with the genetic make-up of the animal from which it was derived (Oudin, 1960), it is quite possible that the reported differences in peptide patterns originate in areas of the γ-globulin molecule which are not part of the antibody-combining site. It thus seems that in order to use successfully the peptide-map techniques of the type described here to obtain additional information about the chemical nature of the biologically active areas of the antibody, they will have to be combined with additional methods such as the selective tagging of the combining sites (e.g., Pressman and Roholt, 1961; Wofsy *et al.*, 1962).

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Isolation and Characterization of Allergens from Ragweed Pollen. II*

T. P. KING, P. S. NORMAN,† AND J. T. CONNELL

From The Rockefeller Institute, The Johns Hopkins University School of Medicine, and The Roosevelt Hospital

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The predominant allergen from ragweed pollen has been characterized as a protein of 37,800 molecular weight by physicochemical and immunochemical procedures. The active protein constitutes about 6% of the proteins in the pollen extract and it has been designated as antigen E. Antigen E can be isolated in four chemical forms. These forms have identical molecular weights and amino acid compositions but they differ in their charges. On removal of antigen E from pollen extract by precipitation with specific rabbit antiserum, a 40-fold decrease of the allergenic activity was observed by direct skin tests of the supernatant on eight sensitive patients. The finding suggests that antigen E represents at least 90% of the allergenic activity in ragweed pollen.

We have previously reported on the isolation of a highly allergenic protein fraction from ragweed pollen (King and Norman, 1962). The active fraction was designated as IV and was isolated from pollen extracts by ammonium sulfate precipitation followed with DEAE-cellulose[1] and Sephadex chromatography. Immunodiffusion studies of fraction IV with rabbit anti-ragweed sera showed that it contained a principal component which was called antigen E. However

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[1] Abbreviations used in this work: DEAE, diethylaminoethyl-; TEAE, triethylaminoethyl-; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

data were not available to decide on (1) how much of the total allergenic activity in ragweed pollen is represented by fraction IV and (2) whether or not the allergen in fraction IV is antigen E.

This paper will present evidences that the allergen in fraction IV is antigen E and that antigen E can be isolated in different chemical forms (IV-A, -B, -C and -D). The two principal forms (IV-B and -C) have been characterized as proteins since amino acid analysis yielded quantitative recoveries of the samples. The two forms are homogeneous by column chromatography, ultracentrifugation, starch-gel electrophoresis, and immunochemical procedures.

Antigen E is found to represent at least 90% of the allergenic activity in ragweed pollen as shown by the decrease of skin-test activity of pollen extract after precipitation of antigen E with specific rabbit antiserum. The importance of antigen E in hay fever of man is further substantiated by its high activity on tests of 184 patients.

In addition to the previously reported esterase, phospho-, mono-, and diesterase activities, ragweed pollen is found to have α- and β-pyranosidases for D-glucose, D-galactose, and L-arabinose. None of these enzymic activities is associated with the allergenic activity.

Materials

The low ragweed pollen of the 1960 and 1961 crops was furnished by the Division of Biologic Standards, U. S. Department of Health, Education and Welfare.

p-Nitrophenyl-β-D-glucopyranoside and *o*-nitrophenyl-β-D-galactopyranoside were products of California Corp. for Biochemical Research. *p*-Nitrophenyl-pyranosides of α-D-glucose, α-D-galactose, and α- and β-L-arabinoses were prepared according to published procedures (Montgomery *et al.*, 1942; Porter *et al.*, 1953; Feier and Westphal, 1956). All samples have melting points and optical rotations in good agreement with the published values.

TEAE-cellulose (exchange capacity, 0.86 meq/g) and Sephadex G-100 (140–400 mesh) were from Bio-Rad Laboratories and Pharmacia Co., respectively.

Rabbit antisera specific for the purified protein were prepared by immunizing rabbits intramuscularly with 2-ml aliquots of an alum suspension of the purified protein. The alum suspension was prepared by adding a solution of the purified protein (2.3 mg) in 13.5 ml of 0.0083 M Tris + 0.0050 M HCl to a mixture of 10% potassium alum (2.75 ml) and 0.943 N NaOH (1.75 ml). The *p*H of the mixture was 6.8. After a resting period of 3 months each rabbit received intravenously a booster shot of a 1-ml solution of purified protein (0.12 mg/ml) in 0.1 M Tris-HCl buffer (*p*H 7.9). Rabbits were bled on the tenth day.

Rabbit antiragweed serum No. 90 was kindly supplied by Dr. R. Wodehouse of the Lederle Laboratory.

Isolation of the Allergens from Pollen.—Most of the isolation procedure was given in the previous paper. Since modifications were made, sections of the procedure are given again.

The pollen (200 g), after ether defatting, was extracted with 1 liter of water for 3 hours at 25°. The active substances were precipitated from the extract by addition of solid ammonium sulfate to 0.9 saturation. The precipitate was dissolved in 70 ml of 0.10 M Tris and 0.06 M HCl (*p*H 7.9) and the solution was depigmented by passage through a Sephadex G-25 column (95 × 4 cm) equilibrated with 0.025 M Tris and 0.015 M HCl (*p*H 7.9). The first peak, fraction A, was concentrated to 90 ml by pressure filtration as described under Methods. The concentrate (82 mg/ml) was separated into fractions C and D by DEAE-cellulose chromatography as in the earlier procedure. Fraction D was concentrated to 20 ml by pressure filtration. The concentrated fraction D (61 mg/ml) was chromatographed on a Sephadex G-100 column (400 × 2.5 cm). Prior to application of the sample to the column, the concentrate was equilibrated by dialysis with the column buffer which was 0.40 M $(NH_4)_2SO_4$ + 0.05 M Tris + 0.04 M HCl (*p*H 7.4). The column was built in two 200-cm sections connected in series and was operated at a flow rate of 22–28 ml/hour. Fractions of 10 ml volume were collected. The major allergen fraction IV emerged at the effluent volume of 1141–1240 ml. This was concentrated to 12 ml (18 mg/ml) and equilibrated by dialysis with a buffer of 0.10 M Tris and 0.06 M HCl (*p*H 7.9).

Fraction IV was separated into immunochemically related fractions by chromatography on a TEAE-cellulose column (22 × 1.6 cm) with a linear NaCl gradient, using reservoirs containing 500 ml of 0.10 M Tris + 0.06 M HCl (*p*H 7.9) and 500 ml of 0.10 M Tris + 0.07 M HCl + 0.20 M NaCl (*p*H 7.8). The column was initially equilibrated with 0.10 M Tris + 0.06 M HCl. Fractions of 8 ml volume were collected at a flow rate of 40 ml/hour. The desired fractions were pooled, concentrated, then equilibrated by dialysis with 0.10 M Tris + 0.06 M HCl buffer. The dialyzed concentrates were stored at 4°.

For analytical scale experiments on TEAE-cellulose, a column (22 × 0.9 cm) was used. The gradient was obtained using 125 ml of each buffer given above. The flow rate was 12 ml/hour and 2-ml fractions were collected.

Methods

A. *Pressure Filtration.*—Solutions were concentrated to desired volumes by pressure filtration through Visking 8/32 tubing (Berggård, 1961). At a pressure differential of about 75 cm Hg, the filtration rate was about 35 ml/hour per meter of tubing. Under these conditions, there was a slight loss (about 5%) of the allergenic protein.

