

Studies on *Alternaria* allergens

III. Isolation of a major allergenic fraction (ALT-I)

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The allergens present in crude Alternaria alternata extract were fractionated by sequential ammonium sulfate precipitation, DEAE cellulose ion-exchange chromatography, preparative flat-bed electrofocusing, and Sephadex G-100 gel filtration. The carbohydrate-rich fraction isolated (Alt-I) was heterogeneous by polyacrylamide gel disc electrophoresis and by thin-layer electrofocusing. By preparative polyacrylamide gel electrophoresis the Alt-I fraction could be further separated into at least five subfractions, all of which contained similar allergenic activity. Significant elevations in IgE antibody to Alt-I were found in 21/25 patients with asthma having positive skin tests to crude Alternaria extract but were not found in 15 patients with non-IgE-mediated asthma. Alt-I appears to contain glycoproteins (7% N) and is a major allergenic fraction of Alternaria.

Alternaria is one of the more common atmospheric mold spores in the United States and a major etiologic agent for fungal-induced allergic rhinitis and extrinsic (IgE-mediated) asthma in man.¹ In a previous study we established radioallergosorbent test (RAST) procedures to identify *Alternaria* allergens in vitro² and demonstrated that major allergenic materials were eluted in void-volume fractions when crude *Alternaria* extracts were gel filtered through calibrated Sephadex G-25 columns. Subsequent studies indicated that commercially available *Alternaria* extracts varied widely in allergenic content and that allergenic

activity could not be correlated with the protein content of the extract.³ In the present study we describe the further isolation and partial characterization of a major *Alternaria* allergenic fraction we have designated as "Alt-I."

EXPERIMENTAL PROCEDURES

The experiments reported were performed with *Alternaria* extracts prepared in our laboratory from a single lot of commercial dry, defatted *Alternaria* powder (Lot M1-33, Greer Lab., Inc., Lenoir, N. C.). The source material for this lot was a strain of *Alternaria alternata* obtained from the American Type Culture Collection, Rockville, Maryland (No. 6663). The mold was cultured in Czapek's medium² supplemented with both maltose and dextrose (15 gm each/L) in place of sucrose and trypticase (10 gm/L). Stationary cultures (5-L flasks) were incubated at 20° to 25° C for 20 days; thimerosal was added to a final concentration of 1:10,000 (w/v) 1 day prior to harvest to kill the organism. Mold pellicles were isolated by filtration, homogenized in a Waring blender, and extracted ×3 in acetone to defat. The pellicles were filtered, air dried for 2 hr, oven dried at 37° C for 2 days, and then powdered by ball-milling for 2 hr.

DEAE cellulose (DE-52) was from Whatman, Ltd., and Sephadex G-75 and G-100 were from Pharmacia Fine Chemicals (Piscataway, N. J.). Spectrapor 3 casing (3,500 mw cutoff) was used for all dialysis steps and was from Spectrum Medical Industries, Inc. (Los Angeles, Calif.)

Flat-bed preparative electrofocusing (PEF) was performed using Sephadex G-75 as the support⁴ with a pH

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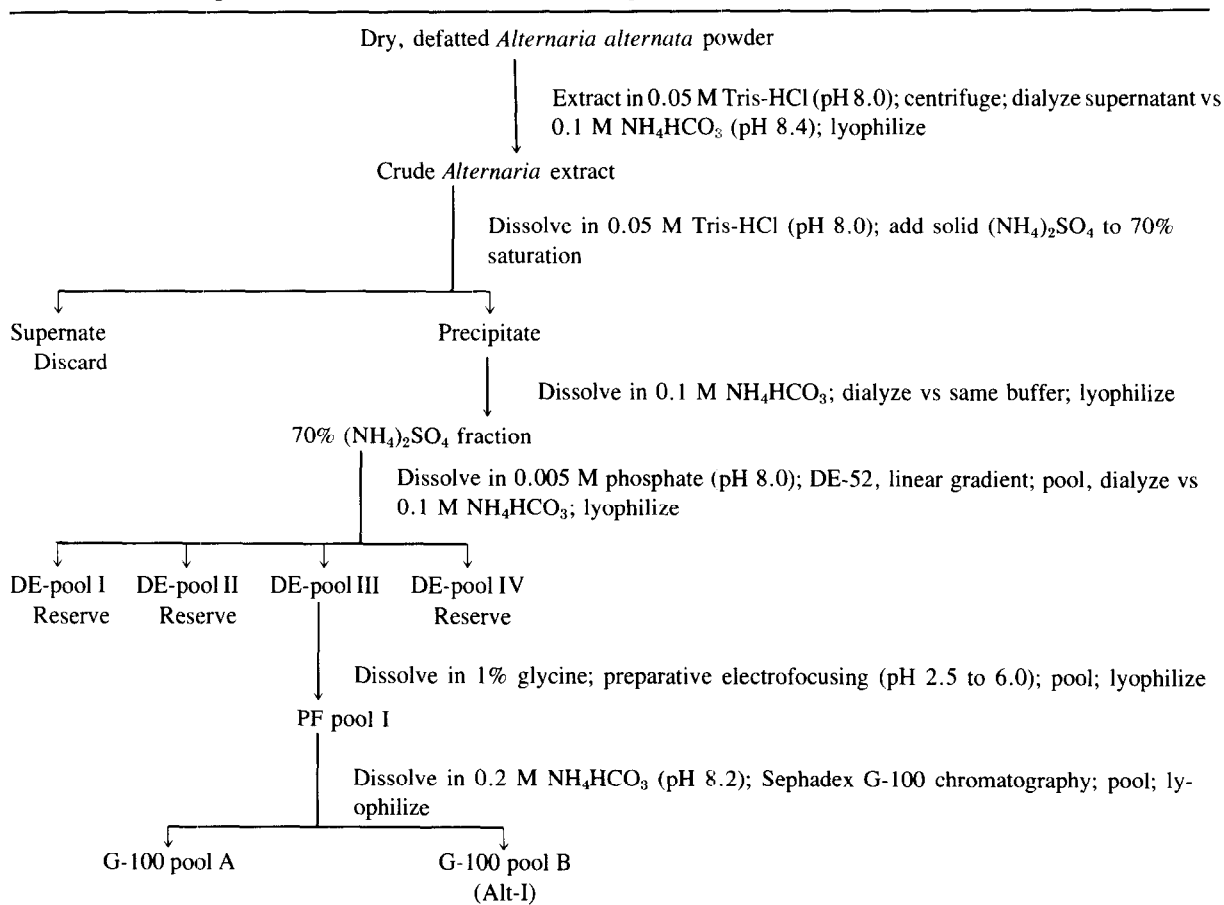
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TABLE I. Flow diagram for isolation of *Alternaria* allergens*



