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Ragweed extract (RW) and antigens E, K, BPA-R and Ra₃ were characterized by electrophoresis on starch and polyacrylamide gels. RW extract and antigen E were also subjected to crossed immunoelectrophoresis. All the antigens contained isomers and contaminating proteins. RW separated by starch gel electrophoresis was skin tested and allergens unrelated to antigens E, BPA-R, Ra₃ and K were found.

THE CHARACTERIZATION OF SOME OF THE ANTIGENS AND ALLERGENS IN RAGWEED POLLEN

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Introduction

THE PURPOSE OF THIS STUDY was to evaluate the use of starch gel electrophoresis (SGE) and polyacrylamide gel electrophoresis (PAGE) as methods for separating and characterizing the proteins in ragweed (RW) extracts and purified antigen preparations and to determine the antigenicity and allergenicity of the proteins after separation.

The protein composition of aqueous extracts of RW pollen has been the topic of many investigations concerned with the isolation of proteins responsible for RW hypersensitivities. Several of the RW allergens have been isolated by ion exchange chromatography, which separates proteins on the basis of their ionic charge, and by gel filtration, which separates proteins according to their molecular size. However, the purity of the RW allergens, antigens E, K, Ra₁, Ra₅ and BPA-R has been determined primarily by immunodiffusion techniques which vary with the specificity of the antiserum.¹ Lapkoff and Goodfriend² detected five protein antigens in RW extract whereas Gussoni³ observed 14 antigens by immunodiffusion with RW antiserum. These variations in immunoassay results were also found in our laboratory. Some rabbits made antiserum which produced seven or eight immunoprecipitin bands while other rabbits produced up to 33 immunoprecipitin bands with the same immunization protocol and antigen preparation.

Marsh et al⁴ demonstrated that SGE was a good method for separating the allergens in the extracts of seven grass pollens and that skin tests could be

performed with pieces cut from the starch gels. The procedures of Marsh et al⁴ were modified to obtain the optimum separation of the RW proteins. The purified antigens were analyzed by SGE and PAGE and differences were noted in the various preparations which could not be detected by the immunologic methods used.

Materials and Methods

Pollen Extracts

Antigen E samples were gifts kindly supplied by Dr. T.P. King of the Rockefeller University, Dr. H.S. Sievert of the Abbott Laboratories, Mr. Jack Center of the Center Laboratories and Dr. C.H. Kirkpatrick of the National Institutes of Health. Antigens K and Ra₃ samples were obtained from Dr. C.H. Kirkpatrick and the Worthington Biochemical Corp. Antigen BPA-R samples were gifts from Dr. B. Griffiths, Laboratory Centre for Disease Control, Ottawa, Canada.

Undeffatted RW pollen (*Ambrosia elatior*), Lot No. Rm. 6600001, RA-009985, was purchased from Miles Laboratory. Fraction A was prepared by the methods of King et al^{5,6} with minor modifications. This fraction, which contained no sulfate in spot tests with Ba(OH)₂, was concentrated by lyophilization to 45.8 mg protein/ml.

The (RW) extract used to immunize rabbits was prepared from 80 g of the pollen used above. The pollen was defatted seven times with 500 ml ethyl ether, air dried and stirred overnight at 4°C in 400 ml of saline. The mixture was filtered through Whatman No. 1 and washed with six 50 ml aliquots of water. The final extract was centrifuged at 12,000 g for 15 minutes at 4°C and lyophilized to a final volume of 100 ml and 28.1 mg protein/ml. The extract was sterilized through a 0.45 micron filter and stored frozen at -20°C until used.

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Rabbit Immunization

Emulsions were prepared with phosphate buffered saline (PBS) and Freund's adjuvant as suggested by Crowle and Jarrett.⁷ The emulsions consisted of 1.5 ml of PBS, the antigen (6 mg of RW extract, 0.15 mg of Antigen E and BPA-R), 1.5 ml of Freund's adjuvant (Difco) and 3.0 ml of mineral oil (viscosity 185-195). The mixture was sonicated for three minutes in an ice bath to produce an emulsion which was stable for several weeks. Complete Freund's adjuvant was used for the first injections. All subsequent injections were with incomplete Freund's adjuvant. Each rabbit was injected with 2.0 ml of emulsion using 0.5 ml in the toe and foot pads, 0.5 ml intramuscularly into each hind leg and 0.5 ml subcutaneously at several places in the back. The animals were bled after three weeks and given booster injections at monthly intervals. One week after each booster injection 50 ml of blood was taken from the orbital plexus.

The serum was tested by immunoelectrophoresis (IEP) with fraction A and once the desired titer and specificity were obtained the animals were bled weekly for four weeks. The animals were rested one month between each of four bleedings.

Protein Determination

Total protein was determined by the Lowry⁸ modification of the Folin-Ciocalteu method.