B. *Chemical Analyses.*—Total solid contents of solutions were determined by weighing dried aliquots of solutions in tared platinum shells (Craig, 1960). The solutions were freed of nonvolatile buffer salts by dialysis against 0.05 M NH_4HCO_3.

Arabinose content was analyzed by the orcinol-ferric chloride method (Volkin and Cohn, 1954). The published procedure was modified for determination in the range of 0–10 μg of total arabinose. Aliquots of the column effluents were used directly without prior hydrolysis.

For total nitrogen and amino acid analyses, solutions containing the samples were desalted on a Sephadex G-25 column (2 × 50 cm) equilibrated with 0.2 M acetic acid, and lyophilized. Amino acid analyses were carried out according to published procedures (Moore and Stein, 1963; Spackman *et al.*, 1958).

C. *Sedimentation Analyses.*—Samples were studied for their homogeneity by equilibrium ultracentrifugation (Yphantis, 1962) at rotor speeds of 24,630 and 35,600 rpm. The studies were carried out at two concentration levels, 0.1% and 0.025%, in a buffer of 0.1 M NaCl + 0.004 M KH_2PO_4 + 0.035 M K_2HPO_4 (*p*H 7.7). Partial specific volume was calculated from the determined amino acid composition (Cohn and Edsall, 1943). Velocity experiments with 1% solutions in the same buffer were also made at 50,740 rpm.

D. *Starch-Gel Electrophoresis (Smithies, 1959).*—The gel was prepared with 67 g of hydrolyzed potato starch (Connaught Laboratories, Toronto, Canada) in 450 ml of 0.025 M Tris + 0.015 M HCl (*p*H 7.9). The buffer in the electrolyte compartments was 0.10 M Tris + 0.06 M HCl. Approximately 20-μl aliquots of sample solutions (concentration 1%) were used. Electrophoresis was carried out at an initial voltage of 250 v and 35 ma for 16 hours, then the gel was stained with 0.2% Amido Schwartz black dye solution. The gel at *p*H 6.3 was prepared with a buffer of 0.004 M Na_2HPO_4 + 0.016 M NaH_2PO_4.

E. *Reduced-carboxymethylated and Oxidized Derivatives.*—Protein samples were reduced and carboxymethylated in 0.10 M Tris + 0.06 M HCl containing 8 M urea, according to the procedure of Anfinsen and Haber (1961) with a modification that the product was isolated from the reaction mixture by chromatography on Sephadex G-25 equilibrated with 0.05 M NH_4HCO_3.

Performic acid oxidation of protein was carried out according to the procedure of Schram *et al.* (1954).

F. *Immunological Analyses.*—Allergenic activity of samples was determined by direct skin tests on sensitive patients as described in the previous publication. To improve the stability of dilute solutions of allergens, all samples were diluted with isotonic saline buffered at *p*H 7.9 with Tris-HCl and containing 0.05% human serum albumin (Pentex, Inc., Kankekee, Ill.). Only the minimal amounts of materials required to give positive reactions are reported.

Antigen concentrations were estimated by double diffusion on agar-gel plates with rabbit antiragweed serum or antiserum specific for the test antigen. The experimental details are given in the previous publication (King and Norman, 1962). Concentrations of unknowns were determined by comparison of their precipitin line positions with that of a standard curve. With the antisera available, the sensitive range of concentration for analysis of antigen E was 0.02–0.20 mg/ml. Eluents from chromatograms were analyzed either directly or after dilution to the desired concentrations. The antigen and antiserum solutions were added to wells as two 10-μl aliquots in an interval of 14–20 hours. After 40–48 hours the precipitin line positions were measured from enlarged photographic copies of gel plates. To compensate for variations due to temperature and gel composition, standards were run together with the unknowns on the same plates. The accuracy of this method is within 20% of the expected values.

Quantitative precipitin analysis in liquid medium was carried out using 0.20 ml of antigen solution in 0.10 M Tris + 0.06 M HCl and 0.20 ml of antiserum. After mixing, the solution was allowed to stand at 25° for 0.5 hour then at 4° overnight. The precipitate collected by centrifugation was washed with two 1-ml aliquots of 0.15 M NaCl, then dissolved in 1 ml of 0.1 N NaOH containing 2% of Na_2CO_3. The amount of immune precipitate was estimated from its absorbancy at 280 mμ. The supernatants were tested for antibody or antigen excess by double diffusion on agar plates. Antibody nitrogen in sera was estimated from the amounts of immune precipitate formed at the equivalence zone. The absorbancy units found in the precipitate were converted into μg of antibody nitrogen using a factor established with normal rabbit γ-globulin (Kabat and Mayer, 1961). The supernatants were tested for their remaining allergenic activities by direct skin tests on sensitive patients. For this purpose the supernatants were diluted on the basis of total amount of antigen originally present.

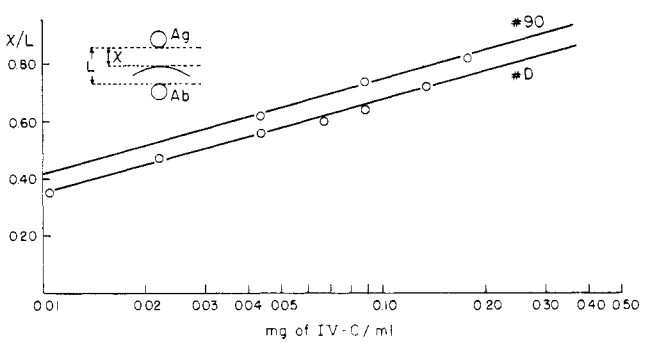

FIG. 1.—Relationship of precipitin line position with antigen concentration on immunodiffusion. Serum No. 90 is a rabbit antiragweed pollen serum and serum No. D is a rabbit antiserum specific for antigen E.

Serologic titers of human sera toward the purified protein were determined by the tanned-cell-hemagglutination method of Arbesman *et al.* (1960) with the modification that unheated sera were diluted in buffer containing 0.1 M EDTA. A solution containing 1 mg of the purified protein per ml was used to coat the tanned cells.

G. *Glycosidase Activity Determinations.*—Assays were carried out at 25° with a system containing 0.1 ml of enzyme solution and 2 μmoles of the appropriate *p*-nitrophenylglycoside in 1.9 ml of 0.15 N NaOAc + 0.15 N HOAc (*p*H 4.65). At intervals an 0.5-ml aliquot was withdrawn and diluted with 0.5 ml of 0.5 M $NaHCO_3$ + 0.5 M Na_2CO_3. The liberated *p*-nitrophenol was estimated by its absorbancy at 400 mμ. The rate of liberation of *p*-nitrophenol follows first-order kinetics. The rate constant (k_1) was linearly proportional to the enzyme concentration under the conditions used. A unit of activity is defined as the amount of enzyme required to give a k_1 value of 0.023 $hour^{-1}$.

RESULTS

Quantitative Estimation of Antigen by Immunodiffusion.—In our previous study the separation of the different components in ragweed pollen was guided qualitatively by the agar-gel-diffusion analysis of antigens with rabbit antiragweed serum, using the double-diffusion technique of Ouchterlony. Thus the most active fraction IV was found to contain a predominant antigen which has been designated as E. To improve the isolation of fraction IV it would be advantageous to have some simple means of estimating the antigen concentration.

