

## Extraction and analysis of coffee bean allergens

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*(Received 17 November 1977; accepted for publication 1 December 1977)*

### Summary

Workers in the coffee industry can develop occupational allergic disease upon exposure to dust associated with coffee manufacturing. Since controversy exists as to the source or chemical nature of these allergens, the mouse model of reaginic antibody production was used to assess the potential sources of allergens in samples obtained from a local coffee manufacturing plant. Mice were immunized with extracts of coffee dust and beans and the resulting reaginic antibody response determined by the passive cutaneous anaphylaxis reaction. Cross-reacting allergens were detected in samples of coffee dust, cleaner can debris and green coffee beans, but not in chaff or roasted coffee beans. None of the allergens detected in coffee samples cross-reacted with extracts of castor beans, although these extracts contained the potent castor bean allergen. Green coffee bean allergens partially purified by gel filtration were heterogeneous with respect to molecular size, although quite similar in their reactivity with reaginic antiserum. These results suggest that the green coffee bean is the major source of allergen in coffee manufacturing plants. This allergen is heterogeneous with respect to size and heat lability, and is immunochemically different from the castor bean allergen.

### Introduction

Workers in the coffee industry, especially those involved in the handling of green coffee beans, may develop occupational asthma, rhinitis or dermatitis upon exposure to dust present in coffee manufacturing plants (Bernton, 1973; Figley & Rawling, 1950). Although the evidence suggests that these workers are hypersensitive to allergens in coffee dust, there is considerable controversy as to their origin. Early studies suggested that castor bean may be a source of allergen, through contamination of coffee bean sacks which had been used to transport castor beans (Figley & Rawling, 1950). Later investigations reported that patients allergic to green coffee beans reacted

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with citrus fruits and castor beans (Freedman, Krupey & Schon, 1961; Freedman *et al.*, 1962, 1964b; Freedman, Shulman & Krupey, 1964a; Siddiqi & Freedman, 1963) and that this cross-reaction was due to the presence of chlorogenic acid in these different vegetable products (Freedman *et al.*, 1961, 1964b). Contrary to these findings are reports which suggest that the green coffee bean protein rather than chlorogenic acid is the major allergen in the green coffee bean (Layton *et al.*, 1961; Layton, Greene & Panzani, 1965a; Layton, Panzani & Greene, 1963; Layton *et al.*, 1965b). The current investigation was undertaken to analyse the allergenic potential of suspect allergens, to assess possible cross-reactivity between coffee and castor bean allergens and to purify coffee allergens.

## Materials and methods

### *Extraction of allergens*

The following samples were generously donated by a local coffee manufacturing plant: green coffee beans (Ethiopian), roasted coffee beans, debris removed from the green beans (cleaner can debris), chaff and two samples of coffee dust (a and b) collected at the coffee plant in the area where sacks containing green beans were opened. A sample of raw castor beans, Hale variety, was generously donated by NL Industries Incorporated, La Mesa, California. Prior to extraction, all bean samples were first homogenized for 2–3 min in a Waring blender at low speed. 100 g of each sample were extracted in 1 litre of 0.01 M phosphate-buffered 0.15 M NaCl at pH 7.2 (PBS) by homogenization for 2 min at low speed, and for 2–5 min at high speed. Following overnight extraction under constant stirring at 24°C, each extract was filtered through gauze and centrifuged (64,000 g) for 30 min. The upper lipid layer was removed and discarded and the remaining aqueous supernatants concentrated (Amicon UM10) to approximately 20 ml. The concentrated extracts were dialysed exhaustively against PBS (4°C) and then centrifuged (105,000 g) for 1 hr. The castor bean extract was heated at 100°C for 1 hr to destroy the ricin and was recentrifuged (105,000 g) for 1 hr. All supernatants were saved, the dry weight of the aliquots determined after the evaporation of H<sub>2</sub>O and corrected for buffer, and the remainder frozen and stored at –20°C.