*Dry weight yields from 100 gm *Alternaria* powder averaged 12.7 gm for crude *Alternaria* extract; 4.2 gm for the 70% ammonium sulfate-precipitable fraction; 1.4, 0.6, 0.6, and 0.3 gm for the DE-pools I through IV, respectively; 1.8 gm for the PF pool (including weight of ampholytes); and 18 and 11 mg for pools A and B, respectively, from Sephadex G-100.

gradient of 2.5 to 6.0 and ampholyte concentrations of 2% (w/v).

Preparative polyacrylamide gel electrophoresis (PAGE) of Alt-I was performed at 4° C in a cylindrical 15% gel column measuring 4.5 cm in diameter and 2.5 cm in height. The gel was preelectrophoresed twice, first with 10% sucrose in 0.1 M Tris–0.1 M borate buffer (pH 8.3), then with 20 mg ascorbic acid in 0.1 M NaOH (0.5 ml) with 0.1 M Tris–0.1 M borate (pH 8.3). The sample load was 5.8 mg Alt-I with 10% sucrose in 2.5 ml Tris-borate. Electrophoresis was performed at 60 V for 25 min; voltage was then increased to 125 V for the remainder of the 18-hr run, during which fractions of 3 to 5 ml were collected.

The allergenic activities of various samples produced during fractionation of crude *Alternaria* extract were monitored by the RAST procedure.⁵ Selected allergen fractions were incubated with cyanogen bromide-activated filter paper disks for 2 to 12 hr at room temperature, after which residual uncoupled sites were blocked by the addition of 1.0 M ethanolamine (1.0 ml) followed by overnight incubation. Disks were subsequently washed three times with 0.1 M

phosphate buffer (pH 7.4) containing 1% Tween-20. Disks were then incubated for 4 to 18 hr with 0.05 ml of either pooled serum obtained from seven nonimmunized *Alternaria*-sensitive patients (IgE antibody pool) or serum from a nonallergic individual, then washed thrice with 0.15 M NaCl. Disks were then incubated overnight after addition of 0.1 ml of ¹²⁵I-labeled anti-IgE (20 ng), washed thrice with 0.15 M NaCl, and counted in an automatic gamma scintillation counter.

The comparative allergenicities of certain *Alternaria* fractions were tested by a RAST-inhibition procedure using the same IgE antibody pool. The solid-phase *Alternaria* allergen in these experiments was prepared by coupling a 70% (NH₄)₂SO₄-precipitable fraction of crude *Alternaria* extract (see below) to CNBr-activated microcrystalline cellulose particles. The inhibition procedure was performed as described previously³ except that a mini-RAST modification was employed.⁶ Briefly, this involved reducing the volume of the solid-phase allergen from 0.5 to 0.05 ml, reducing the reaction volume in each stage of the RAST to 0.25 ml, reducing the quantity of ¹²⁵I-labeled anti-IgE to 5 ng/tube,

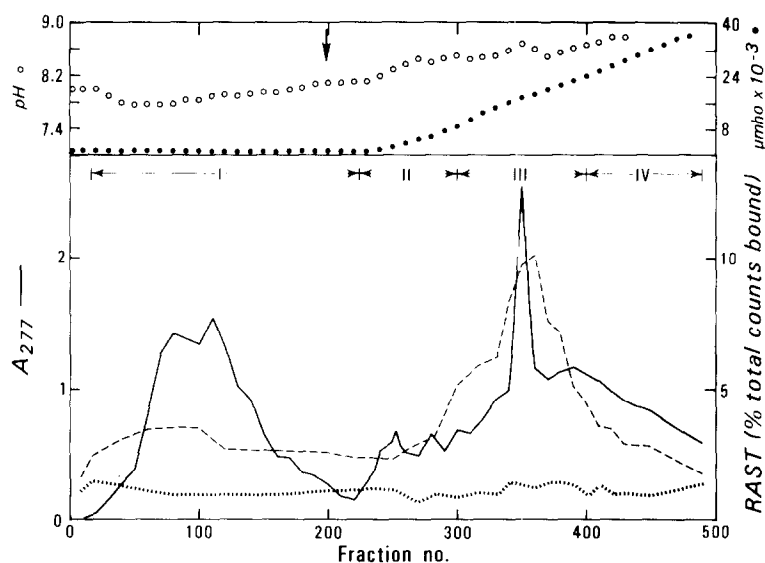


FIG. 1. Chromatography of 70% $(\text{NH}_4)_2\text{SO}_4$ -precipitable fraction on a column (4×50 cm) of DE-52. Fractions were monitored for pH ($\circ$), conductance in μmho ($\bullet$), and absorbance at 277 nm (—). A linear salt gradient was begun at the point indicated by the arrow. Selected fractions were monitored for allergenic activity by RAST using serums from both a nonallergic person (heavy broken line) and *Alternaria*-sensitive persons (dashed line). Fractions were subsequently combined into four pools (I through IV) as indicated.

and rotation of the reaction tubes at an 80° angle at 22 rpm rather than tumbling the tubes end over end.