The double-diffusion technique of Ouchterlony may be adapted for the quantitative estimation of antigens as there is a linear relationship between the precipitin line position and the logarithm of the antigen concentration (Crowle, 1961; Darcy, 1960). Figure 1 shows this relationship for antigen E with an antiragweed serum and an antiserum specific for it. The final product of purification IV-C is taken to be the standard of antigen E. This technique is used in the present study as it is simple yet it provides an estimation and unambiguous identification of the antigen.

Isolation of Fraction IV.—Several modifications in isolation were made following the observations that the allergen is most stable in the *p*H range of 6–8.5 and that the lyophilization of solutions of allergen promotes polymer formation. In the present study only pressure filtration was used to concentrate samples. These observations on the stability and polymerization of allergen will be dealt with more fully in other sections.

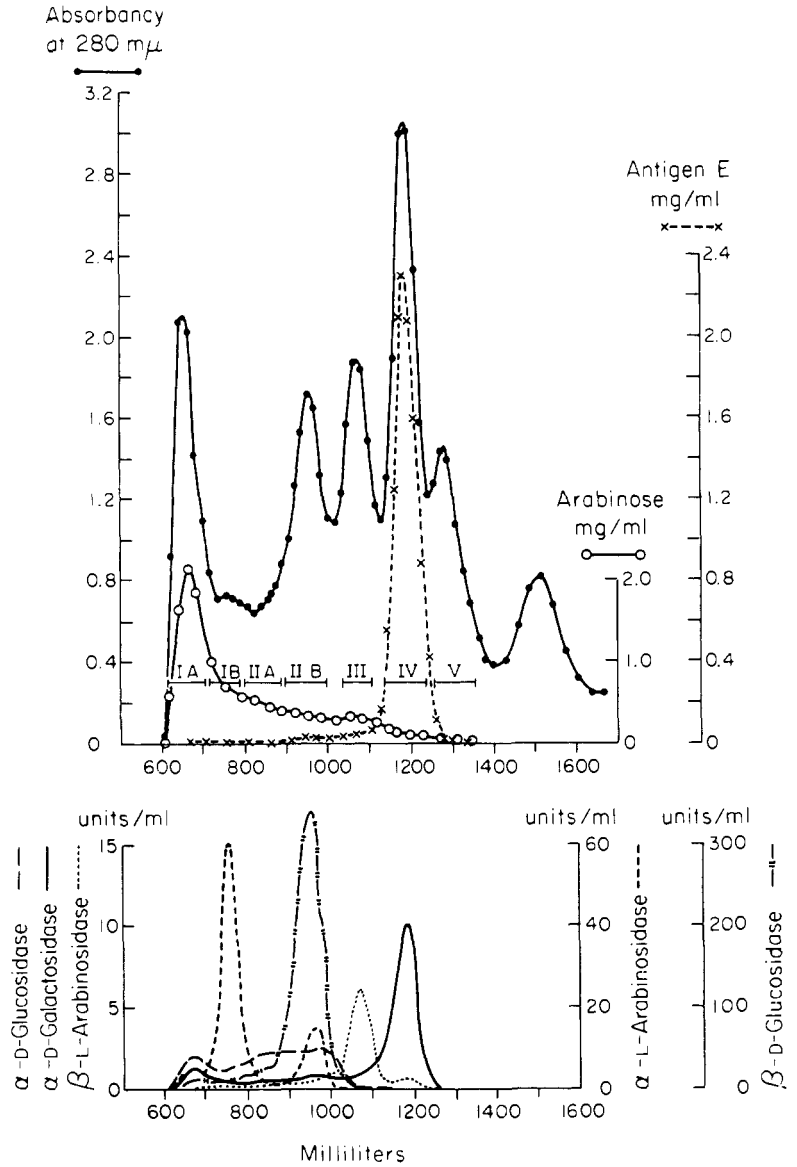

FIG. 2.—Chromatography of fraction D on Sephadex G-100 column (400 × 2.5 cm). The sample load was 1.1 g, and the eluent was a buffer of 0.40 M $(NH_4)_2SO_4$ + 0.05 M Tris + 0.04 M HCl. Antigen E concentration and glycosidase activities were determined by procedures described in text.

The active substances were precipitated from aqueous extracts of ragweed pollen with ammonium sulfate at 0.9 saturation. The precipitate was depigmented by passage through a Sephadex G-25 column to yield fraction A. The choice of 0.025 M Tris-HCl buffer (*p*H 7.9) as eluting agent in place of water used in the earlier experiments has given a better final yield of fraction IV due to the aforementioned instability in acid solutions. Fraction A was separated into fractions C and D by stepwise-elution chromatography on DEAE-cellulose given in the previous publication.

Chromatography of fraction D on a Sephadex G-100 column gave fraction IV together with other fractions. The selectivity of G-100 is more favorable for the separation than that of G-75, used previously. This favorable selectivity has given a better separation and yield of fraction IV as indicated by the analysis of antigen E in Figure 2. The distribution of several glycosidases from the pollen is also given in the figure. The α-D-galactosidase activity in fraction IV is a contaminant which was removed in the subsequent step of purification. Most of the galactosidase was present in fraction C from the DEAE-cellulose chromatography.

Isolation of Fractions IV-A, -B, -C, and -D.—When fraction IV was chromatographed on TEAE-cellulose at *p*H 7.9 with a linear sodium chloride gradient, separation into fractions IV-A, -B, -C and -D was accomplished (Fig. 3A).[2] Fraction IV-A is not a well-defined peak in the figure given, but a distinct shoulder in its position was present on several occasions. The immunochemical identities of these fractions are demonstrated by the antigen E analyses in Figure 3A. The analyses were carried out with rabbit antiragweed serum and with specific rabbit anti-IV-C serum. Identical results were obtained. These fractions were equally active by direct skin tests on sensitive patients (Fig. 9).

The charge differences among fractions IV-B, -C, and -D shown on chromatography were reflected by starch-gel electrophoresis as well. In gels containing *p*H 7.9 Tris-HCl buffer and *p*H 6.3 phosphate buffers, they migrated as negatively charged ions with fraction IV-D having the fastest mobility, IV-B the slowest, and IV-C the intermediate. Fraction IV-C migrated

[2] DEAE-cellulose may also be used for this separation, but TEAE-cellulose is preferred as it permits the use of a buffer of higher ionic strength.