Immunoelectrophoresis and Immunodiffusion

IEP was performed by a modification of the micro slide technique of Scheidegger⁹ as described by Gelman.¹⁰ The agarose gel was composed of 2 g of agarose (Marine Colloids, Inc.), 4 g polyethylene glycol (M.W. 6000-7500), 20 ml 0.42 M Tris-HCl buffer pH 8.7, 4 ml 1M Na₂CO₃ and 170 ml of water. Polyethylene glycol was used to increase the sensitivity of the precipitin reaction.¹¹ The electrode chamber contained 0.42 M Tris-HCl buffer pH 8.7. Electrophoresis was for 45 minutes at 10 ma per frame. Micro-double diffusion tests were performed in the above agarose gel by the Ouchterlony procedure as described by Gelman.¹⁰ The antigens and antisera were allowed to diffuse in a humidity chamber at room temperature for one to two days before examination. Staining was with amido black.¹⁰

In crossed IEP the proteins were first separated by electrophoresis in agarose gel in one dimension. A strip containing the separated proteins was removed from the first gel, placed onto an antibody containing agarose gel and again electrophoresed at 90° to the first dimension. The procedure of Laurell¹² was modified to utilize the Metaloglass Model 92-1 apparatus.¹³ The protein separation gel was prepared by dissolving agarose to a concentration of 1% in a barbital buffer containing 1.86 mM calcium lactate, pH 8.6, 0.1 ionic strength. This solution was poured

between two clamped plates separated on three sides by a 1/16" plastic spacer. One of the glass plates was precoated with 0.3% adhesive agar and dried before use. The gel area was 7" x 3 3/4" x 1/16". After the agarose had gelled completely, the glass plates were separated and a trough 2 cm x 1 mm was cut in the center of the gel perpendicular to the direction of electrophoresis. Ten microliters (0.45 mg) of Fraction A containing bromphenol blue was pipetted into the trough and the gel was electrophoresed for one hour at 90V, 72 ma on a water cooled (15°C) plate. After electrophoresis in one dimension a 0.5 x 9 cm strip of agarose containing the separated proteins was removed from the first gel and placed in the center of the antibody containing agarose plate. The antibody gel plate was prepared in the same manner as the protein separation plate except that the gel which contained 1.5% agarose and 2% PEG was heated, cooled to 50°C and mixed 10:1 with 50°C RW antiserum. The proteins were electrophoresed into the antibody gel in the second dimension for nine hours at 80V, 75 ma. The gel was covered with six to eight sheets of filter paper, weighted with glass plates for 15 minutes to remove excess buffer and then soaked overnight in 1.5% NaCl. The gels were washed in water and stained with amido black.

The crossed IEP of antigen E (A601-901-185) was performed after separation of the isomers of antigen E by PAGE. The polyacrylamide gel was placed onto the antibody containing agarose gel and electrophoresed as above.

Starch Gel Electrophoresis (SGE)

Horizontal SGE was performed by a modification of a technique employed by Smithies¹⁴ and Marsh et al.⁴ The gel and electrolyte buffers were 0.042 M and 0.42 M Tris-HCl, respectively. The buffer pH was 8.7 at 25°C and 9.3 at 4°C. Each gel contained 35 g of starch (Connaught Medical Research Laboratories) and 250 ml of 0.042 buffer. The hot starch was degassed under vacuum and poured into a vaseline sealed plexiglass frame placed on a glass plate. The hot starch was then carefully covered with a glass plate to extrude the excess starch and surface air bubbles. After hardening overnight at room temperature, the gel frames were removed to give a gel size of 214 x 125 x 6 mm. Fraction A and/or purified antigen samples were absorbed onto 20 x 6 mm paper wicks (Beckman Instruments, Inc., paper No. 319329) which were inserted 1 cm apart into cuts made across the gel 10 cm from the cathodic end. Bromphenol blue was added to each sample or to a separate wick to follow the electrophoretic migration. The electrophoresis was performed at 4°C. Electrophoresis was begun at 150V, 25 ma for one hour at which time the sample wicks were removed. The electrophoresis was then continued at 250V, 40 ma until the bromphenol blue moved to the end of the

gel, usually six and a half hours. The gel was sliced horizontally into two 3 mm halves. One half was stained for protein according to Smithies¹⁴ and the other half was used for skin tests and immunologic studies.

Polyacrylamide Gel Electrophoresis (PAGE)

A 7% separating gel prepared according to the pH 9.5 procedure of Davis¹⁵ was used for the separation of both the acidic and basic proteins in the RW extract and antigen preparations. The quantity of protein layered onto the stacking gels is indicated in the legend of Figure 3. The electrophoresis was performed at 2 ma per tube. The anodic run was for 80 minutes and the cathodic run was for 265 minutes. The cathodic run was performed in five-inch tubes containing 1.5 ml of separating gel. The gels were

stained with amido black and destained in 7% acetic acid in a Canalcro destainer.

