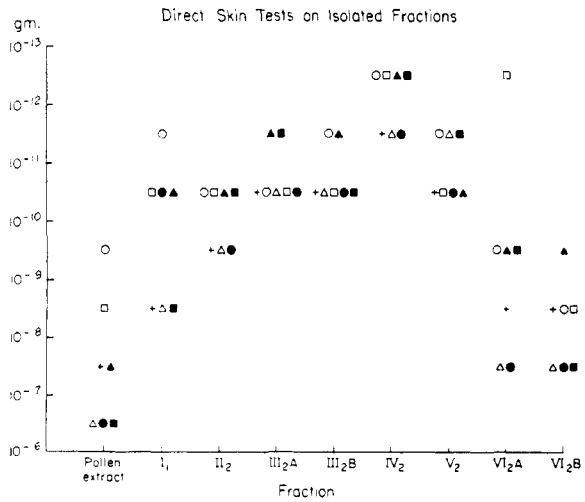

FIG. 7.—Summary of skin test data of the fractions from Figure 5. The symbols represent different patients. The entire test on the patient designated with the □ symbol is described in detail in Table III. Skin tests were negative in seven normal people at a level of 5×10^{-6} g of fraction IV_2.

The only exception in Figure 7 was that in one patient fraction VI_2A was as highly active as IV_2. VI_2B, however, was not highly active. This is anomalous, as it is unlikely that either fraction VI_2A or VI_2B would be entirely free of substances present in the other. However, this did raise the possibility that fraction VI_2 was a second allergen to which an occasional patient is also highly reactive. Rapid screening of nineteen other untreated patients by scratch tests showed three more patients who were as highly reactive to VI_2A as to IV_2.

Because fraction IV_2 was the most reactive allergenic fraction, it was chosen first to be more closely characterized. A summary of the steps required for its isolation is given in the flow sheet (Fig. 8). By antigenic tests, fraction IV_2 was estimated to be enriched about two-hundred-fold in comparison to the extract; by allergenic tests with threefold serial dilutions, fraction IV_2 was enriched about one-thousand-fold. No attempt was made to estimate the yield of the allergenic activity because of inadequacies of the assay. With the antigen assay, it is estimated that the yield of fraction IV_2 was about 30% from the extract. The major loss was found to occur during the isolation of fraction A, when visible amounts of precipitate formed during desalting on the Sephadex G-25 column (Fig. 2). The nature of this precipitate has not been studied.

The analytical data of fraction IV_2 and its amino acid composition are given in Tables IV and V. The amino acids found (uncorrected for decomposition) accounted for 93% of the nitrogen present. The low recovery is within the experimental error and may be due in part to incomplete hydrolysis of the abundant isoleucyl. leucyl, and valyl bonds. An interesting feature of the composition is the abundance of aspartic and glutamic acids, leucine, isoleucine, proline, and hydroxyproline. It remains to be determined

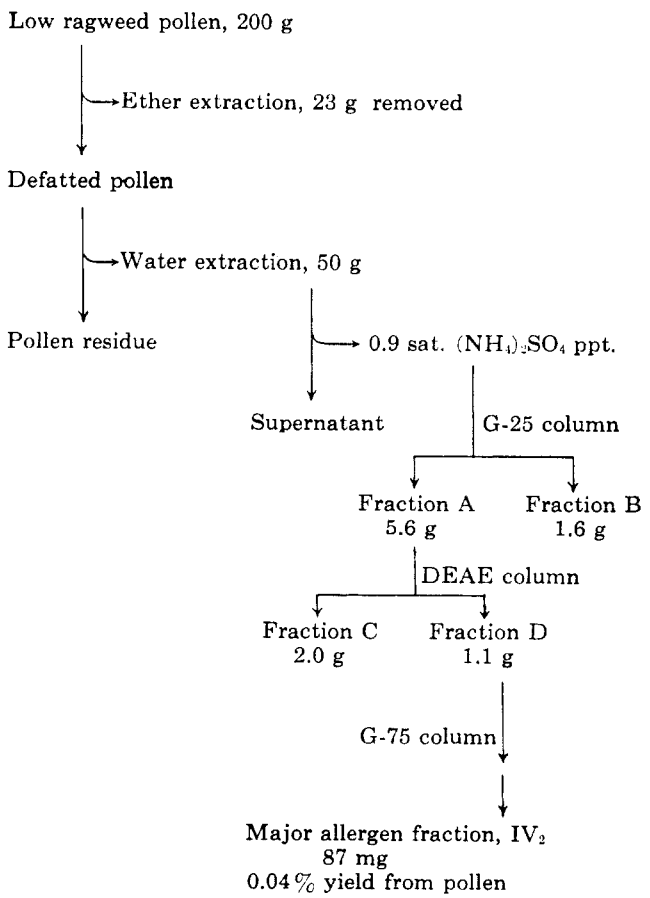

FIG. 8.—Flow sheet for the isolation of the major allergen fraction IV_2.

whether the traces of carbohydrates present are contaminants or actual constituents of the protein.

