

# Rapidly released allergens from short ragweed pollen

## II. Identification and partial purification\*

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*We have identified several basic allergens in 16-min extracts of short ragweed (Ambrosia elatior) pollen and obtained a partially purified fraction (G50 II) using a combination of ion-exchange (CM-Sephadex C25) and gel-filtration (Biogel P30 and Sephadex G50) procedures. G50 II was allergenically and antigenically distinct from known allergens antigen E, Ra3, and Ra5. It gave a highly symmetrical peak on Sephadex G50 (fine), corresponding to a molecular weight of 11,500 daltons, and a single band on SDS polyacrylamide gel electrophoresis under both reducing and nonreducing conditions. However, two cathodic antigens were detectable by both agarose electrophoresis and crossed immunoelectrophoresis. Allergic activity of G50 II, assessed by puncture testing in ragweed-sensitive subjects, showed a characteristic pattern of response independent of patient response to AgE, Ra3, and Ra5. The major antigenic and allergenic component of G50 II is designated Ra6.*

The known allergens of short ragweed (*Ambrosia elatior*) pollen by no means represent the total allergenic complement of the pollen. The first allergens to be characterized were antigen E<sup>1</sup> and antigen K,<sup>2</sup> both of which are acidic proteins existing in several electrophoretic forms. In addition, three basic allergens, Ra3,<sup>3</sup> BPAR (or Ra4),<sup>4, 5</sup> and Ra5,<sup>6</sup> have been purified. Several reports indicate the presence of additional uncharacterized allergens<sup>1, 7-9</sup> that need to be identified and their role in causing clinical symptomatology determined.

In a previous paper,<sup>10</sup> we reported the presence of

several uncharacterized rapidly released allergens in a 16-min ragweed extract while studying the kinetics of release of known allergens in relation to allergenically reactive material. Sixteen-minute extracts were found to contain a large number of basic proteins, including all of the Ra5 but only 1% to 4% of the antigen E and less than 10% of the Ra3 present in the pollen when the pH during extraction was 6.3. Despite the low proportion of antigen E (considered to be the major allergen), the 16-min extracts were highly allergenic. The rapidly released components also contributed a major portion of the biologic activity of conventional (long-term) ragweed extracts, which led to the attempts to identify and purify the rapidly released allergens reported here.

### MATERIALS AND METHODS

#### Preparation of 16-min and 24-hr extracts

Because nondefatted material is difficult to fractionate, both nondefatted and defatted 16-min extracts were prepared to determine whether defatting introduced any changes in the release of antigens from pollen grains. Nondefatted pollen was stirred with physiologic borate-buffered saline,\* pH 8.4 (1:10 w/v) for 16 min at 23° C precisely as

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\*Five parts of the following buffer: H<sub>3</sub>BO<sub>3</sub> (6.184 gm), Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> · 10H<sub>2</sub>O (9.536 gm), NaCl (4.384 gm) to 1 l with water, plus 95 parts 0.15M NaCl.

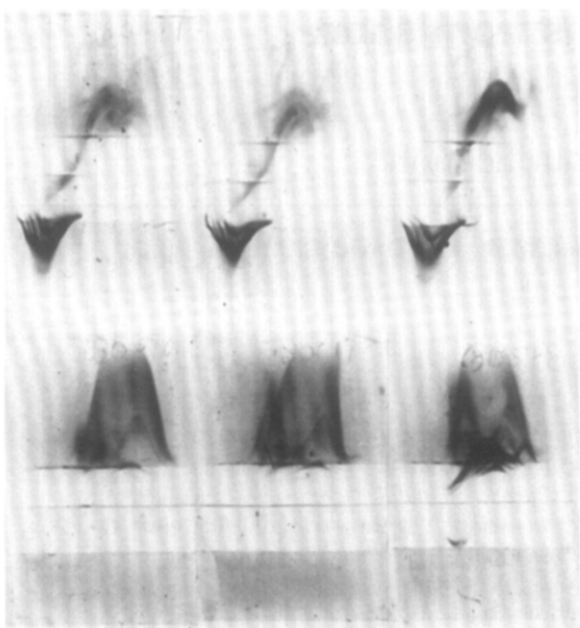


**FIG. 1.** Crossed immunoelectrophoresis of 16-min and 24-hr extracts (40% w/v). *Left to right*, 16-min defatted, 16-min nondefatted, and 24-hr defatted extracts. *Top row*, developed against rabbit anti-16-min extract. *Bottom row*, developed against rabbit anti-24-hr extract. The anode for the 1° dimension is to the right, and 2° dimension is to the top.