Densitometry

The relative densities and positions of the protein bands were determined with a Joyce Loebel Chromoscan densitometer. The starch gels were scanned with a 0.5 x 0.5 mm slit by the optical reflectance method using a 595 nm complementary filter, a 1.0 OD wedge and an A cam. The specimen was scanned at a 3:1 record ratio and the areas under the curve were automatically integrated. The precipitin bands obtained by IEP and crossed IEP were visually counted at 4 times magnification.

Skin Tests

Fraction A was sterilized by filtration through a 0.45 micron Millipore filter and the proteins were



Figure 1. Antigenic proteins in RW fraction A as determined by reaction with RW pollen antiserum. The gel composition and electrophoresis conditions are described in the methods section. A, Immunoelectrophoresis. The wells contained 2 μ l (90 μ g) of fraction A and the troughs contained 50 μ l of RW antiserum. The anode was on the right. B, Crossed immunoelectrophoresis. The anode was on the right during the first electrophoresis and on the top during the second electrophoresis.

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separated by SGE. The gels were cut horizontally into 1 cm strips immediately after the electrophoresis. There were 10 cathodic and 11 anodic segments and each segment was cut into pieces 10 x 5 x 3 mm and stored at 4°C in a Saran wrap covered plate until used either for skin tests or immunodiffusion studies to locate the major antigens. Skin tests were performed on the backs of RW sensitive patients. The back was numbered with a ballpoint pen and a drop of glycerine saline, 1:1, was placed beside each number. The skin was scratched under each drop with a sterile disposable lancet and a starch gel segment was placed on the scratch. Scratches which drew blood were not used and the tests were done at another location on the back. Fraction A 10,000 PNU/ml, antigen E (BB-IND-134) 10,000 PNU/ml and two empty virgin starch gel pieces were tested on each patient in addition to the 21 starch gel segments. The gels were removed after 30 minutes and the glycerine saline

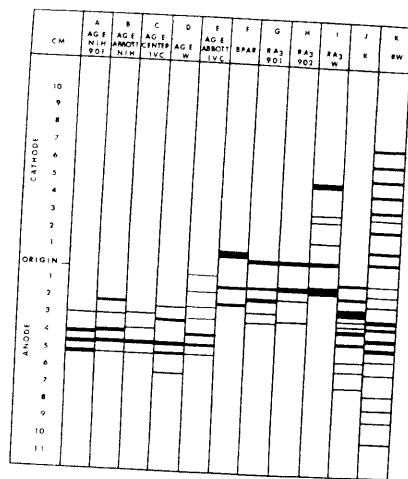


Figure 2. Characterization of the proteins in preparations of antigens E, Ra₃, BPA-R and K by starch gel electrophoresis. The antigen solutions (approximately 0.1 ml) were absorbed onto 6x10 mm paper wicks which were inserted into the starch gels before electrophoresis. The concentration of the antigen solution used in each case is indicated below.

- A. Antigen E, NIH catalog No. A-601-901-185, 5 mg/ml.
- B. Antigen E, Abbott No. 8100, Lot No. 795-8996, NIH-BB-IND 134, 1 mg dried onto the wick.
- C. Antigen E, Center IVc, Lot 00410, 1 mg/ml.
- D. Antigen E, Worthington AGEE 55C495, 5 mg/ml.
- E. Antigen E, Abbott IVc, 1 mg/ml.
- F. Antigen BPA-R, 1.2 mg/ml.
- G. Antigen Ra₃, NIH Catalog No. A-601-901-185, 5 mg/ml.
- H. Antigen Ra₃, NIH Catalog No. A-601-902-185, 5 mg/ml.
- I. Antigen Ra₃, Worthington anti R 535304, 5 mg/ml.
- J. Antigen K, Worthington anti K, 2 mg/ml.
- K. RW extract, fraction A, 45.8 mg/ml.

was blotted dry. A circle was drawn around the wheal and flare with a ballpoint pen. A permanent record of the reactions was made by covering the circles and numbers with a translucent Scotch tape. The wheal and flare reaction of ragweed fraction A was classified as 4+ and the empty starch gel controls were classified as zero. The reactions between zero and 4+ were determined as follows: 2+ - a wheal (with or without erythema) 5 mm in diameter greater than the control starch gel; 3+ - a wheal (with or without erythema) 6 to 10 mm in diameter greater than the control starch gel; 4+ - any wheal greater than 10 mm or equal to the size of the fraction A with or without pseudopods. A 2+ or better response was considered positive.