### *Purification of allergens*

Green coffee bean extract (104 mg) was applied to a Bio-Gel A 0.5 m (100–200 mesh) column (2.5 × 87 cm) equilibrated with PBS. Fractions (3.4 ml) collected dropwise were analysed for protein by ultraviolet (u.v.) absorption at 280 nm. Aliquots of every ten fractions were pooled and assayed for allergenic activity by the passive cutaneous anaphylaxis (PCA) reaction in recipient mice passively sensitized with diluted antiserum to green beans. Fractionation of green coffee bean extract on the Bio-Gel column was repeated, so that a total of 641.6 mg extract was applied to the column. All fractions from each column containing allergenic activity were pooled, concentrated on an Amicon UM10, centrifuged (105,000 g) for 1 hr and the supernatant applied to a Sephadex ultra-fine G-100 column (2.5 × 97 cm), equilibrated with PBS. Fractions (6.8 ml) were collected dropwise and assayed for protein by u.v. absorption at 280 nm. Aliquots of every five fractions were pooled and assayed for allergenic activity, as described above.

### *Production of antiserum*

Six groups containing eight female 8 week old C3H/HeJ mice (Jackson Labs, Bar Harbour, Maine) were immunized with an intravenous (i.v.) injection of 20  $\mu$ g pertussis extract and an intraperitoneal (i.p.) injection of 400  $\mu$ g coffee extract. An i.p. booster injection of 40  $\mu$ g coffee extract was given 4 weeks later. A seventh group of mice was immunized with castor bean extract in the same manner. An eighth group of eight mice was immunized with 1  $\mu$ g green coffee bean extract complexed to 0.5 mg aluminum hydroxide in 0.5 ml every 2 weeks for a total of three injections. 1 week after the last injection, the animals were exsanguinated and blood samples from the individual mice within one group were pooled. The resulting serum was stored at  $-20^{\circ}\text{C}$ .

New Zealand male rabbits (2.25 kg) were immunized via the subcutaneous (s.c.) and footpad routes with either 10 mg green coffee bean, coffee dust or castor bean extracts in Freund's complete adjuvant (FCA). Two booster injections of 10 mg antigen in FCA were given s.c. every 2 weeks, followed by bleedings 12 days after the last injection. The serum was collected and stored at  $-20^{\circ}\text{C}$ . Due to its poor reactivity, castor bean antiserum was fractionated by 40% ammonium sulphate precipitation. Precipitated antibodies were resuspended in, and dialysed against, PBS so that the antiserum was concentrated ten-fold.

### *Passive cutaneous anaphylaxis (PCA) reaction*

Reaginic antibody was assayed by the PCA reaction of Ovary (1964). Diluted antiserum (diluent-PBS) in 0.05 ml was injected intradermally into the back of recipient 6 to 8 week old female CD-1 mice (Charles River). Test animals were challenged i.v. 48 hr later with 0.2 ml extract in 0.5% Evans Blue in PBS and killed 30 min later. A positive response was defined as the blueing of an area at least 5 mm in size on the subcutaneous surface of the skin. All samples of serum were tested at 1:10 dilution in duplicate and any positive sera were diluted and retested in triplicate. PCA titres generally varied at most by one dilution and were expressed as a geometric mean.

### *Absorption of mouse antiserum*

In order to test for cross-reactivity of different fractions of antigens isolated by gel filtration, the antiserum (1:5 dilution) was absorbed in the fluid phase with an equal volume of antigen (4 mg/ml) at  $24^{\circ}\text{C}$  for 2 hr. Controls consisted of antiserum treated with PBS. Samples were centrifuged (10,000 g) and the supernatants tested in the PCA reaction.

### *Immunodiffusion*

Gel diffusion was performed in 0.4% agarose (Sigma Chemical Company) in PBS. Wells were 5 mm in diameter separated by 3 mm distance. Approximately 0.06 ml of antiserum or antigen were added to the wells, as indicated, and the immunodiffusion plate incubated at  $24^{\circ}\text{C}$  for 24 to 48 hr.