described in our publication.<sup>10</sup> Defatted 16-min (at 23° C) and 24-hr (at 4° C) extracts of petroleum ether-defatted pollen were prepared with the borate-saline buffer using techniques analogous to those previously described for 16-min and 4-day extracts, respectively.<sup>10</sup> As previously noted, the initial pH dropped to 6.3 to 6.35 within the first minute of extraction. Filtered extracts were centrifuged and dialyzed for 3 days against large volumes of distilled water using Spectrapore 3 tubing (molecular weight cutoff stated to be 3500 daltons by the manufacturer) and lyophilized.

### Chromatography of 16-min extract

Only defatted pollen extract was used for chromatographic purification, because there were no major differences in the antigenic composition of nondefatted and defatted pollen extract (see Results).

**Depigmentation on DEAE-A25.** Dialyzed 16-min extract (4.54 gm from 80 gm of pollen) in 33 ml 0.05M Tris HCl buffer, pH 8.0, was partially depigmented by passage through diethylaminoethyl (DEAE)-Sephadex A25 (2.5 × 30 cm) equilibrated with the same Tris buffer. The first major protein peak containing most of the antigenic material was dialyzed and lyophilized. Partially depigmented 16-min extract was further fractionated by a combination of ion-exchange and gel-filtration procedures.

**Ion-exchange chromatography on CM-Sephadex C25.** Partially depigmented 16-min extract (500 mg in 13 ml 0.005M sodium phosphate buffer, pH 6.0) was separated on a CM-Sephadex C25 (1.5 × 30 cm) column. Components

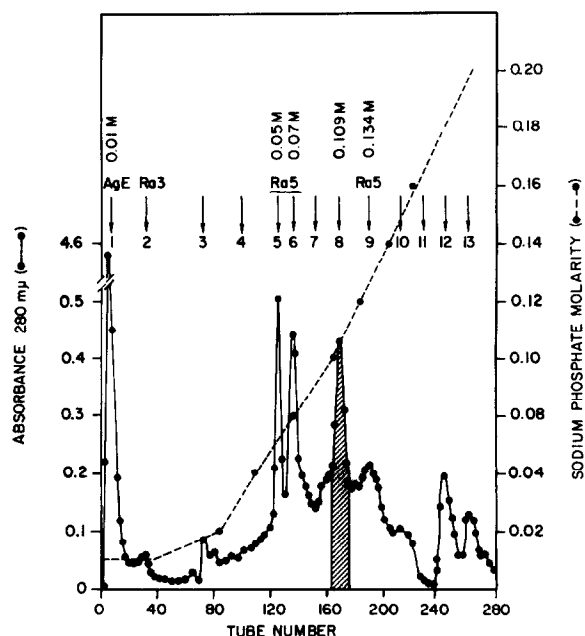


**FIG. 2.** Chromatography of 16-min extract on a CM Sephadex C-25 column (1.5 × 30 cm) eluted with gradient of 0.005 to 0.2M sodium phosphate buffer at pH 6.0. Six-milliliter fractions were collected. The peaks in which antigen E, Ra3, and Ra5 were detected are indicated. *Hatched area*, fraction (C8) selected for further purification.

were eluted with a gradient of increasing molarity of sodium phosphate buffer (0.005 to 0.2M; pH 6.0). Both conductivity and absorbance at 280 mμ were monitored throughout the experiment to determine the molarity of eluted buffer and the presence of eluted protein. The protein peaks were dialyzed and lyophilized.

**Gel filtration on Biogel P30 and Sephadex G50.** One of the protein peaks (C8) from the CM-Sephadex C25 column, which was found to be highly allergenic, was subjected to further purification on a Biogel P30 column (1.5 × 90 cm) and a Sephadex G50 column (1.5 × 90 cm), both of which were equilibrated and eluted with 0.1M  $\text{NH}_4\text{HCO}_3$  at flow rates of 2 to 3 ml/cm<sup>2</sup> cross-sectional areas of the columns.

