

Multiplicity of allergens in peanuts

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Crude peanut protein fractions from raw and roasted peanuts were examined in the RAST with 10 sera from patients showing clinical peanut sensitivity. The radioactive uptake results, which were generally high, did not reveal any distinguishable pattern. Two commercially available peanut proteins, peanut lectin and phospholipase D, gave poor RAST responses. Three purified peanut proteins, α -arachin, conarachin I, and concanavalin A-reactive glycoprotein, all gave significant RAST results that were generally lower than those obtained with the crude extracts. The extent of RAST inhibition obtained with these materials was inversely related to their abundance in the total peanut protein. Crossed immunoelectrophoresis with extracts from raw and roasted peanut indicated the presence of 22 and 10 anodically migrating antigens, respectively. Sixteen IgE binding antigens were revealed for raw peanut and seven for roasted peanut after incubation with a mixed serum from the 10 patients in crossed radioimmunoelectrophoresis (CRIE) using ^{125}I -labeled anti-IgE. CRIE plates treated with individual serum samples showed that all the patients had specific IgE for the major antigen peak, which has been tentatively identified as α -arachin. This major storage protein of peanut, which is known to be particularly heat resistant, may be of greater clinical significance than its apparently low RAST activity would seem to indicate. (J ALLERGY CLIN IMMUNOL 72:61-68, 1983.)

The legume peanut is a food commonly eaten by both children and adults. In some countries its consumption accounts for a significant part of the total dietary protein. Despite its nutritional value, peanut can cause immediate hypersensitivity reactions such as angioedema and asthma in a significant number of allergic patients.¹⁻³ Among foods that produce such reactions, peanut is considered secondary only to milk and eggs in total numbers of patients affected.^{4, 5} A controlled study by Taylor et al.⁶ showed that allergic reactions were not elicited by peanut oil. Examination of a number of peanut products indicated that allergenicity was confined to the protein constituents.⁷

Legume sensitivity, unlike much reported food allergy, is often severe, and peanut sensitivity may sometimes lead to near-fatal anaphylaxis.⁸ Studies by Chua et al.⁹ and Hoffman and Haddad¹⁰ showed that up to 10% of allergic patients may exhibit some sensitivity to peanut. A recent report by Sachs et al.¹¹

detailed the isolation of what was described as a major peanut allergen, reactive in both skin tests and a histamine release assay. The allergenic material did not, however, account for all the allergenicity of peanut extract. Heiner and Neucere¹² used the RAST to show that peanut contains a number of allergens, some of which appear to be particularly heat stable.

The study reported in this paper was undertaken to define the relative allergenic importance of a number of peanut proteins from both raw and roasted peanuts. The proteins examined comprised the major peanut protein fractions, arachin and conarachin,¹³⁻¹⁵ and their respective principal constituents α -arachin¹⁶ and conarachin I,¹³ the homogeneous Con A-reactive glycoprotein,¹⁷ and two commercially available preparations, peanut agglutinin (lectin)¹⁸ and phospholipase D.¹⁹ These proteins, together with CPEs, were examined in RAST, and CRIE²⁰ was used to detect and identify IgE-binding antigens in the peanut extracts.

MATERIALS AND METHODS

Patients' sera

Sera were collected from patients with a clinical history of peanut sensitivity, exhibiting symptoms ranging from asthma and angioedema to acute anaphylaxis after ingestion (Table I). All patients gave positive skin prick tests (wheal diameter >5 mm) to a peanut prick test solution (Hol-

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Abbreviations used

CIE:	Crossed immunoelectrophoresis
Con A:	Concanavalin A
CPE:	Crude peanut extract
CRIE:	Crossed radioimmuno-electrophoresis
PAGE:	Polyacrylamide gel electrophoresis
RAST:	Radioallergosorbent test
SDS:	Sodium dodecyl sulfate

lister-Stier Laboratories, Spokane, Wash.) and to solutions of our own extracts. Phadebas (Pharmacia Fine Chemicals, Uppsala, Sweden) RAST was carried out on the sera, and 10 with classifications of from 1+ to 4+ were selected for examination. A mixed, control serum was prepared from five serum samples from nonatopic individuals.