The ultraviolet spectrum of IV_2 did not reveal any special chromophoric group besides those of tyrosine and tryptophan. Its absorption maximum was at 282 mμ with a shoulder at 292 mμ. The ultracentrifugal analyses of several preparations of IV_2 showed that 85% of the material sedimented as one peak. Ten per cent of the material was slower sedimenting than the main peak, and the remaining 5% was in the form of rapidly sedimenting materials. The molecular weight of the main peak was estimated to be 32,000.

When IV_2 was tested for its immunochemical homogeneity against six different antisera at antigen concentration levels lower than that in Figure 6, a split of the precipitin line E into two closely spaced precipitin lines was observed. (A photograph of these results is not shown in this paper.) This suggested the presence of two antigens in IV_2. Further attempts to resolve IV_2 by zone electrophoresis at *p*H 8.5 gave the result shown in Figure 9. Although the pattern indicated a separation, antigen analyses across the band did not reveal any difference.

In order to form some estimates of the homogeneity of IV_2, amino- and carboxyl-terminal amino acid determinations were carried out. To determine the amino-terminal residue, fraction IV_2 was dinitrophenylated and then hydrolyzed in hydrochloric acid. The ether extract of the hydrolysate and the aqueous phase were both examined by paper chromatography for α-dinitrophenyl-amino acids. Several faintly yellow spots were found, but none was present in a significant quantity (less than 0.01 mole per 32,000 g). If glycine, cystine, tryptophan, or proline were the amino-terminal residue, it could have escaped detection due to decomposition during hydrolysis. Carboxypeptidase degradation showed that leucine

TABLE IV
ANALYTICAL DATA OF THE MAJOR ALLERGEN FRACTION, IV_2

Nitrogen	16.0%
Arabinose	2.4%
Hexosamine	0.3%
Hexuronic acid	0.6%
Ash	0.0%
O.D. at 280 mμ	1.13 for C = 1 mg/ml at *p*H 7.15
$S_{20,w}$	3.0 ± 0.1 × 10^{-13} sec.
$D_{20,w}$	8.0 ± 0.1 × 10^{-7} cm^2/sec.
Partial specific volume	0.719
Estimated molecular weight	32,000
Sulfhydryl group per 32,000 g	0.52 ± 0.05

TABLE V
AMINO ACID COMPOSITION OF THE MAJOR ALLERGEN FRACTION, IV_2

Amino Acid	No. of Residues Found per 32,000 g[a]	No. of Residues to Nearest Integer
Lysine	13.1 ± 0.4	13
Histidine	5.6 ± 0.2	6
Ammonia	23.2 ± 3.7	23
Arginine	10.3 ± 0.5	10
Hydroxyproline	4.5 ± 0.4	5
Aspartic acid	35.7 ± 1.7	36
Threonine	12.4 ± 0.8	12
Serine	15.5 ± 1.5	16
Glutamic acid	20.2 ± 0.9	20
Proline	13.7 ± 1.8	14
Glycine	29.4 ± 1.1	29
Alanine	21.4 ± 0.7	21
Half cystine	5.0 ± 0.2	5
Valine	16.3 ± 0.7	16
Methionine	4.8 ± 0.3	5
Isoleucine	13.3 ± 1.2	13
Leucine	15.7 ± 0.7	16
Tyrosine	3.7 ± 0.4	4
Phenylalanine	10.0 ± 1.0	10
Tryptophan[b]	3.1	3

[a] Average of three determinations. [b] Spectrophotometric analyses.

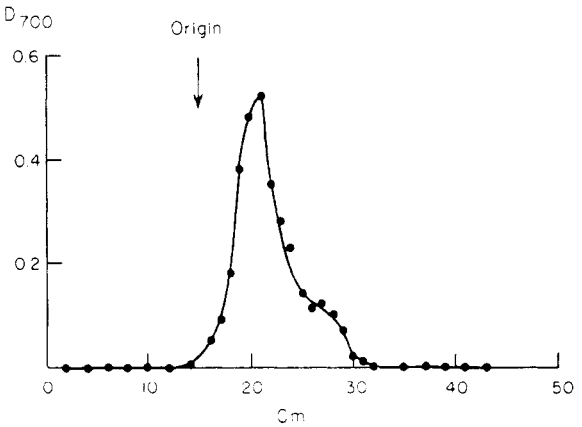

FIG. 9.—Zone electrophoresis of the major allergen fraction IV_2.