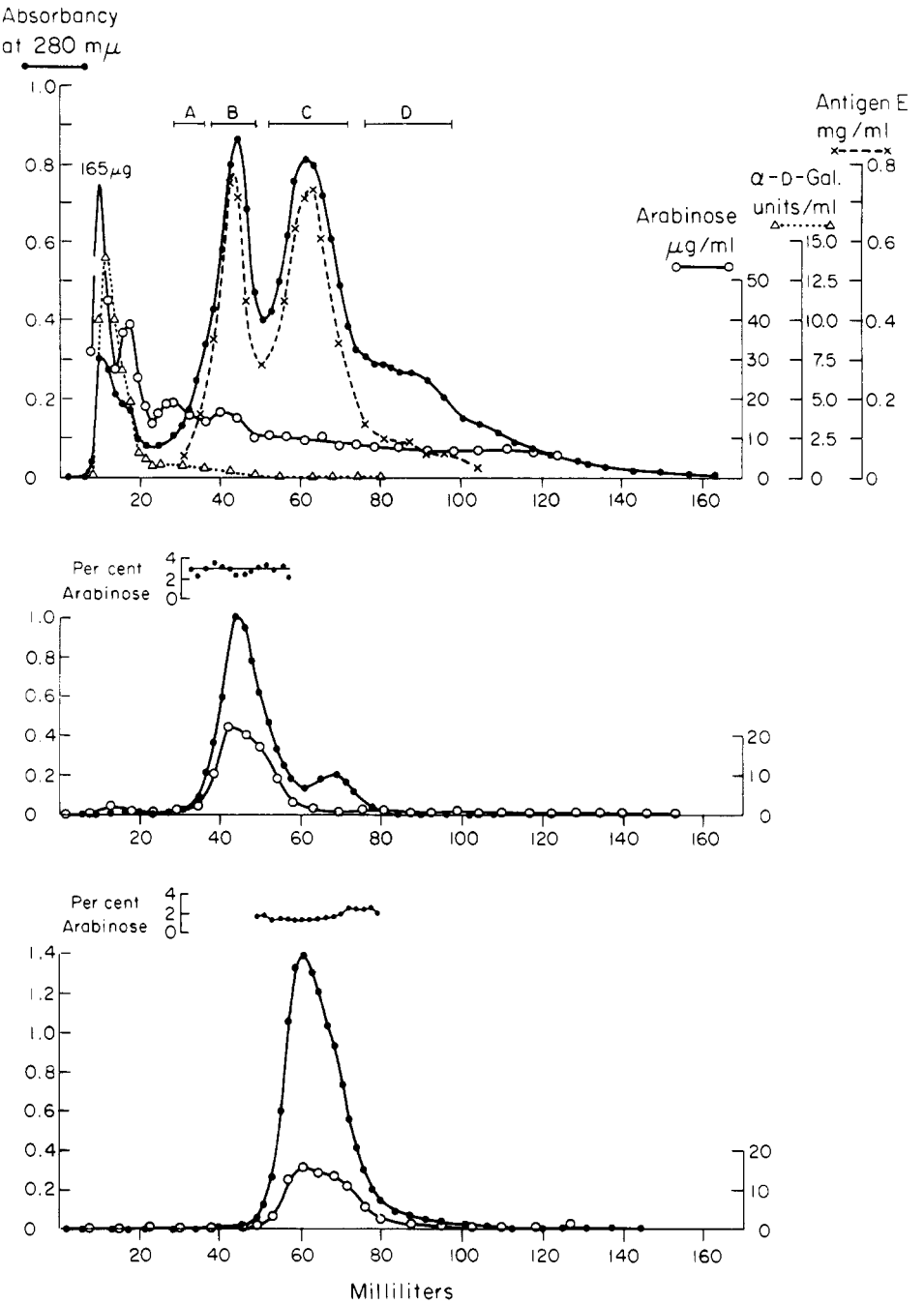

FIG. 3.—Chromatography of fraction IV on TEAE-cellulose column (20 × 0.9 cm). (A) Initial chromatography of fraction IV. The sample load was 36 mg and the eluent was a buffer of 0.10 M Tris + 0.06 M HCl with a linear gradient of NaCl. (B) Rechromatography of fraction IV-B. The sample load was 14 mg. (C) Rechromatography of fraction IV-C. The sample load was 23 mg. Recoveries of samples were quantitative in all chromatograms.

as a single spot while IV-B and -D were contaminated with some IV-C. These electrophoretic differences were also shown by immunoelectrophoresis. (Photographs of these electrophoretic experiments are not shown.)

Fraction IV-D is probably a mixture containing disulfide or sulfhydryl isomer(s) of IV-C; on treatment of fraction IV-D with mercaptoethanol, half was converted to IV-C as judged by chromatography on TEAE-cellulose.

The yields of these fractions and their preceding ones are given in Table I; the isolation was carried out with 200 g of ragweed pollen. The contents of antigen E of these fractions are also given in the table. Good recoveries of antigen E were observed in all isolation steps with the exception that at the stage from fractions A to D there was a 2-fold decrease of antigen E. This was found in several experiments as estimated with both antiragweed and specific anti-IV-C-sera. This decrease is believed to be due to an anomaly in antigen E estimation rather than an actual loss in the chromatographic procedure. The anomaly is probably caused by the presence of another precipitin system which affects the precipitin line position of antigen E. Therefore antigen E accounts for about 6% of the proteins in pollen extract.

Chemical Characterization of Fractions IV-B and -C.— The rechromatographies of fractions IV-B and -C are illustrated in Figure 3B and C. The fractions emerged at their expected effluent volumes. The

TABLE I
YIELDS OF VARIOUS FRACTIONS FROM EXTRACTION OF POLLEN (200 g)

	Total Solid	Antigen E (mg)	Amount Fraction as Antigen E (%)
Extract	53,000[a]	440	0.9
Fraction			
A	7,460 (4,820)[b]	430	5.7
D	1,220	230	19.0
IV	205 (240)	179	87
IV-A		9	
IV-B	46 (58)	46	100
IV-C	66 (89)	66	100
IV-D		34	

[a] The solid contained 4700 mg of protein, as determined by nitrogen analysis of the precipitate from extract on addition of 9 volumes of 10% trichloroacetic acid. [b] Values in parentheses are yields from a separate experiment by the initial precipitation of pollen extract at 0.4–0.8 saturation with $(NH_4)_2SO_4$.

assymetries of the peaks were caused by the high loads applied to column. The recovery of samples as estimated by absorbancy was quantitative in both cases. The two samples have identical absorbancy coefficients at 280 mμ, $A = 1.13 \pm 0.03$ for $c = 1$ mg/ml.

The two fractions differed in their arabinose contents as shown in the figures, 3% and 1.3% in IV-B and -C, respectively. Further work showed that the arabinose was present as an impurity. Fractions IV-B and -C, when isolated by precipitation from pollen extract with ammonium sulfate at 0.4–0.8 saturation, contained only 0.6% and 0.5% of arabinose, respectively.

Fractions IV-B and -C have identical amino acid compositions within experimental error (Table II).

TABLE II
AMINO ACID COMPOSITIONS OF FRACTIONS IV-B AND IV-C

Amino Acid	No. of Residues Found/ 37,800 g of IV-B[a]	No. of Residues Found/ 37,800 g of IV-C[a]	No. of Residues to Nearest Integer[b]
Lysine	17.8 ± 0.6	17.6 ± 0.4	18
Histidine	5.8 ± 0.2	6.3 ± 0.2	6
Ammonia[c]	30.2 ± 2.1	31.3 ± 2.1	31
Arginine	15.8 ± 0.2	15.9 ± 0.1	16
Aspartic acid	48.1 ± 1.0	48.9 ± 1.0	49
Threonine[d]	17.3 ± 0.2	17.6 ± 0.5	17
Serine[d]	25.3 ± 1.0	26.1 ± 1.0	26
Glutamic acid	24.6 ± 0.5	24.7 ± 0.6	25
Proline	15.3 ± 1.0	15.1 ± 0.3	15
Glycine	36.6 ± 0.6	38.1 ± 1.1	37
Alanine	30.3 ± 0.4	31.2 ± 1.0	31
Half-cystine[e]	6.8 ± 0.3	7.2 ± 0.6	7
Valine[f]	23.0 ± 0.4	24.0 ± 0.4	24
Methionine	6.8 ± 0.2	6.8 ± 0.2	7
Isoleucine[f]	20.0 ± 0.3	20.2 ± 0.1	20
Leucine	21.2 ± 0.4	21.3 ± 0.3	21
Tyrosine	3.9 ± 0.2	3.9 ± 0.2	4
Phenylalanine	12.4 ± 0.4	12.5 ± 0.3	12
Tryptophan[g]	6.2	6.1	6

[a] Average of six determinations, four 22-hour hydrolysates and two 90-hour hydrolysates. Both samples contained 17.1 ± 0.3% nitrogen. [b] Average of values from IV-B and -C. [c] Corrected for ammonia from decomposition of serine and threonine. [d] Corrected for decomposition by extrapolation to zero time. [e] Determined as cysteic acid (one analysis) and as carboxymethylcysteine (two analyses). [f] Average of 90-hour hydrolysates only. [g] Spectrophotometric determination.