The cross-allergenicity of Alt-I subfractions was also tested by RAST inhibition. Crude *Alternaria* extract (2.78 mg dry weight) and the various subfractions (13 to 62 μg protein by biuret assay) were each incubated with 10 CNBr-activated filter paper disks. After ethanolamine blocking and washing steps the disks were used in a RAST inhibition assay. Specificity of inhibition was tested by including short ragweed-coupled disks that were tested with an IgE serum pool from five ragweed-sensitive individuals.

Serum IgE antibodies to Alt-I were measured in selected individual patients by the mini-RAST. These serums were collected as part of a study conducted at the University of Wisconsin on the association of human leukocyte antigen haplotypes with asthma. Patients were classified as having intrinsic asthma or extrinsic asthma due to *Alternaria* sensitivity based on a history of asthma limited to or substantially more severe during July to October for three or more years and a positive skin test to *Alternaria* extract (10 mm or greater wheal diameter after intradermal injection of 0.01 ml at a dilution of 100 protein nitrogen units/ml).

The total hexose content of certain fractions was measured by a phenol-sulfuric acid procedure⁷ using glucose as a standard. The protein content of selected fractions was measured by spectrophotometric absorbance at 225 or 277 nm, by a Coomassie blue G assay,⁸ or by the biuret method⁹ using human serum albumin as a standard. The nitrogen contents of purified fractions were measured by the micro-Kjeldahl procedure.¹⁰ The amino acid compositions of purified fractions were measured in a Beckman Model 119 automatic analyzer.¹¹

Polyacrylamide gel disc electrophoresis (disc PAGE) was performed in 7% gels with alkaline buffers¹² and also in 15% gels containing urea-acetic acid buffer (urea PAGE).¹³ In addition, we performed PAGE using 0.1 M Tris-0.1 M borate buffer (pH 8.3) in 15% gels (Tris-borate PAGE). An estimate of molecular weight of Alt-I was attempted by PAGE in 8% gels containing 1% sodium dodecyl sulfate (SDS) with and without the addition of 0.04 M dithiothreitol (DTT) (SDS-PAGE).¹⁴ Thin-layer electrofocusing (TLEF) was performed in 5% gel slabs.¹⁵ Gels were stained with Coomassie blue and in selected instances with Schiff's reagent.¹⁶

RESULTS

Multiple pilot experiments involving relatively small quantities of *Alternaria* extract were conducted over several months to maximize the yield of purified allergens. The final fractionation scheme for isolation of Alt-I involved sequential ammonium sulfate precipitation, anion-exchange chromatography, preparative electrofocusing, and gel filtration. The complete scheme is shown in Table I. Dry, defatted *Alternaria* powder was extracted 1:20 (w/v) in 0.05 M Tris-HCl (pH 8.0) overnight at 4°C with continuous stirring. The pH was maintained above 7.4 by addition of NaOH (22.7 ± 10 mEq per 100 gm powder). The crude extract was isolated by centrifugation; the pellet was washed twice with additional extraction buffer. The crude extract was then dialyzed against 0.1 M NH_4HCO_3 (pH 8.4) and lyophilized. Dry crude *Alter-*

TABLE II. Protein, hexose, and nitrogen content of pool A and pool B (Alt-I) from Sephadex G-100

	Pool A	Pool B (Alt-I)
Protein by biuret assay ($\mu\text{g/ml}$)	992	760
Hexose ($\mu\text{g/ml}$)	1,781	382
Nitrogen (%)	5.2	7.0

naria extract was subsequently dissolved in 0.05 M Tris-HCl at room temperature; solid $(\text{NH}_4)_2\text{SO}_4$ was added to 70% saturation, and the mixture was magnetically stirred for 2 to 3 hr. The mucoid precipitate was isolated by filtration on a Buchner funnel using No. 4 Whatman paper; to facilitate dissolution the precipitate and filters were soaked overnight at 4° C in 0.1 M NH_4HCO_3 , after which the resulting solution was dialyzed against the same buffer and then lyophilized.

The 70% $(\text{NH}_4)_2\text{SO}_4$ -precipitable fraction was subsequently dissolved (3 mg/ml) in 0.005 M phosphate buffer (pH 8.0) and dialyzed overnight against the same buffer. When the sample was applied atop a DE-52 column as usual, we observed shrinkage and channeling of the column bed. A modified batch chromatography step was used to circumvent this problem. The quantity of DE-52 required to fill a 4 × 50-cm column was calculated and approximately 60% of this amount was equilibrated with 0.005 M phosphate buffer and used to pour the column. The remaining 40% was mixed with the dissolved 70% $(\text{NH}_4)_2\text{SO}_4$ -precipitable fraction (maximum sample = 3.5 gm), which was then layered atop the column and allowed to settle. The supernatant containing sample was then eluted at a flow rate of 30 to 35 ml/hr, after which the column was eluted with additional 0.005 M phosphate until the 277-nm absorbance of the eluate fell below 0.200. A linear salt gradient was then applied using 0.1 M phosphate–1.0 M NaCl (pH 8.0) as the limit buffer. Fractions were monitored for 277-nm absorbance, pH, conductivity, and allergenic activity. The elution profile is shown in Fig. 1. A large absorbance peak with modest allergenic activity appeared in the fall-through fractions. Peak allergenic activity was associated with the largest of a series of absorbance peaks eluting after the salt gradient was applied, over a conductance range of 15,000 to 22,000 μmho . Fractions were pooled as indicated in Fig. 1 (I through IV), dialyzed against 0.1 M NH_4HCO_3 , and lyophilized; the bulk of the allergenic materials were in pool III.