Peanut preparations

Raw shelled peanuts of the Virginia Bunch variety were supplied by the Peanut Marketing Board, Kingaroy, Queensland. To facilitate manual removal of the skins, peanuts were heated gently (45°/5 min). Skinned peanuts were finely ground and defatted by Soxhlet extraction with petroleum ether 40° to 60°. Defatted peanut flour was extracted in the following ways:

1. *CPE-raw*. This was obtained by stirring the flour for 4 hr with 0.1M ammonium bicarbonate solution (pH 8.0), 1:9 w/v. Insoluble debris was removed by centrifugation at 5° (20,000 rpm/30 min). The supernatant was filtered through glass wool and lyophilized.

2. *CPE-roast*. This was obtained by similar treatment of peanuts which had been heated, prior to defatting, to simulate commercial roasting (145°/1 hr).

3. *Crude arachin*. Crude arachin was prepared from peanut flour by extraction with 0.01M phosphate buffer, 10% w/v in sodium chloride and 0.02M in sodium azide (1:9 w/v). After stirring for 4 hr the debris was removed by centrifugation as above. The supernatant was made to 40% saturation of ammonium sulfate and kept at 2° overnight. The resultant precipitate was redissolved in the minimum volume of phosphate buffer and dialyzed against distilled water before lyophilization. Arachins were prepared from both raw and roasted peanut flours.

4. *Crude conarachin*. This was precipitated from the crude arachin supernatant by bringing the solution to 85% saturation of ammonium sulfate. Precipitation was complete after 16 hr at 2°. Conarachin was collected by centrifugation (3500 rpm/30 min), redissolved in buffer, and lyophilized after dialysis against distilled water. A crude conarachin was also prepared from roasted peanuts.

5. *α-Arachin*. This was prepared by a modification of the method of Neucere.¹⁶ Crude arachin preparation from raw peanut (500 mg) was dissolved in 0.1M phosphate buffer (pH 8.0) containing 0.02% sodium azide (1:20 w/v), and insoluble material was removed by centrifugation. The clear solution was allowed to stand for 18 hr at 2° and the

resultant cryoprecipitate collected by centrifugation (20,000 rpm/30 min/2°). The precipitate was redissolved in the phosphate buffer and applied to a column, 1.6 by 30 cm, of DEAE-Sephacel (Pharmacia) anion exchange resin that had been equilibrated with the buffer. Protein was eluted by a linear gradient, over 400 ml, of sodium chloride (0M to 0.5M) in the phosphate buffer. Fractions of the eluate corresponding to the major peak of absorbance at 280 nm emerged at 0.45M sodium chloride and were collected, dialyzed against distilled water, and lyophilized.

6. *Conarachin I*. Conarachin I was prepared from crude conarachin derived from raw peanut (500 mg) by a procedure adapted from that of Yu.²¹ The crude conarachin was dissolved in 0.01M phosphate buffer (pH 8.0) containing 0.02% sodium azide (1:20 w/v). The solution was chilled and kept overnight at 2° to precipitate any residual arachin, which was removed by centrifugation. The supernatant was made to 65% saturation with solid ammonium sulfate and stirred at 25° for 1 hr, and the precipitate was collected by centrifugation (10,000 rpm/20 min). The solid was redissolved in buffer and dialyzed overnight against buffer. The dialysate was then made to 40% saturation of ammonium sulfate and stirred at 2° for 1 hr. The resultant precipitate was removed on the centrifuge and the clear supernatant kept overnight at 2°. The chilling produced a settled precipitate from which the supernatant was decanted for dialysis against the phosphate buffer. The dialysate was applied to a DEAE-Sephacel column and treated as described for α-arachin. The major peak, eluting at about 0.2M sodium chloride, was collected, dialyzed, and lyophilized.

7. *Con A-reactive peanut glycoprotein*. This was separated by dissolving CPE-raw (250 mg) in 0.1M ammonium bicarbonate solution. The sample was applied to a column (1 by 12 cm) of Con A-Sephacel (Pharmacia) equilibrated with the buffer. Elution with buffer was continued until the absorbance at 280 nm of eluate returned to zero. The bound material was then eluted with a 0.2M solution of α-methyl D-mannopyranoside in the bicarbonate buffer. The eluate was dialyzed against distilled water and lyophilized. Eluted material (50 mg) from a number of runs was combined, dissolved in 1 ml of 0.1M ammonium bicarbonate buffer, and applied to a column (1.6 by 30 cm) of Sephadex G-75 (Pharmacia). The major peak of absorbance at 280 nm, eluting just after the void volume, was collected, dialyzed, and lyophilized.