### Preparation of antisera

Pools of anti-16-min extract or dialyzed anti-24-hr sera were prepared by hyperimmunizing two groups of three rabbits with either partially depigmented 16-min extract or dialyzed 24-hr extract in complete Freund's adjuvant over a period of 6 mo. Each rabbit was injected with 20 mg extract intramuscularly at 7-day intervals and approximately 40 ml blood taken 7 days after every third injection. After 6 mo, the booster injections were reduced to 5 mg, and the rabbits were given injections and bled on alternate weeks. Sera having the highest antibody titers were pooled. Mono-specific antisera to antigen E and Ra3 have been described previously.<sup>10</sup> Anti-Ra5 was prepared by immunizing a goat with Ra5 coupled with ovalbumin using carbodiimide, fol-

TABLE I. Properties of ragweed extracts

| Extracts (10% w/v) | Nondialyzable solids ( $\mu\text{g/ml}$ ) | Antigen content ( $\mu\text{g/ml}$ ) |      |      | Skin reactivity mean wheal diameter (mm)* |
|--------------------|-------------------------------------------|--------------------------------------|------|------|-------------------------------------------|
|                    |                                           | AgE                                  | Ra3  | Ra5  |                                           |
| Nondefatted 16-min | 5,200                                     | 9.0                                  | 8.3  | 28.9 | 4.8                                       |
| Defatted 16-min    | 5,900                                     | 9.1                                  | 8.3  | 28.9 | 4.7                                       |
| Defatted 24-hr     | 9,000                                     | 489                                  | 89.3 | 31.3 | Not tested                                |

Standard deviations for measurements of nondialyzable solids and antigen contents were  $\pm 5\%$  to  $10\%$ .

\*Average wheal diameter for puncture tests in 57 ragweed-allergic subjects using antigens at  $300 \mu\text{g}$  nondialyzable solids per milliliter.

lowed by boosting with pure Ra5, both emulsified in Freund's complete adjuvant.

### Immunochemical analyses

**Determination of molecular weight by gel filtration.** A Sephadex G50 (fine) column ( $2.5 \times 90 \text{ cm}$ ) was calibrated with the following molecular weight markers: cytochrome C, ragweed Ra3, myoglobin, soybean trypsin inhibitor, ragweed Ra5, and rye grass group II. This column was then used to determine the molecular weight of G50 II using the procedure of Andrews.<sup>11</sup>

**Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS).** SDS polyacrylamide gel electrophoresis of ragweed fractions was carried out in 15% polyacrylamide gels<sup>12</sup> under reducing and nonreducing conditions, using the same molecular weight marker proteins as used in the gel-filtration experiment.

**Immunodiffusion (ID) and crossed immunoelectrophoresis (CIE).** ID analyses were carried out by the method of Scheidegger et al.<sup>13</sup> Quantitative radial ID was performed according to Mancini et al.<sup>14</sup> CIE was carried out by the method of Axelsen et al.<sup>15</sup> Briefly, proteins were separated in 1% agarose (Litex HSB) in Tris-veronal (TV) buffer at pH 8.6 at 10 V/cm for 30 min. A longitudinal strip containing the separated proteins was cut and then placed on a second plate containing agar with appropriate antiserum in TV buffer. The proteins were subjected to further electrophoresis, at right angles to the first dimension, for 18 to 20 hr at 2 V/cm. The gel was washed, dried, and stained.

**Agarose electrophoresis.** Agarose electrophoresis at pH 8.6 was carried out as described in our previous paper.<sup>10</sup>

### Skin testing

Puncture testing was carried out on ragweed-allergic subjects as described previously.<sup>16</sup> Dialyzed 16-min and 24-hr extracts were tested at  $300 \mu\text{g/ml}$  and highly purified AgE and Ra3 and partially purified Ra5 at  $10 \mu\text{g/ml}$ . Partially purified antigens were diluted for testing to give an absorbance of 0.03 or 0.01 at  $280 \text{ m}\mu$  (approximately 30 and  $10 \mu\text{g/ml}$ , respectively, assuming  $E_{1\%}^{1\text{cm}} = 10$ ). Diluent buffer (physiologic phosphate NaCl at pH 7.5 containing 0.03% human serum albumin and 0.4% phenol) was used as negative control.