Results

Proteins in Fraction A

The electrophoretic properties of the RW pollen antigens were investigated by IEP and crossed IEP (Figure 1). Twenty-five bands were detected on the IEP slide. The proteins were more easily seen by crossed IEP where 18 cathodic and 15 anodic bands were counted. Most of the cathodic proteins moved further than the anodic proteins in the antibody containing gel indicating that the antiserum had a lower titer for the basic proteins than for the acidic proteins. Fraction A was separated by PAGE and SGE into 13 anodic and 9 cathodic bands (Figures 2 and 3). The basic protein in cathode segment 7 stained more intensely than the other proteins with amido black stain but then decolorized and after two hours was completely destained. A densitometry scan of a RW starch gel showed that 76% of the total protein in RW extract migrated anodically at pH 9.3. A densitometric scan of a RW polyacrylamide gel showed 78% of the total RW protein on the anode at pH 9.5.

Antigens E, BPA-R, Ra₃ and K

The purified antigen preparations were analyzed by IEP with RW antiserum. The number of precipitin bands produced by each of the antigen preparations is shown in Table I. When the antigen preparations were used as immunogens the antisera formed additional precipitin bands not seen by reaction of the antigens with RW antiserum (Table I). Considerable rabbit variation also occurred in the production of antiserum. For example one rabbit immunized with antigen E (A601-901-185) produced an antiserum which produced only one band with antigen E and with RW extract. Other rabbits produced three or more precipitin bands with RW extract, indicating that the antigen E preparation contained low concentrations of non antigen E proteins which were immunogenic in some rabbits but not others.

The antigen preparations were fractionated by SGE and PAGE. The results in Figures 2 and 3 show

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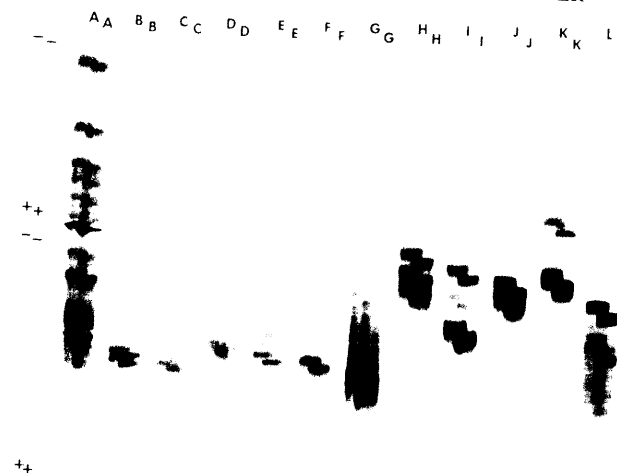


Figure 3. Characterization of the proteins in preparations of antigen E, Ra₃, BPA-R and K by polyacrylamide gel electrophoresis. The anodic gels are on the bottom and the cathodic gels are on the top. The quantity of antigen placed on the stacking gel of each run is indicated below.

	Anode (ug)	Cathode (ug)
A. Fraction A	458	1909
B. Antigen E, A-601-901-185	50	208

C. Antigen E, A-601-902-185	50	208
D. Antigen E, Abbott 8100	12.5	83
E. Antigen E, Center 00410	50	100
F. Antigen E, Worthington	50	250
G. Antigen E, Abbott IVc	100	200
H. Antigen BPA-R	60	60
I. Antigen Ra ₃ , A-601-901-185	125	500
J. Antigen Ra ₃ , A-601-902-185	100	100
K. Antigen Ra ₃ , Worthington	125	125
L. Antigen K, Worthington	75	500

that the protein separation and the number of bands produced by each of the antigen preparations were essentially the same by the two methods. The antigen preparations varied from batch to batch and showed major differences in the number of proteins and the location of the major bands. Some of the antigen E preparations produced four protein bands whereas

other preparations produced five, six and 10 bands. The 901 and Abbott 8100 antigen E preparations appear to contain both the B and C forms of antigen E whereas the other preparations contain mainly the C form of antigen E and other antigens.¹⁶

A study was performed to determine if the four protein bands seen after the PAGE of antigen E

TABLE I. NUMBER OF ANTIGENS PRESENT IN PURIFIED ANTIGEN PREPARATIONS.

Antigen	Source	IEP	
		Antigen with RW antiserum	RW extract with antiserum to specific antigen
Antigen E	A601-901-185	3	
	A601-902-185	3	4
	Abbott 8100	1	
	Worthington	2	
	King IVc	2	
Antigen Ra ₃	King IVc	1	
	Abbott IVc	3	
	Center IVc	2	4
	A601 901-185	2	1
	A601-902-185	3	
Antigen K	Worthington	2	
	Worthington	3	
Antigen BPA-R	Griffiths	1	4
			4

(A601-901-185) were due to the antigenically different proteins detected by IEP (Table I) or to the antigenically similar proteins described by King.^{16,17} The bands cut from an unstained antigen E gel were compared with one another in a double diffusion test with antigen E antiserum. A line of identity was produced by the major protein in each of the four PAGE bands, thus confirming the results of King^{16,17} who previously reported on the isomers of antigen E. A similar double diffusion study was performed with the PAGE bands cut from BPA-R and Ra₃ (A-601-901-185) gels. With BPA-R antiserum the BPA-R bands each contained two proteins and the major protein was identical in all four segments. RW antiserum produced only one band with a line of identity in all four Ra₃ segments. King¹⁶ found that antigen E occurred in several molecular sizes and the isomers in BPA-R and Ra₃ preparations also may be due to aggregates of monomers.