8. *Peanut phospholipase D*. This was purchased from Sigma Chemical Co., St. Louis, Mo.

9. *Peanut agglutinin*. This was purchased from P-L Biochemicals, Inc., Milwaukee, Wisc.

RAST examinations

These were carried out as per the Phadebas procedure. Allergen discs were prepared by coupling, via the cyanogen bromide activation procedure of Ceska et al.,²² the various extracts and fractions (100 µg/disc) to 6 mm diameter discs punched from Whatman grade 42 filter paper (Whatman, Inc., Clifton, N.J.). Phadebas peanut allergen discs were used, as purchased, for comparative purposes.

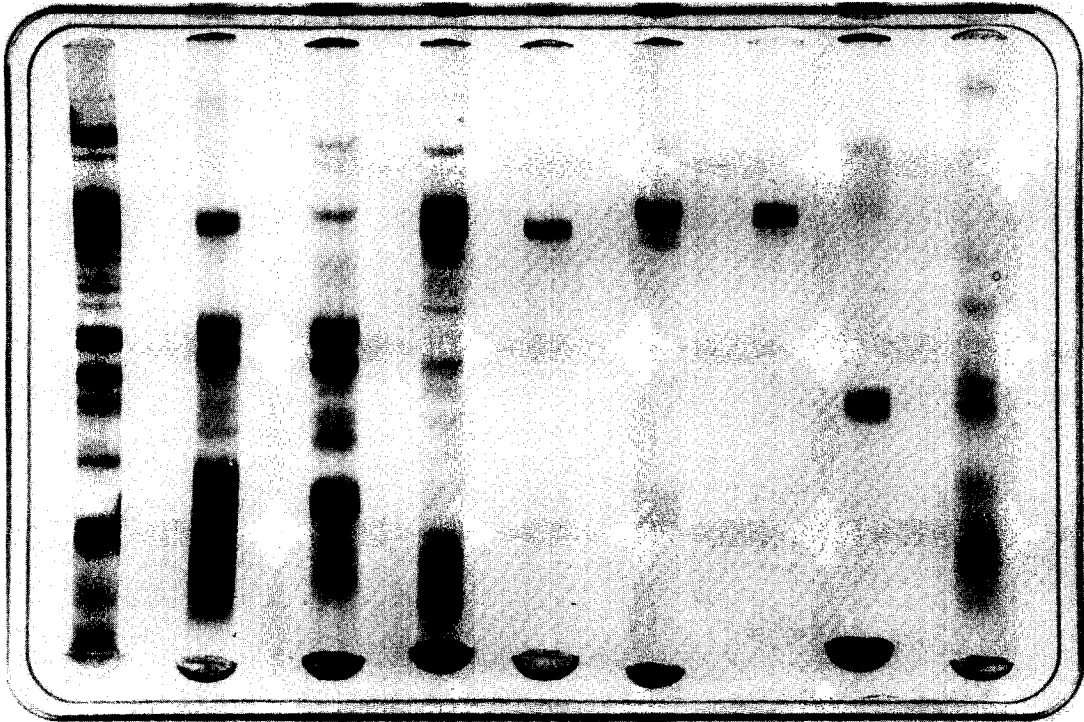


FIG. 1. SDS electrophoresis of peanut extracts and preparations (*left to right*) CPE-raw, CPE-roast, crude arachin from CPE-raw, crude conarachin from CPE-raw, α -arachin, conarachin I, Con A-reactive glycoprotein, peanut agglutinin, peanut phospholipase D. The SDS-proteins applied at the top migrated downward toward the cathode. The molecular weight range of the bands in these gels ranges from about 80,000 daltons near the top to around 10,000 at the bottom.

TABLE I. Peanut-sensitive patient data for serum samples used in this study

Patient	Sex	Age (yr)	Symptoms after peanut ingestion	Other food sensitivity (RAST positive)
LR	F	9	Angioedema	Egg, corn, soybean
PJ	M	10	Asthma, angioedema, anaphylaxis	Legumes, egg
AM	F	5	Angioedema	Cereals
AK	M	13	*	Egg
CP	F	4	*	Egg
JF	M	25	*	Egg, yeast
RD	M	17	*	Wheat, oat
CC	M	15	*	Milk, egg, wheat
MM	F	33	*	Egg, fish
MX	M	9	*	—

*These peanut-sensitive patients were identified by skin prick test and atopic history. In the absence of challenges, precise identification of symptoms due to peanut was not possible, but these patients attributed symptoms of asthma, wheezing, urticaria, or rhinitis to ingestion of peanuts.