Pool III was subsequently fractionated by flat-bed PEF with a pH gradient of 2.5 to 6.0 and ampholyte concentration of 2%. Pool III (7 to 13 mg/ml) was

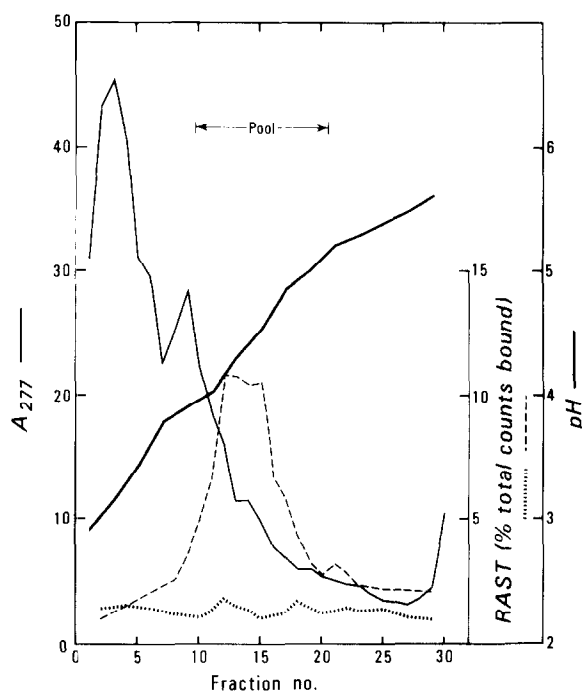


FIG. 2. Preparative flat-bed electrofocusing of pool III from DE-52 chromatography step. The nominal pH gradient was 2.5 to 6.0. Fractions were monitored for pH (heavy line) and absorbance at 277 nm (lighter line). Allergenic activity was monitored by RAST using serums from both a nonallergic person (heavy broken line) and *Alternaria*-sensitive persons (dashed line). Fractions were subsequently pooled as indicated.

dissolved in 1% glycine and dialyzed overnight at 4° C against the same buffer. The sample was applied in a trough approximately 16 to 18 cm from the anodal wick (maximum total sample adequately separated = 330 mg). Separation was complete in 18 hr when a constant power of 7.5 W was applied. Several rust-colored bands of pigment were seen, particularly in the third of the gel nearest the anode. The gel bed was partitioned into 30 segments by means of a template. A small quantity of each fraction was eluted with 0.3 ml distilled water for pH measurements; the remainder of each fraction was eluted with approximately 10 to 15 ml 0.1 M NH_4HCO_3 . Fractions were monitored for absorbance at 277 nm, pH, and allergenic activity; the results are shown in Fig. 2. Allergenic activity was localized to the pH range of 4.05 to 4.50, with a peak of 4.20; these fractions were pooled as indicated and lyophilized.

The pool from preparative electrofocusing (including carrier ampholytes) was dissolved in 0.2 M NH_4HCO_3 (pH 8.2) and gel filtered over a calibrated 2.5 × 50-cm Sephadex G-100 column equilibrated with the same buffer (maximum sample weight including ampholytes = 1,100 mg). Fractions were

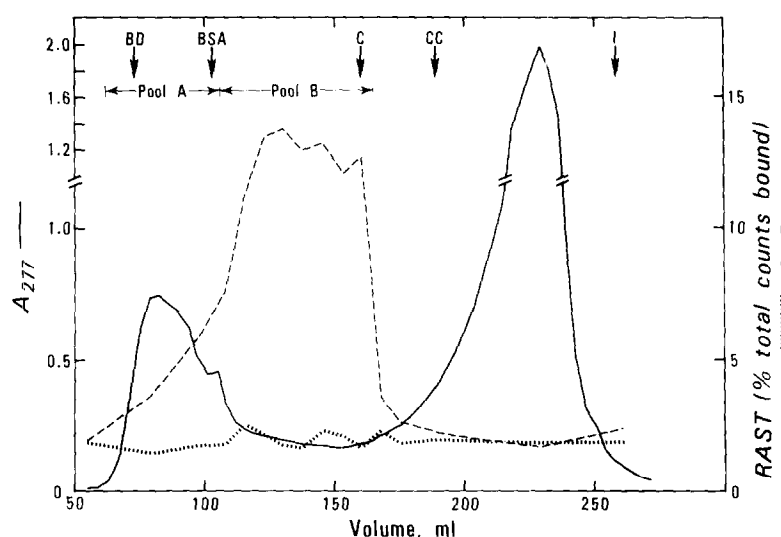


FIG. 3. Chromatography of preparative electrofocusing pool on a column (2.5 × 50 cm) of Sephadex G-100. Elution volumes of calibration markers are indicated by *arrows*, including blue dextran (BD), bovine serum albumin (BSA), chymotrypsinogen (C), cytochrome *c* (CC), and ^{125}I (I). Spectrophotometric absorbance at 277 nm is indicated by the *solid line*; allergenic activity was monitored by RAST using serums from both a nonallergic person (*heavy broken line*) and *Alternaria*-sensitive persons (*dashed line*). Fractions were pooled as indicated.

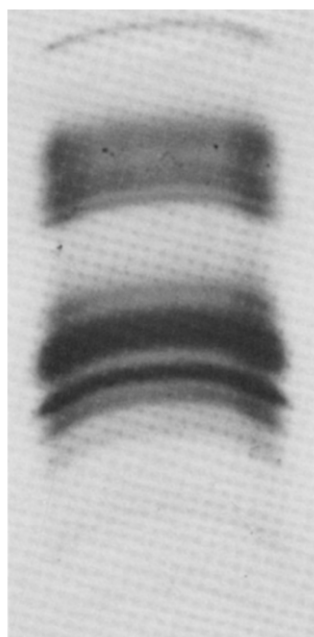


FIG. 4. Thin-layer electrofocusing of the Alt-I fraction in 5% polyacrylamide gel slabs; the anode is at the top. The pH gradient was 2.5 to 6.0; Coomassie-stained bands appeared only in the area between pH 4.0 to 4.5.

monitored for absorbance at 277 nm and for allergenic activity; the elution profile is shown in Fig. 3. A small absorbance peak possibly possessing weak allergenicity eluted at the column void volume; a second larger absorbance peak eluted near the internal col-