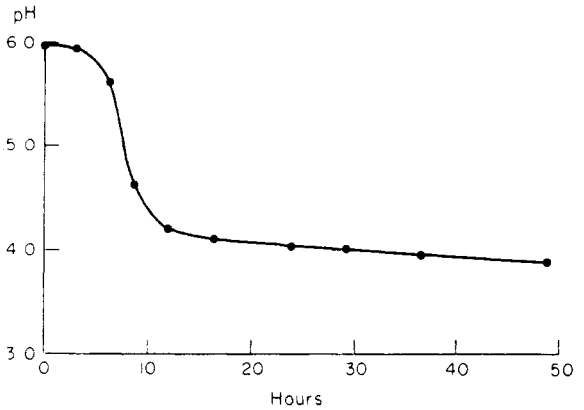

FIG. 10.—Hydrogen ion release of a 13% pollen extract at 25°.

was released in the largest amount, 0.61 mole per 32,000 g after 4 hours (Table VI). A possible interpretation of this result is that fraction IV_2 consists of only 60% of one protein.

DISCUSSION

In a preliminary study of the stability of the aqueous extract of ragweed pollen, a gradual loss of pollen antigens together with the appearance of turbidity was noted when the extract was allowed to stand at 25° (Fig. 2, lower row left). When the pollen extract was acidified to *p*H 4, a heavy precipitate formed and the antigen pattern of the supernatant was found to be similar to that of the aged extract, with the exception of antigen C, which did not change (Fig. 2, lower row right). This similarity suggested that the loss of the antigens in the aged extract was caused by a slow liberation of hydrogen ions. This was found to be the case when the *p*H of the extract was followed (Fig. 10). A sudden drop in *p*H of the extract occurred after 5 hours, when its natural buffering capacity (15 mM phosphate) was exhausted.

TABLE VI
CARBOXYPEPTIDASE A DIGESTION OF THE MAJOR ALLERGEN FRACTION, IV_2

Amino Acid	Mole Released per 32,000 g	
	After 1.5 hr.	After 4.0 hr.
Aspartic acid	0	0.07
Threonine	0.10	0.16
Serine, asparagine, glutamine	0.26	0.40
Glycine	0.12	0.22
Alanine	0.15	0.27
Valine	0.09	0.19
Methionine	0.04	0.04
Isoleucine	0.22	0.30
Leucine	0.41	0.61
Tyrosine	0.01	0.01
Phenylalanine	0.11	0.13

An origin for this hydrogen ion release is suggested by the finding of an esterase in the extract, as shown by direct assays with a synthetic substrate, *p*-nitrophenylacetate. The specific activity of the esterase was increased about three-hundred-fold in the course of the isolation of the allergens (Table II). The crude extract also exhibited phosphomonoesterase and phosphodiesterase activities when assayed with *p*-nitrophenylphosphate and bis-*p*-nitrophenylphosphate as substrates. Since there was no concomitant increase in phosphate concentration with the hydrogen ion release, it would seem unlikely that these enzymes caused the observed *p*H change. Determination of proteolytic activities with fraction A at *p*H 7.6, 6.3, and 4.0 gave negligible results.

The presence of phosphomonoesterase and phosphodiesterase (Nakamura and Becker, 1951) and various other enzymes (Paton, 1919) in ragweed pollen extract has been reported, but their effects on the allergenic activity of the extract have not been studied. It has been reported that the allergenic activity is unstable outside the *p*H limits of 4.5 to 7.5 (Batchelder and Gonder, 1941). The presence of an esterase activity then offers an explanation why ragweed pollen extract loses its allergenic activity on storage (Stull and Sherman, 1939).

The natural substrate of this esterase is still unknown but is apparently of low molecular weight, as a solution of fraction A in 15 mM phosphate (*p*H 5.9) did not show a marked change in *p*H on storage. With the removal of the unknown substrate, the allergenic activity of fraction D was stable even after storage in *p*H 7.4 buffer at 4° for 6 months.