## **Results**

### *Detection of coffee allergens*

Extraction of dust or bean samples with PBS yielded varying amounts of soluble material (Table 1). The weight recovery varied from 3.0% for roasted coffee beans to

**Table 1.** Extraction of coffee beans and related materials

| Sample             | Percentage extracted |
|--------------------|----------------------|
| Coffee dust (a)    | 0.4                  |
| Coffee dust (b)    | 0.6                  |
| Roasted beans      | 3.0                  |
| Green beans        | 1.9                  |
| Cleaner can debris | 0.6                  |
| Chaff              | 2.2                  |
| Castor bean        | 0.5                  |

0.4% for a dust sample. Analysis of these extracts for allergens was done by the immunization of mice with antigen and pertussis extract and the detection of specific IgE antibody in recipient mice challenged with 1 mg antigen in 0.2 ml 0.5% Evans Blue solution. Because some extracts elicited a non-specific blueing in unsensitized recipient mice at this dosage, lesser doses of material were tested in order to determine the optimal dose that would no longer elicit non-specific blueing. Since the greatest non-specific blueing was elicited by the extracts of roasted coffee bean, green coffee bean and chaff, only 100  $\mu$ g of these materials were used as the PCA challenge dose (Table 2). The least non-specific blueing was observed with extracts of cleaner can debris and castor bean and therefore 800  $\mu$ g of these samples could be used as a challenge dose in the PCA reaction. In order to conserve the castor bean extract, a 400  $\mu$ g dose was used.

Once optimal challenge doses of antigen for the PCA reaction were established, the antisera to coffee extracts were tested for reagenic antibody. The results (Table 2) demonstrate that the extracts varied considerably in their ability to elicit reagenic antibody production. Reagenic activity was demonstrated in the antisera to coffee dust and green coffee bean. All other fractions obtained from the coffee plant (roasted coffee bean, cleaner can debris and chaff) were clearly not active in eliciting reagen production. The castor bean extract was very allergenic and elicited the highest PCA titre of all extracts tested.

**Table 2.** Reagenic antibody response of mice immunized with coffee or castor extracts

| Antigen             | Challenge dose ( $\mu$ g) | PCA titre* |
|---------------------|---------------------------|------------|
| Coffee dust (a)     | 200                       | 40         |
| Coffee dust (b)     | 200                       | 80         |
| Roasted coffee bean | 100                       | < 10       |
| Green coffee bean   | 100                       | 80         |
| Cleaner can dust    | 800                       | < 10       |
| Chaff               | 100                       | < 10       |
| Castor bean         | 400                       | 2560       |

\* Geometric mean of pooled antiserum tested in triplicate.

**Table 3.** Reaginic antibody response of mice to green coffee or castor bean extracts

| Immunizing agent    | PCA titre after antigen challenge |             |
|---------------------|-----------------------------------|-------------|
|                     | Green coffee bean                 | Castor bean |
| Dust (a)            | 80                                | < 10        |
| Dust (b)            | 80                                | < 10        |
| Roasted coffee bean | < 10                              | < 10        |
| Green coffee bean   | 80                                | < 10        |
| Cleaner can debris  | 640                               | < 10        |
| Chaff               | < 10                              | < 10        |
| Castor bean         | < 10                              | 2560        |