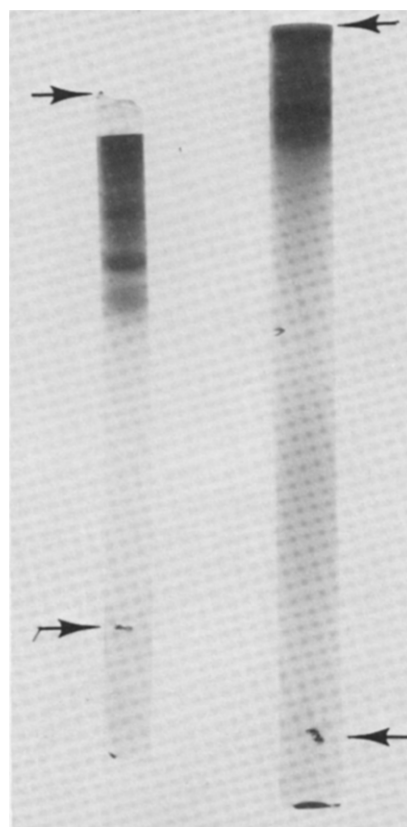


FIG. 5. Tris-borate PAGE, pH 8.3 (*left, anode at bottom*) and urea PAGE, pH 3.2 (*right, anode at top*); Coomassie blue stain. The origin and extent of electrophoresis are indicated by the top and bottom *arrows*, respectively.

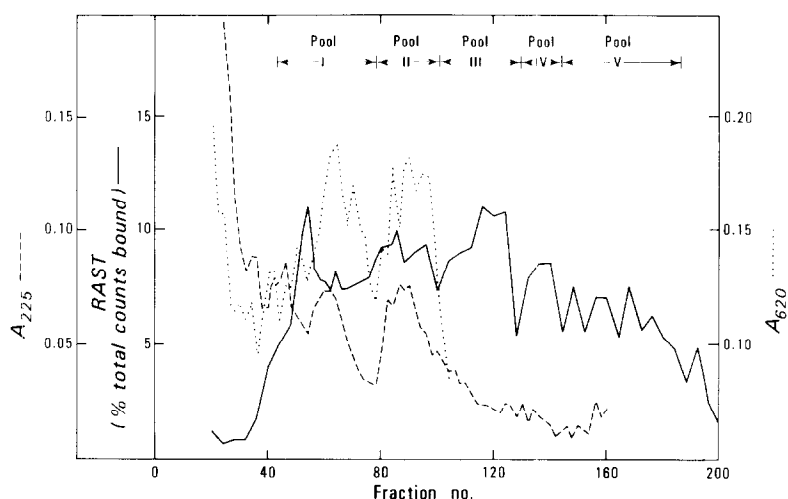


FIG. 6. Preparative electrophoresis of the Alt-I fraction in 15% polyacrylamide gel using Tris-borate buffer (pH 8.3). Fractions were monitored for absorbance at 225 nm (dashed line), for protein content by Coomassie blue G assay (dotted line), and for allergenic activity by RAST using a serum pool from *Alternaria*-sensitive persons (solid line). Serum from a nonallergic person bound approximately 1% total radioactive counts in the RAST (data not shown). The bromphenol blue tracker dye eluted at fraction No. 16; fractions were subsequently pooled as indicated (PE-I through PE-V).

umn volume and probably represented carryover ampholytes from the electrofocusing step. The bulk of the allergenic activity eluted in a broad zone with an apparent molecular weight of approximately 25,000 to 50,000 in an area where there was little absorbance at 277 nm. Fractions were combined into two pools (A and B) as indicated and lyophilized. These pools differed with respect to their protein, hexose, and nitrogen content, as shown in Table II. At concentrations of 1 mg/ml and greater, both pools were light tan in color. Both pools contained appreciable quantities of carbohydrate. Further studies were confined to pool B, and we will subsequently refer to this fraction as Alt-I.

The homogeneity of the Alt-I fraction was tested by a variety of analytical procedures. By TLEF approximately 12 bands were visible in the region of pH 4.0 to 4.5 (Fig. 4). By punching out disks from unstained gel areas with a cork borer, eluting the gel disks with 1 ml H₂O, and coupling the eluate to CNBr-activated filter paper disks, we confirmed that allergenic activity was confined to the area where Coomassie-stained bands appeared. In both Tris-borate PAGE and urea PAGE approximately six major bands were seen (Fig. 5). We again demonstrated allergenic activity only in the regions of the gels where bands appeared, and all bands were associated with allergenic activity. Sharp bands were not seen when the Alt-I fraction was analyzed in disc PAGE or SDS-PAGE; only a broad area of smearing was noted (data not shown). This was

TABLE III. Amino acid analysis of preparative electrophoresis (PE): pools I, II, and III.*

Amino acid	Residues per pool		
	I	II	III
Asp	48	43	43
Thr	49	43	38
Ser	45	41	47
Glu	57	52	54
Pro	57	61	57
Gly	47	50	62
Ala	64	59	54
Val	32	33	33
Met	3	3	3
Ile	9	12	12
Leu	16	22	24
Tyr	12	15	15
Phe	10	15	15
His	17	11	10
Lys	19	19	17
Arg	6	9	10
GlcN	2	0	1

*Nanograms of amino acid were normalized to equal mass prior to conversion to nanomoles to permit comparison of the different pools.

probably due to the relatively high carbohydrate content of Alt-I.