## RESULTS

### Antigenic composition of nondefatted and defatted 16-min extracts

The antigenic compositions of both nondefatted and defatted 16-min extracts were compared in terms of (1) nondialyzable solids, (2) content of known allergens, and (3) allergenic activity (Table I). Nondefatted pollens gave slightly lower yields of nondialyzable solids. Both types of extract were, however, strikingly similar in terms of antigen content and allergenic activity. For comparison, a defatted 24-hr extract was also analyzed, and its content of known allergens was found to be similar to that reported previously for nondefatted 4-day extracts.<sup>10</sup>

The antigenic composition of defatted and nondefatted 16-min extracts and defatted 24-hr extract were also analyzed more extensively by CIE (Fig. 1). All extracts were analyzed at the same pollen equivalence ( $40\% \text{ w/v}$ ). Anti-16-min extract serum detected an identical number and proportion of basic antigens in all three extracts. Much larger concentrations of acidic antigens were seen in the 24-hr extract. Because antiserum toward 16-min extract was not very potent toward acidic antigens, all of the extracts were also analyzed with anti-24-hr extract serum. Again, no difference was observed in defatted and nondefatted 16-min extracts in either acidic or basic antigens, but a much larger number and proportion of acidic antigens was again observed in 24-hr extract. These results show that a very large number and proportion of basic, but relatively few acidic, antigens are released very rapidly from the pollen at pH 6.3. An interesting observation was that the anti-24-hr extract serum reacted very poorly toward basic antigens.

Because the antigenic compositions of defatted and nondefatted 16-min extracts were indistinguishable, defatted pollen extract was used in all further studies. Because of a high proportion of basic antigens in the 16-min extract, cation-exchange chromatography was used as a first step toward purification, followed by gel filtration on Biogel P30 and Sephadex G50.

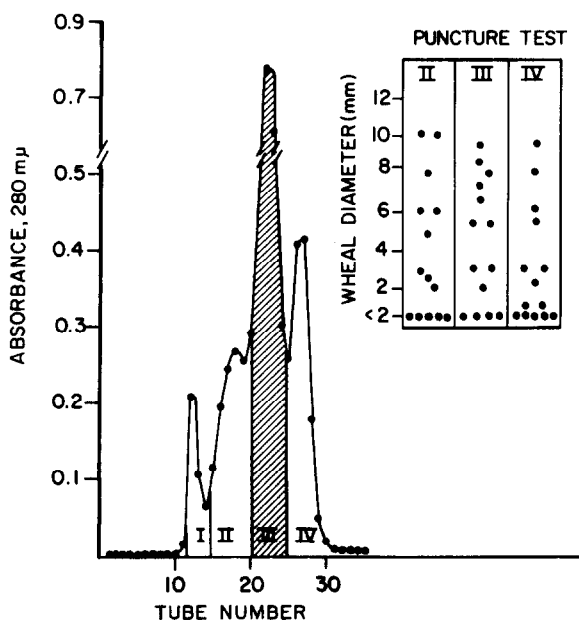


FIG. 3. Chromatography of fraction C8 on a Biogel P30 column (1.5 × 90 cm). Three-milliliter fractions were collected. *Inset*, skin reactivity of 14 patients to components present in individual peaks (tested at  $OD_{280m\mu} = 0.03$ ).

#### Cation-exchange chromatography

Partially depigmented 16-min extract was resolved into four major and several minor protein peaks on CM-Sephadex C25 using a gradient of increasing molarity of sodium phosphate buffer at pH 6.0 (Fig. 2). ID analysis using monospecific antisera localized antigen E in peak 1 and Ra3 in peak 2. Ra5 was found in three peaks (5, 6, and 9), with peaks 6 and 9 being well resolved. This indicates the possibility of at least two distinct isoelectric forms of this allergen. Gleich et al.\* have also made a similar observation. The only major peak devoid of detectable AgE, Ra3, and Ra5 by immunodiffusion analysis was C8, which was eluted with 0.109M buffer. This peak was further purified by gel filtration.

Fig. 3 shows the elution profile of fraction C8 on Biogel P30. Four protein peaks were obtained. The recovery of peak 1 was insufficient for detailed analysis. Peaks 2, 3, and 4 were highly allergenic by skin testing (puncture method). Although our tests revealed a wide distribution of allergenically active fractions, the only peak with allergenic activity obtained in sufficient amounts to be purified further was P30 III. This peak was rechromatographed twice on Sephadex G50, which resulted in a single symmetrical protein peak (G50 II), as shown in Fig. 4. Like fraction C8 from which it was derived, G50 II failed