The amino acids listed account for 99% and 98% of the nitrogen and the weight of the samples, respectively. The samples used for the reported analysis contained 0.5–0.6% of arabinose; identical results were obtained with samples of fractions IV-B and -C containing higher amounts of arabinose.

Both samples were homogeneous by equilibrium ultracentrifugation and have indistinguishable molecular weights of 37,000 ± 2,000. The partial specific volumes were calculated to be 0.72 from their amino acid compositions. Assuming that each mole of the protein contains 6 moles of histidine and 7 moles of methionine, a molecular weight of 37,800 ± 1,000 was calculated from the values found for these amino acids. This value is 15% higher than that of fraction IV reported previously. This discrepancy is caused by an inaccurate estimation of the diffusion coefficient of fraction IV, as the sedimentation coefficients of fractions IV-B and -C ($s_{20,w}$ 3.05 ± 0.10 × 10^{-13} sec) are identical to that of fraction IV. The diffusion coefficients of fractions IV-B and -C are $D_{20,w}$ 7.41 ± 0.20 × 10^{-7} cm^2/sec.

Upon treatment of fractions IV-B and -C with carboxypeptidase A, identical amounts of amino acids were liberated in both cases; the major amino acid released was leucine in the amount of 0.48 mole/37,800 g of protein as was found with fraction IV previously.

No attempt was made to determine the amino terminal amino acid in these fractions, as repeated efforts with their preceding fraction IV did not reveal an end group. The procedures used were the dinitrophenylation and the phenylisothiocyanate methods (Fraenkel-Conrat *et al.*, 1955). Performic-acid-oxidized and the reduced-carboxymethylated fraction IV as well as the native fraction IV were used for these experiments.

Immunochemical Characterization of Fractions IV-B and -C.—On immunization of four rabbits each with 0.37 mg of fraction IV-C, antisera specific for antigen E with antibody nitrogen 100–150 μg/ml were obtained. The specificity of one such antiserum (No. D) when tested with fraction A by immunodiffusion is shown in Figure 4A. One sharp precipitin line formed with the specific serum and only at high concentrations of fraction A was there a possible indication of the serum containing antibodies for antigens other than E. For comparison, the pattern with an antiragweed serum (No. 90) is also given in the figure.

Immunodiffusion analyses of fractions IV-B and -C with these two sera are shown in Figure 4B and C. Presence of contaminating antigens was detected at high concentrations of the fractions when the precipitin line of antigen E disappeared due to antigen excess; two of these contaminating antigens were identified as the major components of fractions III and V.

A rough estimation of the probable amounts of impurities in fractions IV-B and -C may be arrived at from Figure 4A and B. In Figure 4A discernible precipitin lines are still seen with fraction A at a concentration of 0.08 mg/ml, equivalent to 22 mg of pollen per ml. In Figure 4B the lowest concentrations of fractions IV-B and -C showing contaminating antigens are 0.12 mg/ml, equivalent to about 400 mg of pollen per ml. Therefore the amount of contamination at most can be only 5%.

Precipitin analyses of the two fractions in liquid media with an anti-IV-C serum (No. C) are given in Figure 5A and C.

Polymer Formation of Antigen E.—The quantitative estimation of antigen E in the chromatogram of Figure 2 showed that antigen E was concentrated in fraction IV, and that it was also present in the regions

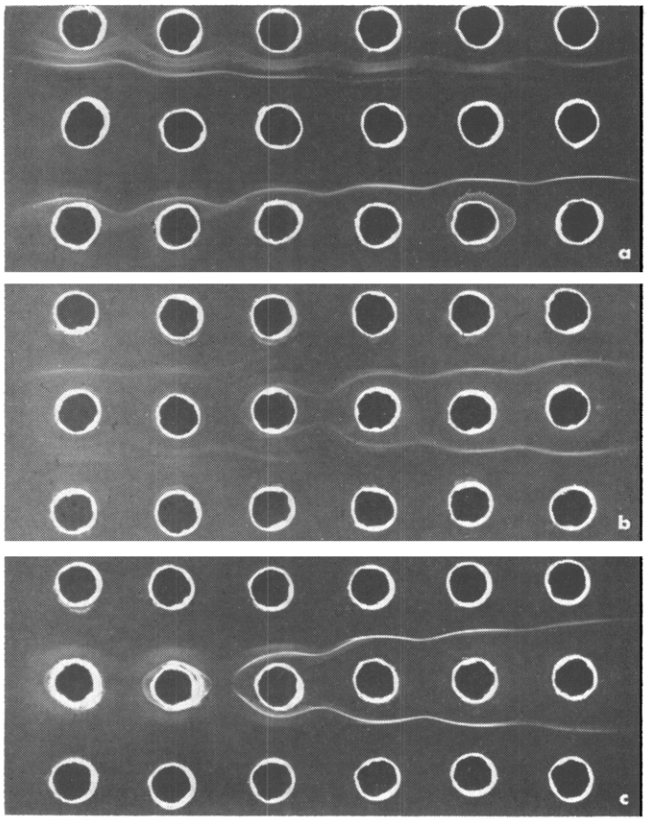

FIG. 4.—Double-diffusion analyses. (A) Comparison of specificities of two rabbit antisera. Top and bottom row wells were filled with serum Nos. 90 and D, respectively. From left to right, middle-row wells were filled with fraction A, 16.2, 8.1, 3.2, 1.6, and 0.8 mg/ml and fraction IV, 0.06 mg/ml. (B) and (C) Top and bottom-row wells were filled with fractions IV-C and IV-B, respectively, in both photographs. The concentrations of the antigen solutions were 5.79, 2.90, 0.58, 0.12, 0.06, and 0.03 mg/ml. Middle-row wells were filled with serum Nos. 90 and D in B and C, respectively.

of substances with higher molecular weights. Further studies showed that this was a result of polymer formation. The reaction occurs readily when the sample has been lyophilized from a solution in 0.01 M NH_4HCO_3 and stored in such a form. Figure 6 gives these results; in Figure 6A the sample was concentrated by pressure filtration and in Figure 6B the lyophilized sample was stored at 4° for 40 days. The recovered fraction IV and the central small peaks in Figure 6B were still capable of precipitation with rabbit antiserum, but the material in the polymer peak had lost this capacity. Attempt to regenerate the native protein by treatment of the polymer with mercaptoethanol was unsuccessful. The presence of polymers in samples may be detected similarly by TEAE-cellulose chromatography, as the polymers are not eluted under the conditions used. Treatment of fraction IV with 8 M urea at *p*H 7.9 also caused a rapid polymerization.