RAST inhibition

The relative IgE-binding capacity of the three purified proteins, α -arachin, conarachin I, and Con A-reactive peanut glycoprotein, was measured by the method of Gleich *et al.*²³ Mixed patient serum (100 μ l) was incubated overnight at 2° with 100 μ l of RAST incubation buffer (Phadebas) containing 0.1 to 50 μ g of the suspected inhibi-

tor. Fifty-microliter aliquots of these serum mixtures were added to RAST discs prepared from CPE-raw and the RAST then performed according to the Phadebas procedure. Serum diluted with buffer only was used as a control and the determinations were carried out in duplicate. The inhibition was calculated by dividing the respective radioactive counts bound to an inhibited disc by the counts obtained with the

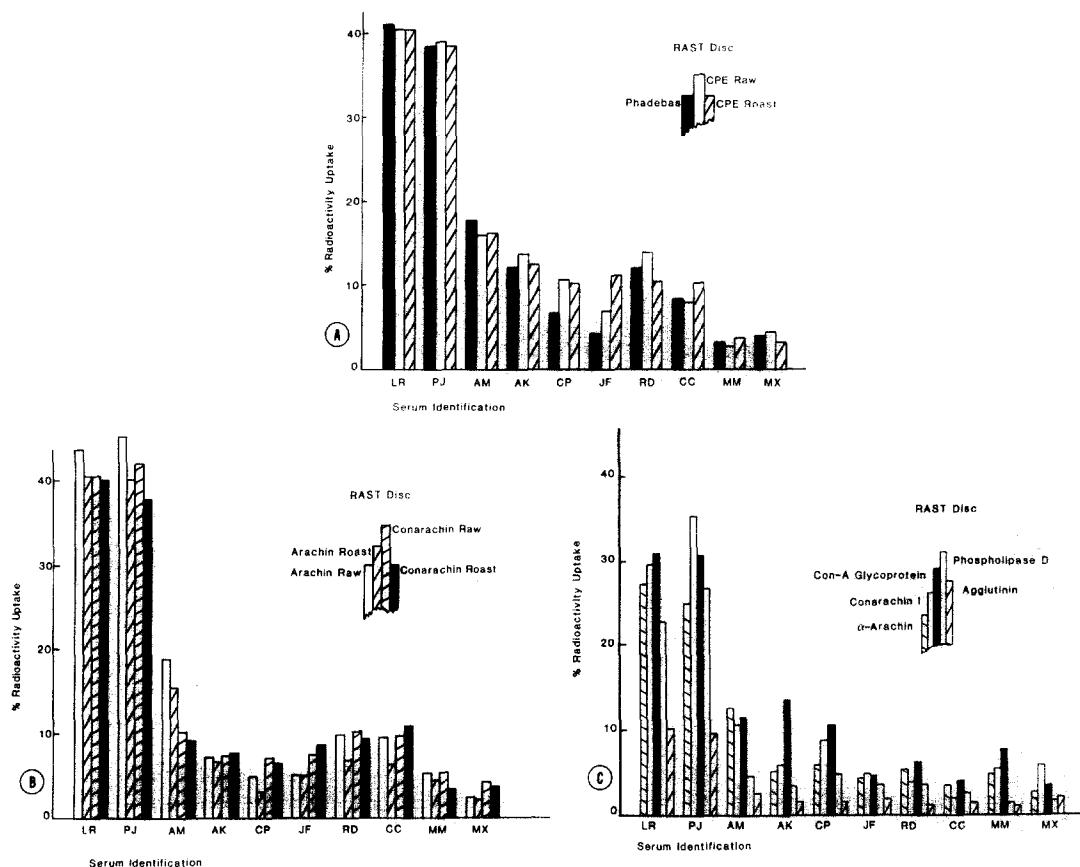


FIG. 2. RAST examination of peanut extracts and proteins for the presence of specific IgE antibodies in serum samples from 10 peanut-sensitive patients.

control, expressed as a percentage. A semilogarithmic plot enabled an estimate of the concentration of inhibitor required for 50% inhibition to be made.