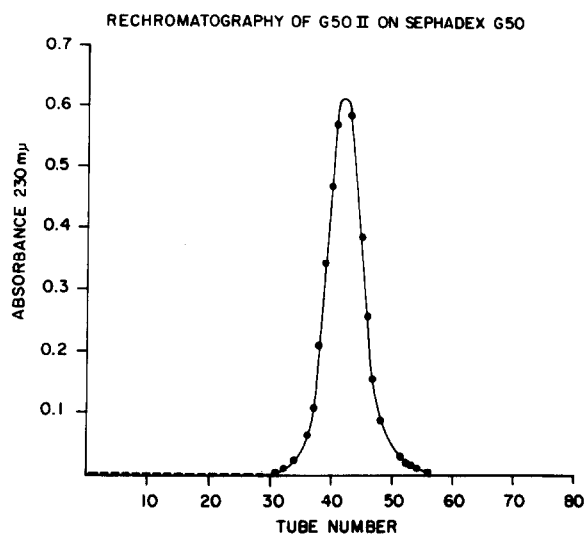


FIG. 4. Rechromatography of fraction G50 II on a Sephadex G50 column (1.5 × 90 cm).

to react with monospecific antisera to AgE, Ra3, and Ra5 in ID analysis.

#### Allergenic activity of G50 II

Fraction G50 II was tested by the puncture method in 22 ragweed-sensitive individuals who gave positive skin tests to both 16-min and 24-hr extracts (Fig. 5). Seventeen of 22 patients (77%) gave mean wheal diameters ranging from 2 to 6.5 mm. In addition, these individuals were also tested with AgE, Ra3, and Ra5. Higher frequencies of response to both Ra3 (90%) and Ra5 (40%) were observed than those previously reported.<sup>17, 18</sup> This is probably because individuals with very high sensitivity to 16-min as well as 24-hr extracts were selected for testing. Also, response to Ra5 may include reactions to impurities found to be present in this particular preparation by CIE analysis (unpublished observations). Each patient showed a characteristic spectrum of sensitivity to each of the individual allergens. For example, several individuals were highly sensitive to Ra3 but did not react to G50 II, and others reacted to G50 II but not to Ra5. Thus sensitivity to G50 II was independent of sensitivity to the other three allergens.

#### Characterization of G50 II

A highly symmetrical peak was observed on final chromatography of G50 II (Fig. 4), indicating homogeneity with respect to molecular size. Using the calibrated Sephadex G50 (fine) column, G50 II was estimated to have a molecular weight of 11,500 daltons ± 10%. A similar precision of molecular weight estimation by gel filtration was reported by An-

\*Gleich GJ et al: Personal communication.

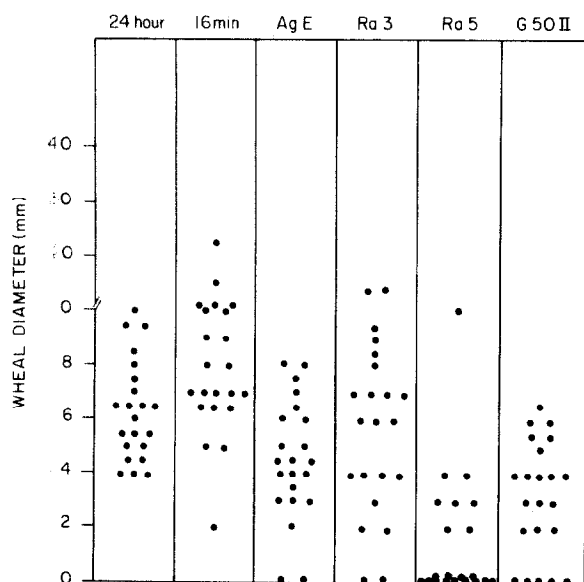


**FIG. 5.** Scattergrams of wheal reactions in 22 ragweed-sensitive subjects tested with 24-hr extract, 16-min extract, antigen E, Ra3, Ra5, and G50 II by the puncture method. Concentrations were 300  $\mu$ g/ml for the crude antigens and 10  $\mu$ g/ml for the purified antigens.

draws.<sup>11</sup> By SDS polyacrylamide gel electrophoresis, a molecular weight of 8,000 daltons was obtained for both reduced and nonreduced G50 II.

The purity of G50 II was further assessed by agarose electrophoresis and by CIE. Both techniques detected two highly basic components in differing proportions. Agarose electrophoresis showed that the more basic fraction was present in a larger amount than the other component. However, in CIE (Fig. 6) the most basic component reacted only weakly with antiserum to 16-min extract as seen by the faint peak "shooting off" the plate. On the other hand, the second relatively less basic component reacted very strongly, even though it was present in lower amount, indicating that it may be much more immunogenic in rabbits.