In an attempt to confirm the isomeric nature of the antigen E preparation a polyacrylamide gel of the antigen E was analyzed by crossed IEP. The presence of electrophoretically different but antigenically related populations of molecules should produce coalescing loops whereas gradually varying heterogeneous antigen molecules should produce skewed loops.¹⁸

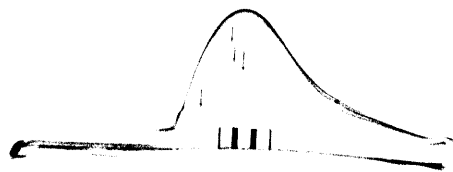


Figure 4. Crossed immunoelectrophoresis of antigen E. Antigen E, A601-901-185, was separated by PAGE as described in Figure 3. The unstained polyacrylamide gel was placed onto an agarose gel containing 10% RW antiserum and electrophoresed perpendicular to the first electrophoresis for nine hours at 75 ma. The anode was at the right for the PAGE and at the top in the agarose gel electrophoresis.

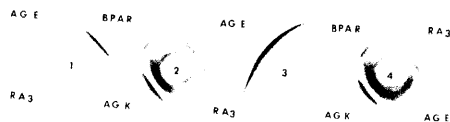


Figure 5. The immunologic relationships of antigens E, BPA-R, Ra₃ and K. The antigen concentrations were as follows: antigen K, 1 mg/ml; antigen Ra₃, A601-901-185, 0.05 mg/ml; antigen E, A601-901-185, 1.25 mg/ml; BPA-R 0.3 mg/ml. Antiserum to BPA-R was in well 1, RW antiserum was in wells 2 and 4, and antigen E antiserum was in well 3. Each well contained 8 μ l of solution.

The heavy precipitin loop shown in Figure 4 is made up of four bands which probably represent the combined locations of the antigen E bands on the polyacrylamide gel. The other six or seven loops are believed to be due to other proteins in the antigen preparation.

The proteins in the Ra₃ and BPA-R preparations migrate differently by SGE than by PAGE. By SGE all three Ra₃ preparations and the BPA-R preparation had a major band in the first cathode segment. However, by PAGE the only preparation with major cathodic proteins was the Worthington Ra₃ preparation. This difference may be due to a change in the running pH of the starch gels since a warming of the starch gel during the run would decrease the pH and cause the anodic proteins to move toward the cathode.

Several of the antigen preparations contained proteins which migrated the same distance in the polyacrylamide gels. To determine whether these proteins were antigenically related, double diffusion tests with antiserum specific for RW, antigen E and BPA-R were used with antigens E, BPA-R, Ra₃ and K (Figure 5). Because the immunologic relationships are crucial to suggestions for clinical use of antigens, some of the patterns will be reviewed. The reactions around well 1 show that the BPA-R antiserum is not monospecific since it reacted with antigens BPA-R, E and K but not Ra₃. There is a faint extension of the band between anti-BPA-R and BPA-R as well as an apparent joining of this band with that found by antigen E and anti-BPA-R. This apparent spur could be interpreted as a partial identity. The bands produced between antigen K and anti BPA-R are too weak to interpret. However, from well 4 it is obvious that the K preparation contains a small amount of BPA-R which produces a line of identity with the major BPA-R band. Well 2 shows the extension of the BPA-R band to be inhibited by the antigen E band, suggesting the presence of common antigen determinants between BPA-R and antigen E. Ra₃ is a major contaminant in the antigen K preparation but appears to be unrelated to antigen E. The reactions around well 3 indicate that antigen E is distinct from Ra₃, K and BPA-R. The weak band formed with BPA-R may be due to a small amount of antigen E or some other antigen in the BPA-R preparation. Well 4 shows that Ra₃ is not related to either BPA-R or antigen E. The antigen K preparation appears to contain Ra₃, BPA-R and E, but the major band in antigen K is unrelated to the BPA-R and Ra₃ bands. Antigens K and E, however, appear to share common antigen determinants with RW antiserum but not with antiserum to antigen E. A weak line of partial identity was observed in Figure 5 well 1 between the BPA-R antiserum, BPA-R and antigen E (1.25 mg/ml). The weak band between antigen E and the BPA-R antiserum did not occur when antigen E was diluted to

200 μ g/ml, the concentration used by Griffiths.¹⁹ If antigens E and BPA-R share a common polypeptide chain with similar dimensions to the heavy chain of antigen E,²⁰ one would anticipate a stronger immunologic relationship than shown in Figure 5. Also, absorption of the BPA-R antiserum with antigen E should remove the BPA-R globulins from the antiserum. The results in Figure 6 show that the reaction of BPA-R antiserum with antigen E is limited to a common minor protein in the antigen E and BPA-R preparations. When the BPA-R antiserum was absorbed 1:1 with antigen E at 200 μ g/ml and 100 μ g/ml only two closely associated bands were formed and neither band contained proteins in common with antigen E. The antigen E and BPA-R bands crossed in a pattern of nonidentity. A similar pattern of non-identity between antigen E and BPA-R was observed when fraction A (750 μ g/ml) was substituted for the BPA-R in Figure 6.