Because analytical Tris-borate PAGE produced the sharpest separation of Alt-I components, we elected to use preparative Tris-borate PAGE for further study

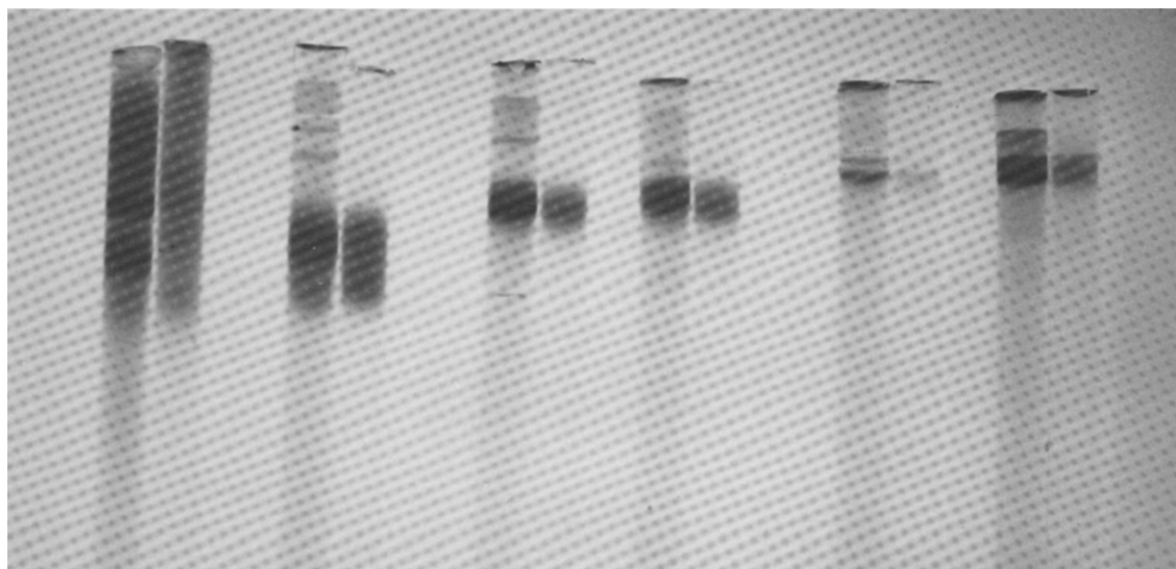


FIG. 7. Tris-borate PAGE of preparative electrophoresis pools from Alt-I; the anode is at the bottom and the origin is at the top. The member on the left of each gel pair is stained with Coomassie blue; the member on the right is stained with Schiff's reagent. The gel pairs, from left, are Alt-I, PE-I, PE-II, PE-III, PE-IV, and PE-V.

TABLE IV. Cross-inhibition RAST assay of purified *Alternaria* allergens*

Solid-phase allergen	RAST (% total counts bound)							
	No inhibitor		Inhibitor†					
	Neg. control	Pos. control	Crude	PE-I	PE-II	PE-III	PE-IV	PE-V
Crude <i>Alternaria</i>	0.82	4.65	0.71	2.34	2.57	1.43	3.64	4.16
PE-I	1.15	10.49	2.31	1.81	3.53	4.33	6.36	5.87
PE-II	1.16	9.76	1.27	1.69	1.21	1.29	2.32	1.88
PE-III	1.06	9.81	1.51	2.55	1.76	1.17	1.75	1.25
PE-IV	1.23	7.78	1.17	2.17	1.75	1.74	1.24	1.32
PE-V	1.17	8.40	1.16	2.47	2.49	1.20	1.49	1.55
Short ragweed	0.92	11.74	9.85	10.67	10.58	10.98	10.95	10.76

*Preparative electrophoresis pools (PE-pools) were tested for cross-allergenicity by their ability to inhibit either a crude *Alternaria* RAST or a RAST using purified solid-phase *Alternaria* allergens. Insignificant nonspecific inhibition (less than 16.1%) was seen in a short ragweed RAST assay.

†Quantities of inhibitors tested per tube were 347 μ g crude *Alternaria*, and 7.7, 5.9, 3.4, 1.6, and 3.7 μ g protein by biuret assay for PE-pools I through V, respectively.

of these subfractions. Only two peaks could be identified by the Coomassie blue G assay by spectrophotometric absorbance at 225 nm (Fig. 6). In contrast, using the RAST procedure, not only were these two peaks identified, but the results suggested that at least two additional allergenic peaks were present. We combined selected fractions into five pools (PE-I through PE-V) as indicated in Fig. 6. These pools were dialyzed against 0.1 M NH_4HCO_3 and lyophilized; milligram quantities of each pool were obtained. The homogeneity of each pool was tested by Tris-borate PAGE; duplicate gels were stained with Coomassie blue or Schiff's reagent and are

shown in Fig. 7. By the number of bands produced we estimated that PE-I, PE-II, and PE-III were at least 95% homogeneous by this technique. The amino acid analyses of PE-I, PE-II, and PE-III are shown in Table III; the pools were rather similar in their compositions.

The cross-inhibition RAST assay results using crude and purified *Alternaria* allergens linked to filter paper disks are shown in Table IV. Significant cross-allergenicity was demonstrated among all *Alternaria* fractions; insignificant (less than 16.1%) inhibition was seen in the short ragweed RAST system. In the case of PE pools I through IV the homologous frac-

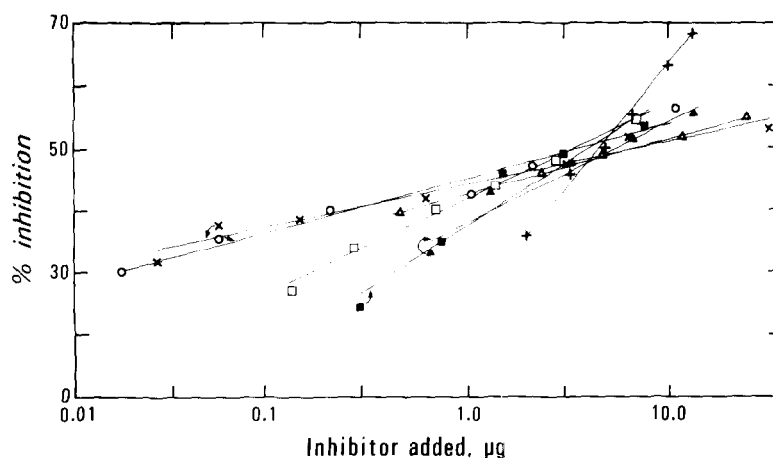


FIG. 8. RAST inhibition assay of the Alt-I fraction and each preparative electrophoresis pool. Solid phase allergen (70% ammonium sulfate-precipitable fraction of crude *Alternaria* extract) was reacted with IgE antibody in the presence of the inhibitors; the correlation coefficient for each least-squares regression line was 0.96 or greater. The quantity of inhibitor added is expressed as micrograms by biuret (PE pools) or as micrograms dry weight (crude extract). $\circ$ = Alt-I; $\times$ = PE-I; $\triangle$ = PE-II; $\square$ = PE-III; $\blacktriangle$ = PE-IV; $\blacksquare$ = PE-V. The inhibition curve produced by crude *Alternaria* extract (+) is also shown for comparison.