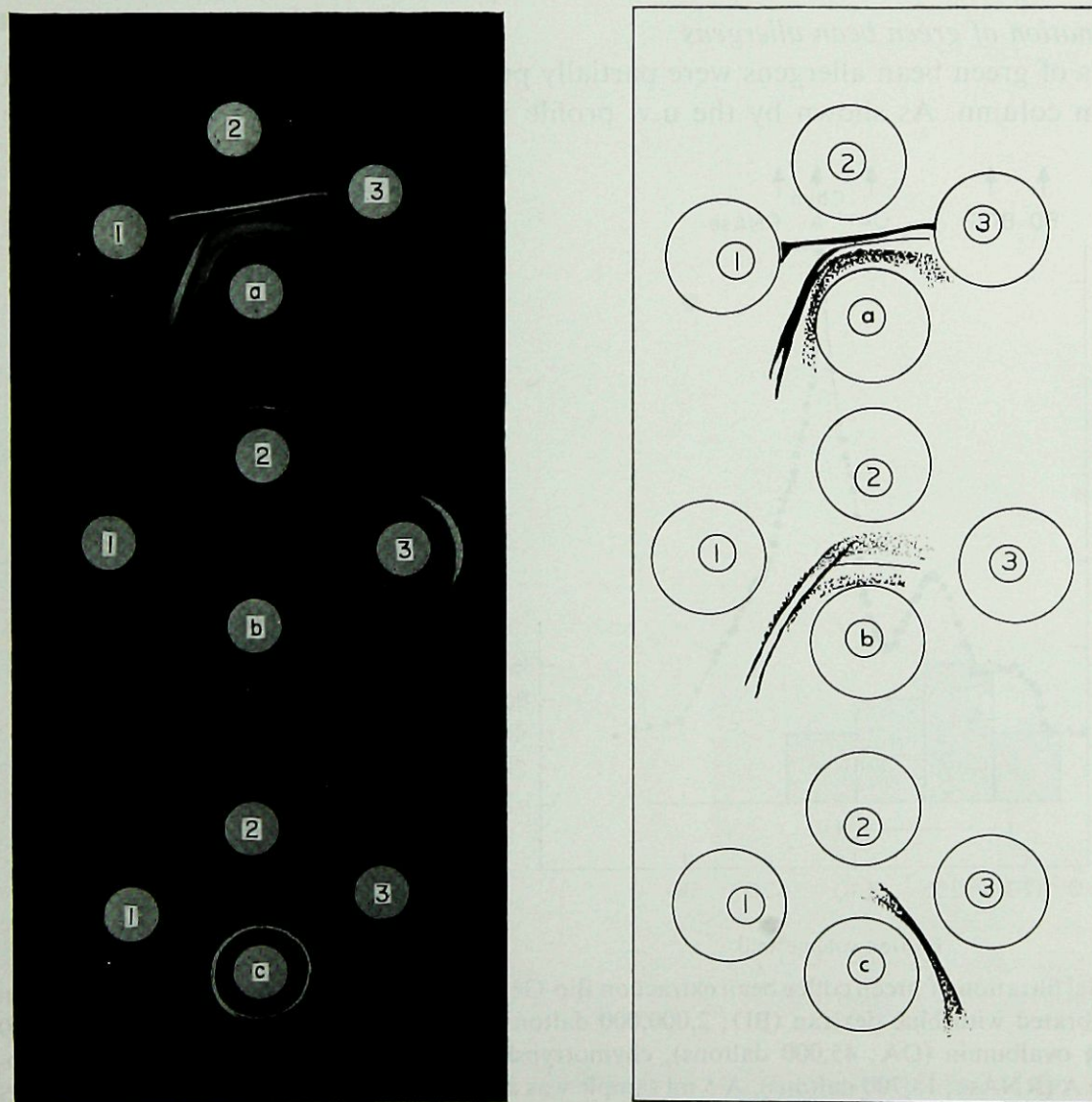


**Fig. 1.** Detection of cross-reacting antigens in green coffee bean, coffee dust and castor bean. (a) Antiserum to green bean; (b) antiserum to coffee dust; (c) antiserum to castor bean; (1) coffee dust (35–350  $\mu\text{g}$ ); (2) green coffee bean (70–700  $\mu\text{g}$ ); (3) castor bean (70–350  $\mu\text{g}$ ).

*Cross-reactivity of allergens*

Mice were sensitized with antisera that reacted with different extracts and then were challenged with green coffee bean or castor bean extracts. As summarized in Table 3, the green coffee bean extract elicited a PCA reaction with the antisera to dust and cleaner can debris, but not with the antisera to roasted coffee bean, chaff or castor bean. Furthermore, there was no cross-reactivity between the antisera to coffee samples and the castor bean extract, in spite of the fact that the castor bean extract contains potent allergens which elicit a significant castor bean-specific PCA reaction. This suggests that castor bean allergens do not contribute, either by contamination or by cross-reactivity, to the allergenicity of products obtained from the coffee bean plant.