Stability of Antigen E in Solutions at Different pH.—As the crude extract of ragweed pollen was known to lose its allergenic activity and most of its antigens in acid solutions (King and Norman, 1962), a detailed study on the stability of antigen E in fraction IV was therefore undertaken. Fraction IV was dissolved in buffers of different *p*H. After 5 and 17 days at 22–26°, the solutions were adjusted to *p*H 7.9, then assayed for the remaining allergenic activity and antigen E content. The results are given in Figure 7. In the region of *p*H 6–8.5 the sample is most stable; at other

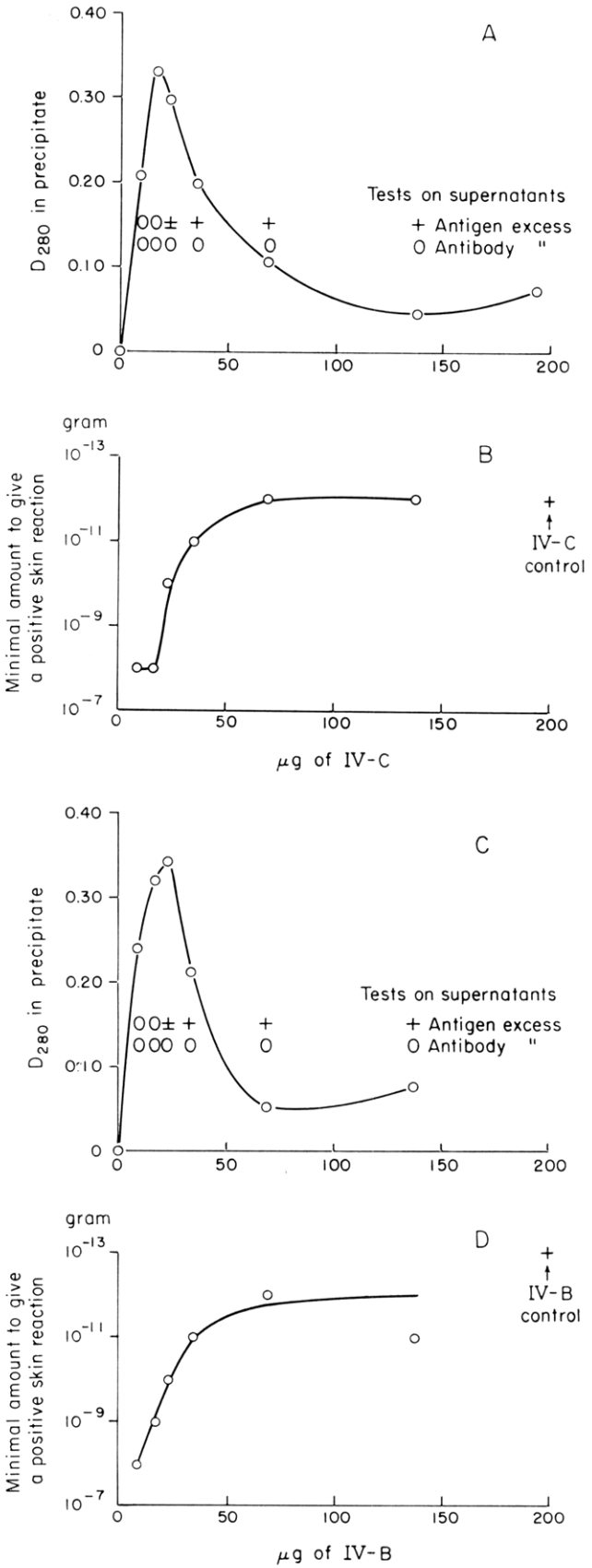

FIG. 5.—Precipitin analyses of fractions IV-B and -C with a specific rabbit serum in liquid media. (A) and (C) The precipitin curves of fractions IV-C and -B, respectively. The antiserum No. C was concentrated 3-fold before use by precipitation with $(NH_4)_2SO_4$ at 0.5 saturation and redissolution in one-third of the original volume with 0.10 M Tris + 0.06 M HCl. (B) and (D) Direct skin tests of the supernatants from Fig. 5A and C, respectively.

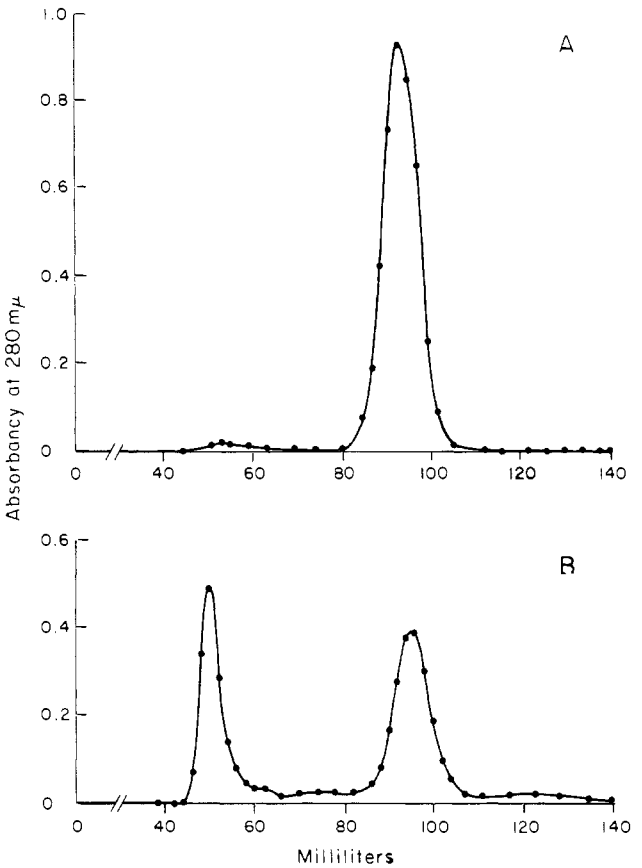

FIG. 6.—Chromatography of fraction IV-C on Sephadex G-100 (200 × 0.9 cm). The eluent was the same as in Fig. 2. (A) A sample (8.5 mg) of fraction IV-C which was concentrated by pressure filtration was used. (B) A sample (6.7 mg) of fraction IV-C which was lyophilized from 0.05 M NH_4HCO_3 and had been stored at 4° for 40 days was used.