tion was the best inhibitor, raising the possibility that these fractions contained not only cross-reacting allergens, but individual allergens as well. Finally, we determined the qualitative and quantitative cross-reactivity among the various PE pools in a mini-RAST inhibition assay; results are shown in Fig. 8. Appreciable cross-allergenicity among the PE pools was also shown in this experiment. However, the slopes of the inhibition curves produced by the Alt-I and PE fractions differed significantly from that produced by crude *Alternaria*, suggesting that not all allergenic activity in crude *Alternaria* extracts was contained in the Alt-I fraction.

Up to this point the allergenic activity of the various *Alternaria* fractions had been defined only by a RAST-inhibition procedure using serums from seven nonimmunized *Alternaria*-sensitive patients. We wished to determine whether a larger series of persons who exhibited positive skin-test responses to crude *Alternaria* extracts would also react to the Alt-I fraction by the RAST. We immobilized Alt-I by reacting 2 mg Alt-I with 250 mg CNBr-activated microcrystalline cellulose; this polymer was used to test serums from patients having either intrinsic (non-IgE-mediated) asthma ($n = 15$) or nonimmunized patients with extrinsic (IgE-mediated) asthma and positive skin tests to *Alternaria* ($n = 25$). Results are shown in Table V. RAST binding produced with serums from 15 intrinsic asthmatics ranged from 0.10% to 0.29% total counts, and serums from three nonallergic control persons bound 0.10% to 0.33% total counts. However, 21/25 serums from *Alter-*

naria-sensitive asthmatics bound over 1% total counts. We concluded that the Alt-I fraction contained a major allergen or allergens present in crude *Alternaria* extract.

DISCUSSION

There have been few previous attempts to isolate and characterize *Alternaria* allergens. However, the finding that the Alt-I fraction is relatively carbohydrate-rich is in keeping with previous findings with partially-purified *Alternaria* materials. By pretreatment of *Alternaria* pellicles with alcohol and precipitation of the crude *Alternaria* extract thereof with cold acetone, LeBeau¹⁷ was able to remove 96% of extractable nitrogen from *Alternaria* pellicle extracts without appreciable loss in skin-test reactivity. He concluded that allergenic activity was associated closely with the carbohydrate portion of the extract. Sternberger et al.¹⁸ noted that deproteinization of crude *Alternaria* extracts did not reduce the allergenicity of the extract, as judged by scratch tests in mold-sensitive patients. Bonilla-Soto et al.¹⁹ treated 10% *Alternaria* extracts with hot phenol (50% v/v), then added 95% ethanol to the aqueous phase. The resulting precipitate was dissolved in 0.15 M NaCl and was shown to be skin-reactive in *Alternaria*-sensitive patients. In subsequent experiments Bonilla-Soto²⁰ fractionated crude *Alternaria* extracts over Sephadex G-200 columns. Although these columns were not calibrated, skin test-reactive fractions eluted just prior to the second of two 280-nm absorbance peaks. We estimate that this elution position

TABLE V. Alt-I RAST results in asthmatic patients*

Intrinsic asthma		<i>Alternaria</i> -induced asthma	
Serum No.	RAST	Serum No.	RAST
3	0.18	2	7.81
6	0.13	5	4.13
17	0.13	7	0.18
18	0.16	10	9.56
20	0.20	19	0.52
21	0.16	23	5.07
37	0.11	24	1.14
40	0.14	26	10.33
42	0.13	31	0.47
51	0.10	33	3.23
64	0.29	35	13.52
68	0.16	45	1.52
82	0.10	46	6.60
91	0.25	49	11.10
94	0.13	54	11.18
		55	3.13
		58	2.69
		61	4.46
		62	1.26
		63	0.56
		71	4.92
		72	4.63
		75	1.36
		92	1.42
		95	6.71

*RAST results are expressed as percent total counts bound. Serums from three nonallergic control patients bound from 0.10% to 0.33% total counts, while a serum pool from seven *Alternaria*-sensitive individuals bound 5.47% total counts.

would correspond closely to the elution position of Alt-I from the Sephadex G-100 columns used in the present study.

A partially purified *Alternaria* allergen mixture has been prepared by Lynch et al.²¹ by sequential 85% (NH₄)₂SO₄ precipitation of crude *Alternaria* extract, Sephadex G-25 gel filtration, DEAE cellulose ion-exchange chromatography, and Sephadex G-100 gel filtration. When this allergen was coupled to microcrystalline cellulose and used for measurement of serum IgE antibodies by RAST in asthmatic children, the RAST results correlated with skin-test results but not with bronchial-challenge results. These authors determined that this was due to competitive RAST inhibition by IgG antibodies to *Alternaria*, which could be overcome by linking the partially-purified *Alternaria* allergen to a Sepharose-2B support. RAST results with this support correlated with both skin-test and bronchial-challenge results. We did not examine the test serums used in our study for IgG

antibody to *Alternaria*, although our serums were obtained from nonimmunized patients in whom such IgG antibodies would presumably be minimal. Neither did we explore support systems other than filter paper disks and microcrystalline cellulose particles for the RAST. Nevertheless, the RAST systems we utilized provided clear-cut discrimination between major allergenic and nonallergenic fractions. In addition, the microcrystalline cellulose particle RAST permitted the demonstration of significantly elevated serum IgE antibodies to the Alt-I fraction in over 80% of *Alternaria*-sensitive asthmatics.