These results were further supported with rabbit antisera to coffee dust, green coffee bean or castor bean. As shown in Fig. 1, the rabbit antiserum to coffee dust also reacted with green coffee bean antigens and the antiserum to green coffee bean reacted with dust antigens. However, neither antiserum reacted with castor bean antigens, nor did the antiserum to castor bean react with coffee dust or green bean antigens.

*Fractionation of green bean allergens*

Extracts of green bean allergens were partially purified by gel filtration on a Bio-Gel A 0.5 m column. As shown by the u.v. profile (Fig. 2), gel filtration of the coffee

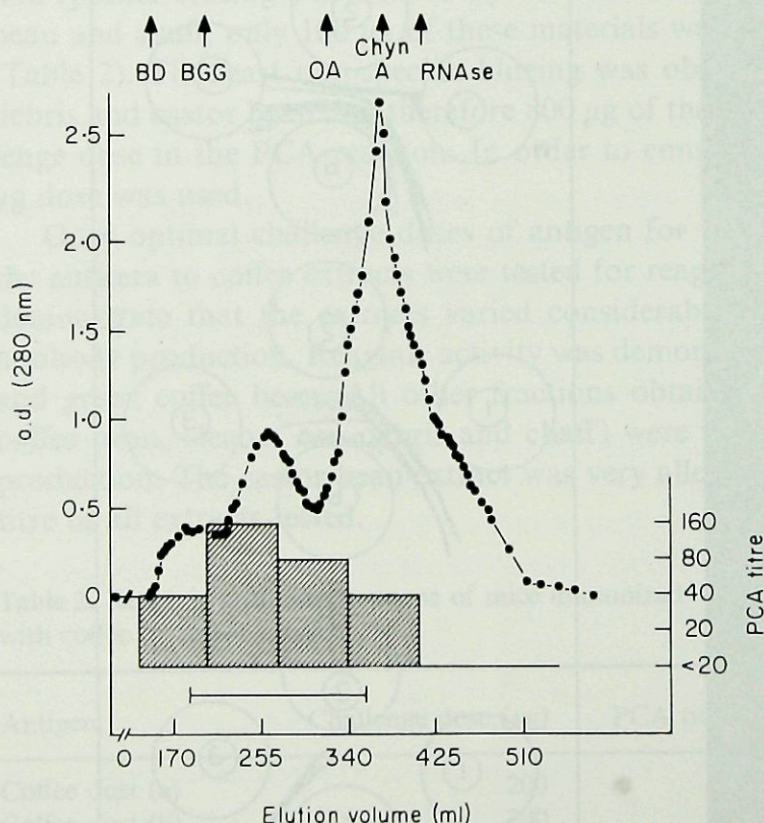


**Fig. 2.** Gel filtration of green coffee bean extract on Bio-Gel A 0.5 m column (2.5 × 87 cm). The column was calibrated with blue dextran (BD; 2,000,000 daltons), bovine gamma globulin (BGG; 160,000 daltons), ovalbumin (OA; 45,000 daltons), chymotrypsin A (Chyn A; 25,000 daltons) and ribonuclease A (RNase; 13,700 daltons). A 5 ml sample was applied to the column equilibrated with PBS at 4°C. 3.4 ml fractions collected dropwise were analysed for protein by u.v. absorption at 280 nm. Aliquots of every ten fractions were pooled and assayed for allergenic activity by the PCA reaction, and those with maximal activity were pooled (as indicated by the brackets), concentrated and applied to the Sephadex G-100 column. (▨) PCA titre; (●) o.d. (280 nm).