There are many conflicting reports in the literature on the chemical nature and molecular size of ragweed pollen allergens. These differences may in part be caused by the different experimental procedures used, but they also indicate the

possibility of a multi-allergen system in ragweed pollen (Sherman and Stull, 1947). Because of this possibility, efforts were made in this study to examine closely all the derived fractions for their allergenic activities and antigen contents. Allergenic activity tests were carried out only on untreated ragweed hay fever patients, as it has been reported (Richter *et al.*, 1958) that treated patients developed an additional sensitivity toward the "dialyzable" fraction of the pollen extract, which was usually inactive in untreated patients.

All the pollen antigens were in fraction A, as described in the Results section. Procedures for the separation of this antigen mixture were devised after some preliminary observations on the electrophoretic mobility and molecular size distributions of fraction A. Chromatography on DEAE-cellulose was chosen because of the probable acidic nature of the pollen proteins. This led to the separation of fraction A into fractions C and D. Fraction D contained several antigens, and these were resolved on the basis of their molecular size differences by chromatography on Sephadex G-75.

Tests of allergenic activities of fractions A and B (Table I) clearly showed that the molecular weights of the allergens must be larger than several hundred, otherwise fraction B would have activities equal to that of A. Also, after chromatography on DEAE-cellulose the activity was concentrated in fraction D. The skin test data (Table III and Fig. 7) of the fractions derived from fractions D by Sephadex G-75 chromatography were not as decisive as those in Table I. The data definitely showed that fraction IV_2 was the most reactive one in all cases tested, but fractions II_2, III_2, and V_2 were also more reactive than the extract. The explanation of the observed activities of these fractions could be that they are contaminated with IV_2 or that they are different allergens of lower activities. At present both possibilities are to be considered. One would expect from the rechromatograms (Fig. 5) that fractions III_2 and V_2 would be contaminated with a few per cent of fraction IV_2, and this is indicated from the antigen analyses (Fig. 6). That amount of contamination in these two fractions could give the observed activities when compounded with the variations in skin testing. The activity in fraction II_2 could be explained by the observation that this fraction contained antigen similar to that in fraction IV_2, as described in the Results section.

Because of one exception in Figure 7 which showed fraction VI_2A to be equally reactive as IV_2, further tests were carried out, and they led to the finding that fraction VI_2A is a different allergen, highly reactive in four out of a total of thirty-two patients tested. If other allergens were present in fractions II_2, III_2, and V_2, their frequencies in eliciting a skin reaction as strong as IV_2 must be less than or equal to that of fraction VI_2A, or otherwise the activity relationship in Figure 7 would not have been observed.

Within the limits of these results, it is possible to conclude that the ragweed pollen contains two allergens, a major one in fraction IV_2 and a minor one in fraction VI_2. The other fractions perhaps are also allergenic but they probably play less significant roles in hay fever hypersensitivity than the two mentioned.

Recently Goldfarb and Callaghan (1961) have also reported the isolation of an allergenic fraction from ragweed pollen. Their fraction probably corresponds to the major allergen in fraction IV_2, as it was reported to have a skin reactive potency similar to IV_2. A detailed comparison of these two different preparations is not possible owing to a lack of information on the physicochemical properties of their sample.

The minor allergen fraction has not been studied thus far with regard to its purity and composition. On the basis of its elution volume on Sephadex G-75 chromatography, its molecular weight is probably about 10,000. Its antigenic determinant group appeared to be different from the major allergen, as the Ouchterlony analysis of the two fractions did not show any fusion of the precipitin lines.

From the results discussed in the above section, the major allergenic activity would seem to be associated with a protein fraction apparently of 32,000 molecular weight. Since fraction IV_2 represents at best no more than 10% of the proteins in ragweed pollen extract, it is of interest to question the cause of its powerful sensitizing effect. The amino acid composition of fraction IV_2 has not revealed any unusual chemical feature which could explain this effect. The traces of carbohydrate found in fraction IV_2 are probably impurities, as at each stage of purification the carbohydrate content decreased while the allergenic activity increased. Therefore, it is unlikely that the allergenic activity resides in the carbohydrate moiety. The relatively large molecular weight of fraction IV_2 also raises the interesting question of how this allergen can be so readily absorbed through mucous membranes as to sensitize the susceptible persons. Perhaps the allergen dissociates into smaller molecular units under suitable conditions.

Finally, it is not to be inferred from the observed close correlation of the allergenic activity with the presence of antigen E that the allergenic and antigenic sites are identical. Indeed the allergenic activity may be due to some small haptenic group attached to a carrier protein which is antigenic in rabbits. These aspects, as well as the homogeneity of fractions IV_2 and VI_2, are being studied further. The application of fraction IV_2 to desensitize ragweed hay fever patients is being carried out.

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