The slopes of the RAST inhibition curves produced by the Alt-I fraction and crude *Alternaria* extract differed significantly, suggesting that only a portion of the total allergenic activity in crude *Alternaria* extracts is contained in the Alt-I fraction. Sternberger et al.¹⁸ demonstrated allergenic activity in the dialyzable fraction (molecular weight presumably less than 10,000) of crude *Alternaria* extracts. Lynch et al.²¹ have described a glycopeptide material isolated from the supernatant fraction obtained by 85% (NH₄)₂SO₄ precipitation of crude *Alternaria* extract. This glycopeptide (3% to 5% N) was capable of eliciting passive cutaneous anaphylaxis in monkeys and, when linked to a Sepharose-2B-human serum albumin polymer to produce a RAST reagent, could identify elevated serum IgE antibodies in asthmatic children sensitive to *Alternaria*. When considered together, these findings suggest that allergenic materials are present in fractions other than Alt-I.

The Alt-I fraction produced in these experiments was "pure" in the sense that it did not contain nonallergenic components. On the other hand, the Alt-I fraction was heterogeneous by both PAGE and TLEF procedures. Such microheterogeneity is not unusual, however, having been noted previously for the purified ragweed allergens AgE and AgK, as well as for the rye group allergens I, II, and III.²² It is possible that the lyophilization procedures employed during the latter steps of the fractionation produced aggregation and/or denaturation of the allergen and contributed to the multiplicity of bands seen in PAGE and TLEF. Additional attempts will be made to further purify the Alt-I fraction in future studies.

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REFERENCES

- Solomon WR, Mathews KP: Aerobiology and inhalant allergens, in Middleton E Jr, Reed CE, Ellis EF, editors: Al-

- lergy: Principles and practice. St. Louis, 1978, The C. V. Mosby Co., p. 899.
2. Yunginger JW, Roberts GD, Gleich GJ: Studies on *Alternaria* allergens. I. Establishment of the radioallergosorbent test for measurement of *Alternaria* allergens. *J ALLERGY CLIN IMMUNOL* **57**:293, 1976.
 3. Yunginger JW, Jones RT, Gleich GJ: Studies on *Alternaria* allergens. II. Measurement of the relative potency of commercial *Alternaria* extracts by the direct RAST and by RAST inhibition. *J ALLERGY CLIN IMMUNOL* **58**:405, 1976.
 4. Radola BJ: Analytical and preparative isoelectric focusing in gel-stabilized layers. *Ann NY Acad Sci* **209**:127, 1973.
 5. Gleich GJ, Yunginger JW: Standardization of allergens, in Rose NR, Friedman H, editors: *Manual of clinical immunology*. Washington D. C., 1976, American Society for Microbiology, p. 575.
 6. Gleich GJ, Adolphson CR, Yunginger JW: The mini-RAST: Comparison with other varieties of the radioallergosorbent test for the measurement of immunoglobulin E antibodies. *J ALLERGY CLIN IMMUNOL* **65**:20, 1980.
 7. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F: Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**:350, 1956.
 8. Sedmak JJ, Grossberg SE: A rapid, sensitive, versatile assay for protein using Coomassie brilliant blue G250. *Anal Biochem* **79**:544, 1977.
 9. Kabat EA, Mayer MM: *Experimental immunochemistry*, ed. 2. Springfield, Ill., 1967, Charles C Thomas, Publisher, p. 559.
 10. Kabat EA, Mayer MM: *Experimental immunochemistry*, ed. 2. Springfield, Ill., 1967, Charles C Thomas, Publisher, p. 476.
 11. Spackman DJ, Stein WH, Moore S: Automatic recording apparatus for use in the chromatography of amino acids. *Anal Chem* **30**:1190, 1958.
 12. Davis BJ: Disc electrophoresis. II. Method and application to human serum proteins. *Ann NY Acad Sci* **121**:404, 1964.
 13. Panyim S, Chalkley R: High resolution acrylamide gel electrophoresis of histones. *Arch Biochem Biophys* **130**:337, 1969.
 14. Fairbanks G, Steck TL, Wallach DFH: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606, 1971.
 15. Awdeh ZL, Williamson AR, Askonas BA: Isoelectric focusing in polyacrylamide gel and its application to immunoglobulins. *Nature* **219**:66, 1968.
 16. Kapitany R, Zebrowski EJ: A high resolution PAS stain for polyacrylamide gel electrophoresis. *Anal Biochem* **56**:361, 1973.
 17. LeBeau LJ: Allergenic fractions of *Alternaria* sp. (Thesis). Chicago, 1952, University of Illinois College of Medicine.
 18. Sternberger LA, Feinberg AR, Feinberg SM, Clarke M, Benaim C, Warren SA: Immunologic and chemical studies on allergenic fungi. I. Purification of an antigen from *Alternaria*. *J ALLERGY* **27**:16, 1956.
 19. Bonilla-Soto O, Rose NR, Arbesman CE: Allergenic molds: Antigenic and allergenic properties of *Alternaria tenuis*. *J ALLERGY* **32**:246, 1961.
 20. Bonilla-Soto O: Mold allergy: Fractionation of allergen by Sephadex G-200 gel filtration. *J ALLERGY* **43**:125, 1969.
 21. Lynch NR, Dunand P, Newcomb RW, Chai H, Bigley J: Influence of IgG antibody and glycopeptide allergens on the correlation between the radioallergosorbent test (RAST) and skin testing or bronchial challenge with *Alternaria*. *Clin Exp Immunol* **22**:35, 1975.
 22. Cunningham S, editor: *NIAID Catalog of Research Reagents, 1978-1980*. DHEW Publication No. (NIH) 78-899. Washington, D. C., 1978, Department of Health, Education, and Welfare, pp. 981-987.