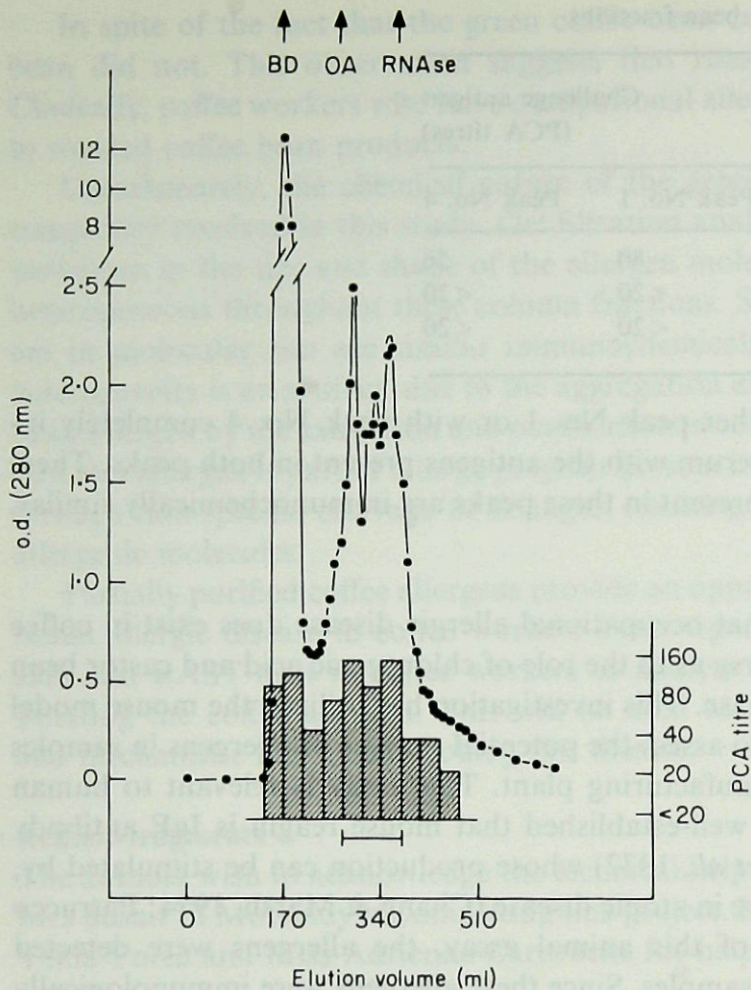


**Fig. 3.** Gel filtration of coffee allergens (obtained from the Bio-Gel column) on Sephadex G-100 column (2.5 × 97 cm). The column was calibrated with BD, OA and RNase. A 5 ml sample was applied to the column and 6.8 ml fractions were collected dropwise. Fractions were assayed for protein by u.v. absorption and aliquots of every five fractions pooled and assayed for allergenic activity. Fractions with maximal activity were pooled (as indicated by the brackets), concentrated and saved. (▨) PCA titre; (●) o.d. (280 nm).

bean extract revealed three peaks. The column fractions were pooled and assayed for allergenic activity by the challenge of mice sensitized with the antiserum to green bean antigens (produced in mice immunized with green coffee bean complexed to  $\text{Al}(\text{OH})_3$ ). Although maximal allergenic activity was associated with the second peak, activity was also present in the first and third peaks.

Fractions containing maximal allergenic activity were pooled, as indicated in Fig. 2, and rechromatographed on a Sephadex G-100 column (Fig. 3). Gel filtration of this extract revealed four peaks of u.v.-absorbing material. The allergenic activity was heterogeneous, since it was detected throughout much of the column eluate. There were several regions of maximal allergenic activity associated with the first, second and fourth peaks.

In order to determine if green coffee bean allergens fractionated on Sephadex G-100 column were antigenically similar, the antiserum to green bean allergens was absorbed with either the large molecular weight material in peak No. 1 (see Fig. 3) and challenged with small molecular weight fraction in peak No. 4, or absorbed with peak No. 4 and challenged with peak No. 1. The results (Table 4) indicate that the

**Table 4.** Cross-reaction of green coffee bean fractions

| Sensitizing antiserum plus<br>absorbing antigen | Challenge antigen<br>(PCA titres) |            |
|-------------------------------------------------|-----------------------------------|------------|
|                                                 | Peak No. 1                        | Peak No. 4 |
| Anti-green bean plus PBS                        | 80                                | 56         |
| Anti-green bean plus peak No. 1                 | < 20                              | < 20       |
| Anti-green bean plus peak No. 4                 | < 20                              | < 20       |

absorption of antiserum with either peak No. 1 or with peak No. 4 completely inhibited the reactivity of this antiserum with the antigens present in both peaks. These results suggest that the antigens present in these peaks are immunochemically similar.