Localizations of Antigens E, Ra₃ and BPA-R in the Starch Gels Used for Skin Tests

The locations of antigens E, Ra₃ and BPA-R in the starch gel segments used for skin testing were determined by immunodiffusion tests so that the role of these allergens as well as other RW allergens could be assessed.

Fraction A was subjected to SGE and the gels were cut into 1 cm sections. Numbering was from the origin to the anode A₁ to A₁₁ and from the origin to the cathode C₁ to C₁₀. With antigen E antiserum the reference antigen E for the skin tests (BB-IND-134) identifies with proteins in segments A₄, A₅ and A₆ (Figure 7A). No antigen E was found in the cathodic segments. Unlike antigen E, the BPA-R and Ra₃ antigens appear to have components which migrated only in the first two segments on each side of the origin in the A₁, A₂, C₁ and C₂ segments of the starch gels. The Ra₃ and BPA-R bands cut from polyacrylamide gels were tested with the Ra₃ and BPA-R starch gel segments to determine if differences in the isomers could be detected with the SGE system. The BPA-R antiserum was not monospecific and reacted with three antigens in the BPA-R preparation (Figure 7B). The BPA-R (A) band closest to the antiserum well crossed the BPA-R band in A₁ in a pattern of nonidentity and produced a line of identity with a protein in C₁ and C₂. Therefore the protein nearest the antiserum well occurs only in the C₁ and C₂ segments whereas the middle band, the major BPA-R band, occurs in both the cathode segments C₁ and C₂ and the anode segments A₁ and A₂. The third band nearest the BPA-R (A) antigen well does not occur in segments A₁, A₂, C₁ and C₂.

The results in Figure 7C show no detectable differences in the isomers. Ra₃ (A) identified with the same protein in A₂ as Ra₃ (B) and Ra₃ (C) identified with the same protein in C₁ and C₂ as Ra₃ (B). An-

tigen Ra₃ is one of the major antigens in segment C₁ and a minor antigen in C₂ and A₂ (Figure 7C). Other studies showed that there was no detectable Ra₃ band in segment A₁ even though this segment did cause a deflection in the Ra₃ reference bands. The Ra₃ antigen appears to be pure in all of the PAGE segments

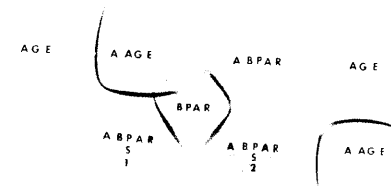


Figure 6. The antigenic relationship of antigens E and BPA-R. Antigen E, A601-901-185, 0.4 mg/ml and BPA-R, 0.3 mg/ml were used. Well 1 contained BPA-R antiserum sorbed 1:1 with AgE. A601-901-185, 100 μ g/ml. Well 2 contained BPA-R antiserum sorbed 1:1 with AgE. A601-901-185, 200 μ g/ml.

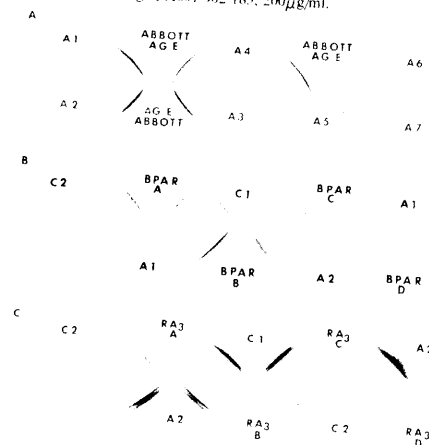


Figure 7. The location of antigens E, Ra₃ and BPA-R in the starch gel segments. Fraction A was separated by SGE as described in the methods section. The starch gels were cut into 1 cm² pieces from the origin to the anode and cathode. Each piece was mashed and a portion added to the indicated outer wells. The location either anode or cathode and the distance from the origin is indicated. The antigen E NIH catalog No. Abbott 8100, antigen E NIH A601-901-185, BPA-R and Ra₃ A604-901-185 concentrations were 0, 5, 1.25, 0.3 mg/ml and 0.2 mg/ml respectively. A. The location of antigen E. Antigen E antiserum was in the center wells. B. The location of antigen BPA-R proteins. Antigen BPA-R antiserum is in the center wells. The antigen BPA-R bands, A, B, C and D were cut from an unstained polyacrylamide gel. The polyacrylamide and starch gel segments were in the antigen wells. C. The location of antigen Ra₃ proteins. RW antiserum was in the center wells. The antigen Ra₃ bands A, B, C and D were cut from unstained polyacrylamide gels. The polyacrylamide and starch gel segments were in the antigen wells.

except Ra₃ (C) which contains two bands. The Ra₃ preparation also contained two proteins by IEP.