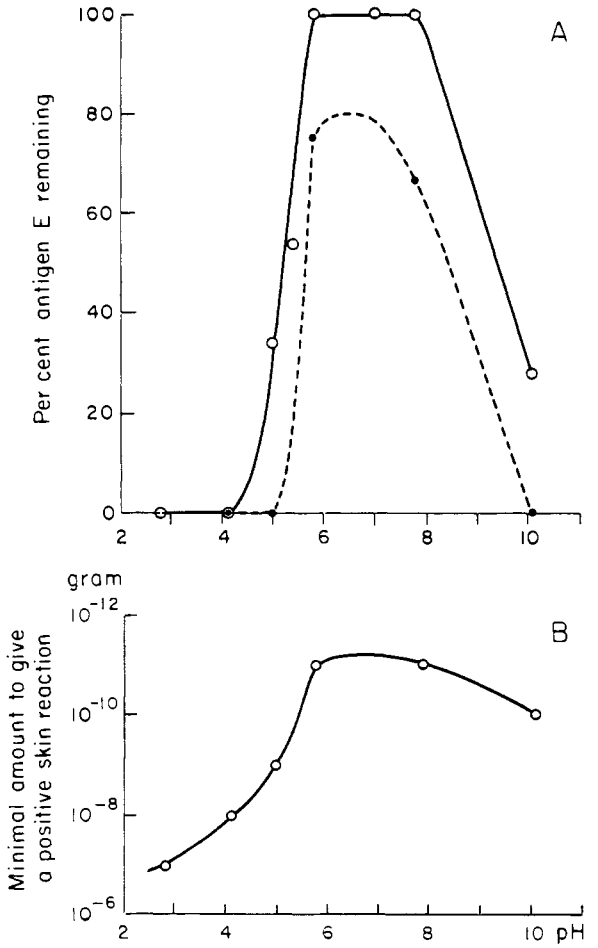

FIG. 7.—Stability of fraction IV in solutions at different *p*H. Fraction IV was made up to a concentration of 1 mg/ml in the following buffers: *p*H 2.8, 0.1 M acetic acid; *p*H 4.1 and 5.0, 0.2 M sodium acetate; *p*H 5.8, 0.2 M sodium phosphate; *p*H 7.9, 0.1 M Tris-HCl; and *p*H 10.1, 0.2 M sodium carbonate. After 5 and 17 days at 23–26°, the solutions were diluted with 0.1 M Tris-HCl buffer for antigen E and allergen assays. Direct skin tests of the solutions from the 17-day experiment on one patient are given. Only slight differences were found between the 5-day and 17-day experiments.

*p*H values there is a simultaneous loss of the allergenic activity and antigen E.

Correlation of the Allergenic Activity with Antigen E.— The supernatants from the precipitation of fractions IV-B and IV-C with a rabbit anti-IV-C serum (Fig. 5A and C) provided a direct test of this correlation. In the region of antibody excess the supernatants should contain relatively little antigen E, and the allergenic activity should be decreased. This was found to be the case as illustrated in Figure 5B and D; the allergenic activity decreased 10,000-fold in the region of antibody excess when compared to that in the region of antigen excess. The minimal amounts required to give positive reactions in two of the five patients tested are given in the figures. Similar results were obtained with the other three patients. A separate set of experiments with another anti-IV-C serum (No. D) also gave similar results when tested with three patients. The geometric mean of the tests on eight patients is a 40,000-fold decrease in activity. Within the concentration range of fractions IV-B and -C where there was an extensive decrease of allergenic activity, only a single precipitin line corresponding to antigen E was seen on immunodiffusion with the specific sera. Therefore the results provide conclusive evidence that antigen E is the allergen.

To ascertain that antigen E is the principal allergen in ragweed pollen, assays of allergenic activities were carried out with ragweed pollen extracts after the precipitation of antigen E with its specific serum. The precipitin pattern of pollen extract with a specific rabbit antiserum (No. D) is given in Figure 8A. Skin tests of the supernatants were carried out on seven patients. The result from one patient is illustrated in Figure 8B; in this case, a 1000-fold decrease in activity was found. The results from all patients are summarized in Table III, together with those from a separate experiment using antiserum (No. C). The decrease in activity was not uniform with all patients. The geometric mean of the tests on eight patients is a 40-fold decrease in activity. These findings will be treated further in the discussion.

Figure 9 shows the accumulated data on skin tests of several antigen E–containing fractions. The observed distribution of activity of each fraction is due to varying degrees of sensitivity in patients. Increased activities of the fractions are observed with the enrichment of antigen E. The importance of antigen E as an allergen in hay fever is supported further by nearly symmetrical distribution of the activity of fraction IV in tests on 184 patients, thus indicating the unlikelihood that it may be inactive in a sizable group of hay-fever patients.

In addition to their high skin sensitivities to antigen E, hay-fever patients also have the normal type of antibodies for antigen E. These antibodies were

TABLE III
THE DECREASE OF SKIN-TEST ACTIVITY OF RAGWEED-POLLEN EXTRACT AFTER PRECIPITATION OF ANTIGEN E WITH RABBIT ANTISERUM

Patients	Precipitated with Serum C (1)[a]	Precipitated with Serum C (2)	Precipitated with Serum D (1)	Precipitated with Serum D (2)	Geometric Mean of Tests
HF	10	10			10
AM	10	100		100	47
ST[b]	10	10		10	10
HP	10	100		100	47
BF[c]		100	1000	1000	470
RC		1000	10	10	47
Y		100	10	100	47
K		10	100	10	22
			Geometric Mean of Tests on All Patients		40

[a] The values given represent the *n*-fold decrease of activity in the region of antibody excess when compared to that in the region of antigen excess. Values in columns (1) and (2) are tests made on different dates. [b] Patient ST has been immunized with fraction IV-C for one season. [c] The complete set of data from patient BF is illustrated in Fig. 8.

detected by the hemagglutination technique with tanned red cells. In Table IV are given the results obtained with sera from forty-seven previously untreated hay-fever patients and control sera from forty patients with other diseases. High titers were found only with sera from ragweed-sensitive patients.

DISCUSSION

The separation of the allergenic activity in ragweed pollen into a highly active fraction and less active fractions was described in our previous work. Studies by other workers (Callaghan and Goldfarb, 1961, 1962; Robbins *et al.*, 1963) have also shown the presence of a

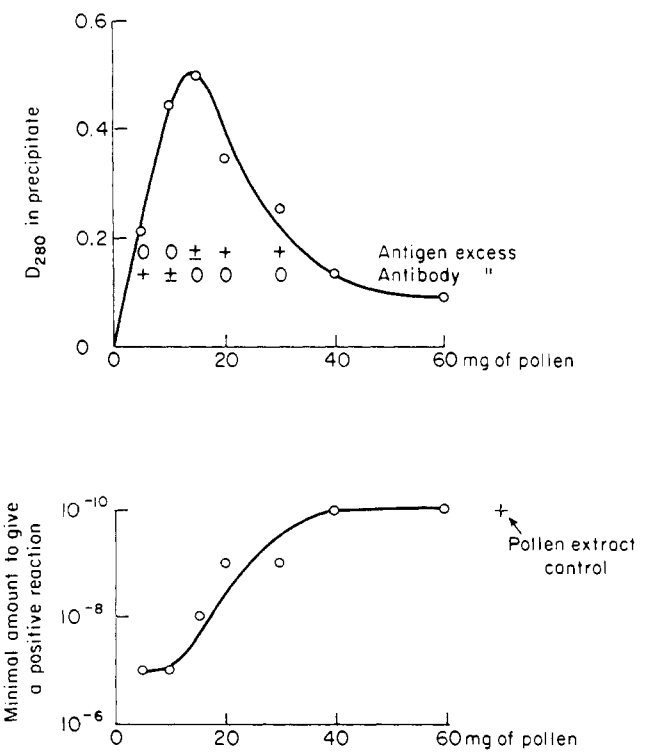

FIG. 8.—Precipitin analysis of ragweed-pollen extract with a specific rabbit serum in liquid media. (A) The precipitin curve of pollen extract. The antiserum No. D was concentrated 2-fold before use as given in the legend of Fig. 5. (B) Direct skin tests of the supernatants from Fig. 7A

TABLE IV
HEMAGGLUTINATION TITERS OF HUMAN SERA TO FRACTION IV

Titer	Number of Patients: Hay Fever	Number of Patients: Other Diseases
0	0	16
1/2	0	18
1/4	2	6
1/8	3	0
1/16	10	0
1/32	8	0
1/64	13	0
1/128	10	0
1/256	1	0

most active fraction in ragweed pollen. A main objective in all these studies is to determine to what extent the most active fraction represents the original activity in pollen. The question is of importance as it will aid in determining the total number of allergens in ragweed pollen. In previous studies a direct answer to the question was not forthcoming due to the difficulties in allergen assay by direct skin test. This objective is now fulfilled as a result of the identification of the predominant allergen as antigen E and the preparation of rabbit antisera specific for this antigen.