## DISCUSSION

We report here partial purification of a new allergen fraction, G50 II, which is apparently homogeneous with respect to molecular weight both by gel filtration and SDS gel electrophoresis. A molecular weight of 11,500 daltons was obtained by gel filtration, but SDS gel electrophoresis showed a single band with estimated molecular weight of 8000 daltons under both reducing and nonreducing conditions. Inasmuch as these values were confirmed in further experiments, it seems likely that G50 II behaves anomalously in one or both assays. Agarose and CIE revealed a major

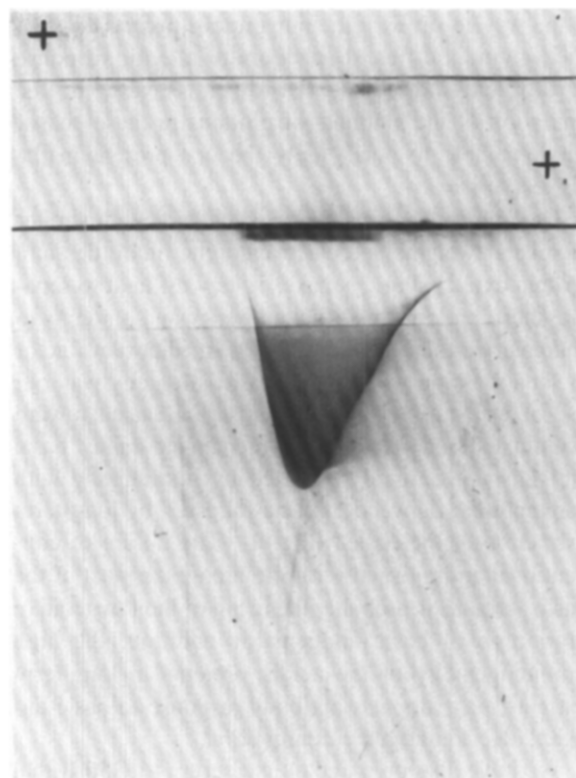


**FIG. 6.** Crossed immunoelectrophoresis of G50 II (15  $\mu$ g) against antiserum to 16-min extract. The anode is to the right in 1° and to the top in 2° dimension.

highly basic component and a minor relatively less basic component. It is interesting that it was the minor component that reacted most strongly with the rabbit antiserum to 16-min extract, indicating that it may be more immunogenic than the major component in rabbits. It will be of interest to see whether the same is true with respect to allergic response in humans.

Fraction G50 II was antigenically distinct from AgE, Ra3, and Ra5, as it failed to react with mono-specific antisera to all three allergens. Furthermore, unique patterns of allergic response to these purified allergens were observed in different ragweed-sensitive individuals. Characteristic "sensitivity spectra" or "allergic fingerprints" are regularly seen for different allergic people's responses to noncross-reacting allergens.<sup>16, 19</sup> Fraction G50 II also showed a much higher cathodic mobility in agarose electrophoresis than previously shown for Ra3 and Ra5.<sup>10</sup> In addition, G50 II was also distinct from Ra4 in terms of pI, molecular weight, and noncross-reactivity with antigen E.<sup>4</sup>

During our fractionation studies, skin testing with various fractions suggested unique allergens in several other peaks. For example, a CM-Sephadex fraction, C8, when chromatographed on Biogel P30 col-

umn, was resolved into four protein peaks (Fig. 3), each giving a different spectrum of allergic response in different individuals. However, because of the limitation of the amounts of proteins in these peaks, we were able to purify only the one present in the highest amount.

The presence of several basic antigens with similar molecular weights and electrophoretic mobilities in a 16-min extract (Fig. 1) makes it very difficult to separate these proteins in sufficient purity and amounts to characterize them further. The most important factor in identification of the new basic allergens was our use of an extremely potent antiserum raised toward 16-min extract. This serum recognized many basic antigens not normally detected by immunizing with conventional ragweed extracts prepared by long-term extraction of the pollen (Fig. 1). The failure of conventional antiragweed serum to recognize most of the basic antigens is probably the result of the low relative proportion of basic antigens. In addition, the high immunogenicity of the acidic proteins in long-term extracts may suppress antibody response to basic antigens due to antigenic competition.

We have also prepared an antiserum to G50 II that will facilitate further purification on a larger scale. The isolation of a further ragweed allergen will facilitate our understanding of the complexity of immune response to ragweed allergens. We are designating the major antigenic and allergenic component in G50 II as Ra6<sup>20</sup> and are in the process of completely purifying and characterizing it.

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