Skin tests using the 21 starch gel segments were performed on 46 ragweed sensitive patients. The percentage of patients who gave a 2+ or better reaction to a given segment is shown in Figure 8. The results show that antigen E is a major antigen in RW pollen. Sixty-one percent of the patients reacted with the control antigen E sample and 74% with the A4 segment. The highest concentration of antigen E in the starch gels occurred in segment A4. Segments A1 and A2, which contained some of the minor proteins in the BPA-R and Ra₁ preparations, produced skin reactions in 52% of the patients. The cathodic segments 1 and 2, which contained the major portion of the Ra₃ and BPA-R proteins, produced skin responses in 30% and 20% of the patients, respectively. Two other areas in the starch gels contained active allergenic proteins. Anodic segments 7 to 9 produced positive skin reactions in 20 to 40% of the patients and contained two precipitin bands with RW antiserum. Cathodic segments 4 to 8 produced positive skin reactions in 30 to 50% of the patients and contained several precipitin bands with RW antiserum. The proteins in these segments have not been characterized.

Some of the patients responded to a few of the starch gel segments while other patients gave positive skin tests to several segments. Six of the patients responded only to the antigen E containing segments A₄ to A₆. Four of the patients gave negative reactions to antigen E and reacted to the following segments only: (1) A₉; (2) A₉ and C₅; (3) C₆, C₇ and C₈; (4) C₄, C₆ and C₇.

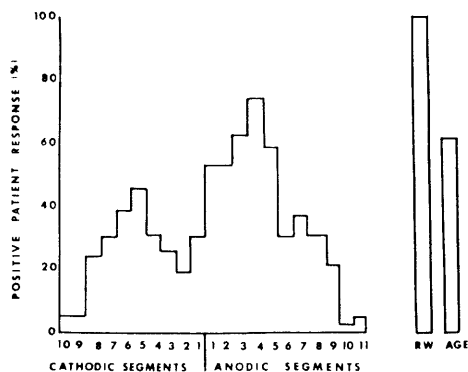


Figure 8. Skin test responses of ragweed sensitive patients to starch gel segments after electrophoresis of fraction A. The skin tests were performed as described in the methods section. Fraction A at 10,000 PNU/ml and antigen E (BB-IND-134) at 10,000 PNU/ml and two empty virgin starch gels were used as standards and controls for testing the 21 starch gel segments.

Discussion

Previously we reported that an extract of RW contained 23 electrophoretically different isozymes and that at least 12 of these enzymes were detectable in the presence of their substrates as separate immunoprecipitin bands.²¹ The present report shows that RW extract contains an additional 33 antigens. Prior to these studies RW pollen was reported to contain up to 14 antigens and only a portion of these proteins was believed to be allergenic.³

This study showed that the methods and antisera were suitable for defining the relationships of the known antigens and in addition could be used to localize the antigens in the starch gel segments. The Antigen E antiserum used in the immunodiffusion studies did not react with antigens K and Ra₃ and produced only one band with RW extract and the antigen E preparation (Figure 5). The anti-Antigen E reacted weakly with the BPA-R preparation and the anti-BPA-R reacted weakly with the antigen E preparation. Since the BPA-R and antigen E were demonstrated to be antigenically distinct (Figure 6), any reactions of apparent relation must be due to cross contamination. The obvious concentration and specificity of the antigen E antiserum used in Figure 5 and Figure 6 can only be interpreted to mean that K, Ra₃ and BPA-R are not related to antigen E. These results are not in accordance with those of King¹⁷ and Griffiths,¹⁹ who showed that antigen E shared common antigen determinants with both antigens K and BPA-R. The Ra₃ preparations were antigenically distinct from antigens BPA-R, E and K. The antigen K preparation was contaminated with antigens Ra₃, BPA-R and E but the major band is obviously different from any of these allergens (well 4, Figure 5). Underdown and Goodfriend²² in cross neutralization studies observed that antigens E and Ra₃ were related. However, the studies presented herein and those of Lichtenstein et al²³ show no cross reactivity between antigens E and Ra₃. Berrens²⁴ disagreed with the hypothesis that RW extract contains several allergens sharing determinants with antigen E and pointed out that shared antigenic determinants by two or more proteins or groups of proteins is just as fortuitous as it would be, for example, among human serum proteins. The coeluting loops on the anode side of the crossed IEP experiment, Figure 1, provide evidence for the presence of two electrophoretically different but antigenically related populations of molecules. These coeluting loops may be due to the presence of isomers, degradation products of a complex molecule or electrophoretically different molecules complexed with a common antigen.¹⁸ The antigenic relationships of the PAGE bands cut from the antigen E, Ra₃ and BPA-R gels show that all three antigen preparations contain isomers (Figures 7A, B and C). The antigen prepara-

tions also contain contaminants and 6 of the contaminants in antigen E are shown in Figure 4.