### Discussion

Although it is well established that occupational allergic disease does exist in coffee workers, there has been controversy as to the role of chlorogenic acid and castor bean allergen contaminants in this disease. This investigation has utilized the mouse model of reaginic antibody production to assess the potential sources of allergens in samples obtained from a local coffee manufacturing plant. This assay is relevant to human IgE-mediated disease, since it is well-established that mouse reagent is IgE antibody (Lehrer, 1976; Prouvost-Danon *et al.*, 1972) whose production can be stimulated by, and can react with, allergens active in atopic disease (Chang & Marsh, 1974; Patrucco & Marsh, 1977). Through use of this animal assay, the allergens were detected primarily in dust and green bean samples. Since these allergens were immunologically similar, the dust allergens were probably derived from the green coffee bean.

The castor bean allergen did not react with any antisera to samples from the coffee plant (including green coffee bean), despite the ability of castor allergen to elicit its own potent reaginic antibody response in mice. These results were obtained not only by utilizing the PCA reaction, but also in immunoprecipitation reactions employing rabbit antisera to coffee dust, green coffee bean and castor bean. This observation suggests that castor bean allergens are immunochemically different from coffee bean allergens and are not present in the samples analysed from this coffee plant. Although this study cannot exclude heat-labile castor bean allergens (all the extracts of castor bean used in animal studies were heat-inactivated to destroy the ricin, which kills experimental animals), another investigation demonstrated that essentially all castor bean allergens are heat-stable (R.M. Karr *et al.*, in preparation).

The fact that no common allergen was demonstrated in coffee and castor beans suggests that chlorogenic acid does not play as significant a role as had been formerly proposed (Freedman *et al.*, 1961, 1964b). In fact, it is highly unlikely that the extracts tested in this study contained chlorogenic acid, since these extracts were extensively dialysed (chlorogenic acid is dialysable) and their u.v. absorption spectrum did not demonstrate its presence.

It is of interest that the antiserum to cleaner can debris did not react with its homologous antigen, but did give a significant PCA reaction with the green coffee bean extract. It is therefore likely that allergens (probably originating from the green coffee bean) are present in cleaner can debris in immunogenic amounts, but not in great enough quantity to elicit a positive PCA reaction.

In spite of the fact that the green coffee bean contained allergens, roasted coffee bean did not. This observation suggests that roasting destroys allergenic activity. Clinically, coffee workers who have occupational allergic disease are not hypersensitive to roasted coffee bean products.

Unfortunately, the chemical nature of the green coffee bean allergen cannot be completely resolved in this study. Gel filtration analysis suggests that there are many variations in the size and shape of the allergen molecules because the activity was so heterogeneous throughout these column fractions. Since fractions significantly different in molecular size are similar immunochemically, it may be that this observed heterogeneity is an artifact, due to the aggregation and complexing of the green coffee bean allergen by the extraction and purification procedures. Alternatively, it is possible that this allergen is part of a large polymer present in the green bean and is solubilized through non-specific cleavage of linkages, resulting in a heterogeneous population of allergenic molecules.

Partially purified coffee allergens provide an opportunity to investigate the occupational allergic disease in coffee workers more vigorously. We are currently utilizing skin and RAST tests in coffee workers to analyse allergen fractions in the hope of isolating the coffee allergen. This will be used to investigate further environmental and mechanistic factors controlling this disease.

### Acknowledgments

The authors wish to acknowledge the technical help of Mrs Katherine McCarron and Mrs Susan El Morshidy in completing this project. In addition, we wish to thank Miss Velda Force and Miss Adrienne Carrouche for help in the preparation of this manuscript.

Supported by NIH grants No. AI-13401 and HL-15092. Presented in part at the annual meeting of the American Academy of Allergy, New York City (1977).

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