Antigen E has been isolated in four chemical forms from extracts of ragweed pollen. The two principal forms have been sufficiently characterized to be classified as distinct proteins. The chemical relationship of these forms is not known for they have identical molecular weights and amino acid compositions but they differ in their charges. The two minor forms are chemically related to the principal forms and they are probably artifacts formed during isolation.

There are three lines of evidence for the identity of the predominant allergen as antigen E. First, the allergenic activity of a fraction invariably rises with the increasing content of antigen E at each stage of purification (Table I and Fig. 9). Second, a parallel decrease of the allergenic activity and antigen E was found on acid and alkali inactivations of fraction IV (Fig. 7). Third, the allergenic activity of fraction IV-B or -C was decreased 10,000-fold after precipitation with rabbit antiserum specific for antigen E (Fig. 5).

With the identity of the predominant allergen as antigen E established, an alternative approach becomes available to determine the contribution of the activity in the purified product to that in ragweed pollen. The approach is to determine the decrease in the allergenic activity of pollen extract after precipitation of its antigen E with a specific rabbit antiserum.

When pollen extract neutralized for its antigen E was tested on eight sensitive patients, the decrease in activity was found to range from 10- to 1000-fold (Table III). The geometric mean of the tests in eight patients is a 40-fold decrease. These results therefore indicate that antigen E constitutes at least 90% of the total activity in pollen. The decrease in the activity of neutralized pollen extract is less than that found with neutralized preparations of fraction IV-B or -C. This difference is most probably due to the activities of the minor allergens in pollen extract. The presence of minor allergens was suggested in our previous work though their contribution to the total activity was not known. The results therefore place an upper limit of 10% on their possible contribution of the activity in pollen.

The findings described above are of interest, as antigen E accounts for about 6% of the proteins in

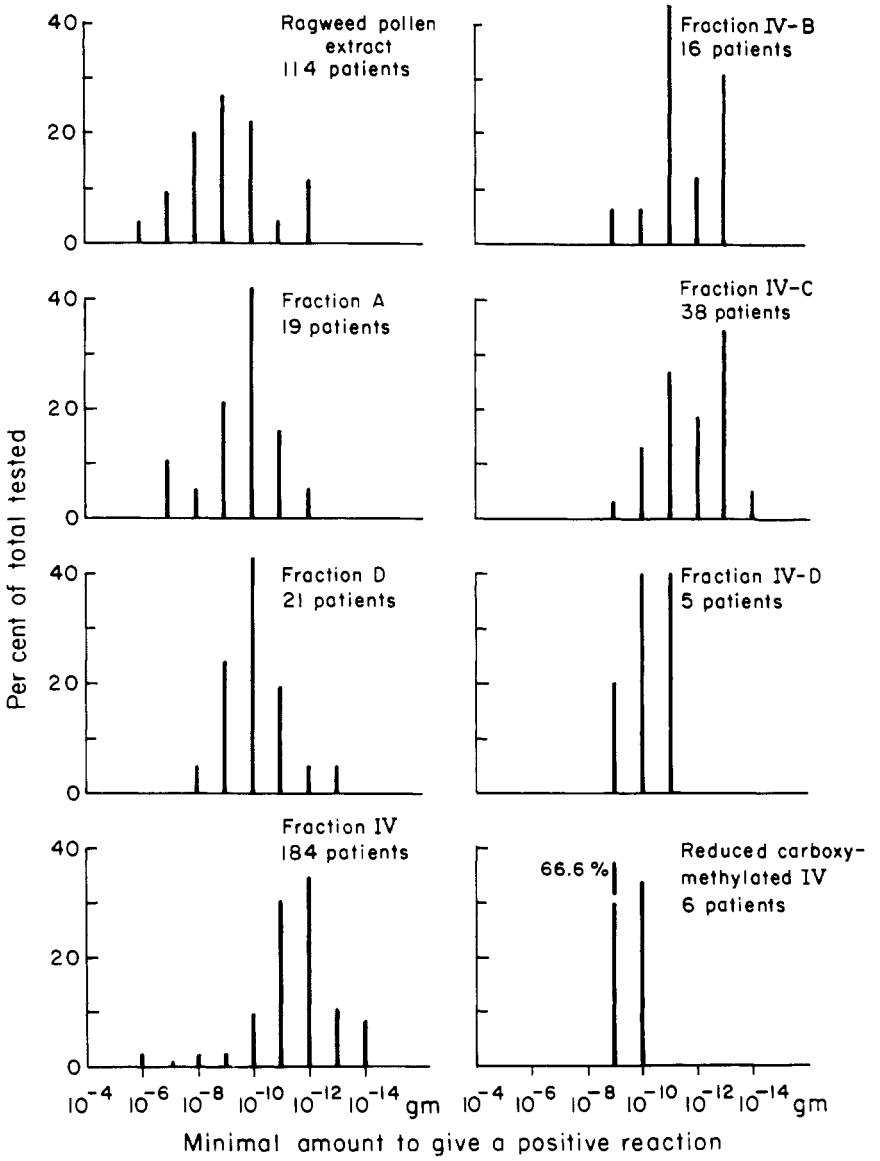

FIG. 9.—The distribution of allergenic activities of several fractions isolated from ragweed pollen. These direct skin tests were carried out in the cities of Baltimore and New York during 1961–63. Allergists routinely standardize pollen extract on the basis of protein nitrogen unit (PNU), one PNU being defined as 10^{-8} g of protein nitrogen. For pollen extract, 7.7×10^{-7} g is equal to 1 PNU and for fraction IV, 6.2×10^{-8} g is equal to 1 PNU.

ragweed pollen yet it constitutes at least 90% of the total activity. These findings warrant further chemical investigations of antigen E. Preliminary studies showed that this protein is resistant toward digestion by trypsin, chymotrypsin, and papain, and that its reduced and carboxymethylated derivative contains about 0.01 of the original activity (Fig. 9).

There are several other attempts on the characterizations of air-borne allergens from rye, timothy, and cocklesfoot grass pollens (Johnson *et al.*, 1958; Malley *et al.*, 1962; Augustin and Hayward, 1962), horse dander (Stanworth, 1957), and mold (Palmstierna *et al.*, 1963). Although not enough chemical data of these allergens are known for comparison, it is of interest to note that they are all reported to be acidic proteins with molecular weights greater than 10,000.

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