The PAGE and crossed IEP studies show that many of the RW antigens are present in low concentrations in the RW pollen. However, these minor antigens may be allergenic in some patients. Marsh et al²⁵ found that antigen Ra₅, one of the minor antigens in RW pollen, produced a marked IgE mediated sensitivity in 22% and 37% of the RW sensitive population in Baltimore and Belleville. The allergic response to the Ra₅ antigen was found to be closely associated with the possession of a major histocompatibility antigen of the HL-A7 cross reacting group comprising HL-A7, W-27, W-22, and W-10. This data suggests that the recognition of some of the minor antigens in RW pollen may be controlled by an immune response gene which is closely associated with the possession of an HL-A7 Creg antigen.

The locations of antigens E, K, BPA-R and Ra₃ in starch gels after electrophoresis were determined so that possible new allergens could be defined. All of the starch gel segments except C₂ produced a positive skin response in 20% or more of the 46 RW sensitive patients and more than 40% of these patients were sensitive to segments C₅, A₂, A₃, A₄ and A₅ (Figure 8). Several cathode segments gave a 2+ or greater response in a significant percentage of patients and these allergens were not immunologically related to the four allergens studied.

Antigen E occurred in starch gel segments A₄ and A₅ and these segments produced positive tests in 76% and 60% of the RW sensitive patients respectively. Segment A₄ may have contained an allergen besides antigen E since four patients reacted with this segment and gave no response to either the antigen E standard or the starch gel segment A₅. The BPA-R and Ra₃ preparations contained major antigens in segments C₁ and C₂ and minor antigens in segments A₁ and A₂ (Figures 7B and 7C). Segments A₁ and A₂, however, were much more allergenic than segments C₁ and C₂ and produced positive skin tests in 63% compared with 35% of the ragweed sensitive patients. This data suggests that the major antigens in the Ra₃ and BPA-R preparations are not the major allergens. However, some of the allergenicity of the A₁, A₂ and A₄ segments may have been due to antigen K. A new preparation of antigen K (A602-901-185) produced 1 major and 5 minor bands by PAGE. The major band was not as acidic as antigen E and localized in segments A₂, A₃ and A₄.

Lichtenstein et al²³ observed that antigen Ra₃ was allergenic in 16 of 23 antigen E sensitive patients and that in two Ra₃ sensitive patients the Ra₃ mediated histamine release from leukocytes was inhibited by antigen E antiserum but not by Ra₃ antiserum. One of the explanations given for the latter response was

the presence of a minor allergenic contaminant in the Ra₃ preparation which does not cross react with antigen E. The results shown in their paper, as well as those shown herein, gave no evidence that the antigen E antiserum reacted with contaminants in the Ra₃ preparation (Figure 5). However, our results agree with Lichtenstein's explanation since the Ra₃ preparation contained two antigens by IEP (Table I) and most of the rabbits immunized with antigen E produced antiserum which reacted with two or more proteins in antigen E.

The results obtained by SGE and PAGE were similar to the results obtained by Cebra²⁶ and McKaba and Norman²⁷ with starch blocks. The one block of Cebra²⁶ and the 2 blocks of McKaba and Norman²⁷ contained 77%, 76% and 63% acidic proteins respectively. The amido black stained starch and polyacrylamide gels used herein contained 76% and 78% acidic proteins respectively. Skin tests performed with starch gels and starch block eluates²⁷ were also similar: positive skin responses were obtained with an eluate from the first Folin peak; RW patients were more sensitive to the anodic proteins than to the basic proteins; some of the patients were sensitive to several of the starch block or gel samples; differences in patient responses to segments from different locations in the starch blocks and gels suggested the presence of different allergens. Four of the patients in the starch gel study failed to react with antigen E or the antigen E containing segments but did respond to other segments. Melam, Pruzansky and Patterson²⁸ reported that four of 12 RW sensitive patients reacted to crude RW extract but not to antigen E. Norman and Lichtenstein²⁹ presented data which showed that some patients may be sensitive to other proteins in RW extract without being sensitive to antigen E but these patients are relatively insensitive to whole pollen extract. While skin sensitivity and clinical sensitivity are not synonymous, the data presented herein give pause to the economically unsound suggestion of treating atopic patients with pure antigens. This study indicates the probability that in RW sensitivity, antigens other than antigen E are clinically relevant in many patients.

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Nature will force her way, and if you try to stifle her by drowning,
 she comes up, not the fairest part of her uppermost.
 George Meredith (from *Diana of the Crossways*)