Antiserum to CPE-raw

Four rabbits were immunized with 1 ml each of a solution containing 5 mg of CPE-raw in 0.5 ml of sterile water mixed with 0.5 ml of complete Freund's adjuvant. Injections were made at multiple sites on the back of the rabbit and were repeated three times at 21-day intervals. A blood sample was taken 12 days after the third injection. Subsequent samples were taken 12 to 14 days after booster injections, containing 2 mg of CPE and incomplete Freund's adjuvant.

SDS-polyacrylamide gel electrophoresis

Electrophoresis was carried out according to the method of Segrest and Jackson,²⁴ with all the peanut extracts and fractions. Samples of 50 μ g were applied to the gels for electrophoresis, after which the proteins were fixed in 12% trichloroacetic acid and stained with Coomassie blue.

Crossed immunoelectrophoresis

Peanut antigens were examined on glass slides, 8 by 10 cm, as outlined by Weeke and Lowenstein²⁵ and adapted to

the LKB 2117 Multiphor apparatus.²⁶ Agarose gels (1% w/v) were made in a buffer of 0.073M Tris, 0.024M diethylbarbituric acid, 0.006M calcium lactate, and 0.003M sodium azide (pH 8.6/25°), which was also the electrophoresis buffer. The first-dimension electrophoresis was made with a 5 μ l sample of CPE (50 mg/ml), placed in a 2.5 mm diameter hole punched at the cathodic end of the gel strip, which had the dimensions 20 by 80 by 1.5 mm. The second-dimension electrophoresis was run into antibody-containing gel (1 ml of rabbit antiserum with 9 ml of melted gel) cast adjacent to the first and measuring 80 by 80 by 1.5 mm. The running conditions were 15° and 28 V/cm for 2.5 hr and 7.5 V/cm for 20 hr in the first and second dimensions, respectively. The slides were pressed, washed, and dried before staining with Coomassie blue. Slides that were to be radiolabeled were dried with cool air and not stained before autoradiography.

Crossed radioimmuno-electrophoresis

Detection of peanut allergens was performed by a method similar to that of Lowenstein et al.²⁷ CIE slides were incubated at 25° for 24 hr with patient serum (0.5 ml diluted to 15 ml with RAST incubation buffer). The slides were washed with 0.9% w/v sodium chloride solution, four times for 10 min, and then incubated with ¹²⁵I-labeled anti-IgE (Phadebas RAST isotope, approximately 250,000 cpm, so-

lution diluted into 15 ml incubation buffer) for 24 hr at 25°. The radiolabeled slides were washed four times for 10 min with 0.9% w/v sodium chloride solution and once with distilled water. The slides were dried and the labeled peaks were detected by enhanced autoradiography as described by Laskey and Mills.²⁸ The film (Kodak X-Omat RP; Eastman Kodak Co., Rochester, N.Y.) was preflashed, placed in contact with an intensifying screen (Cronex Lightning Plus; DuPont Instruments, Wilmington, Del.), sandwiched with the labeled slides in a light-tight cassette, and placed at -78° for periods ranging from 24 hr to 10 days. After exposure the film was developed according to the manufacturer's instructions.

RESULTS

Examination of peanut preparations by SDS-PAGE

Fig. 1 shows the band patterns obtained after reduction and SDS-PAGE of the various peanut preparations. The complexity of the crude extracts and fractions can be seen, with an apparent diminution in the number of bands after roasting. The three apparently homogeneous fractions showed a single main band, although there were faint secondary bands present in the conarachin I sample. Commercial peanut agglutinin appeared to be homogeneous and of the expected molecular weight of 29,000 daltons.²⁹ However, the phospholipase D showed a number of bands, which may be contaminants or subunits, as described for the preparation of Tzur and Shapiro.¹⁹ Comparison of these results with the SDS-PAGE of peanut I¹¹ indicated that none of these materials corresponded with that particular allergen.

Detection by RAST of IgE antibodies specific to peanut proteins

The RAST was used with discs that had been reacted with raw and roasted crude peanut extracts, arachin and conarachin preparations, Con A-reactive glycoprotein, peanut agglutinin, and phospholipase D. In these direct RAST studies, discs were tested with a control consisting of mixed sera from five nonallergic subjects. Radioactive uptakes with control discs made with CPE-raw, CPE-roast, crude arachin, and crude conarachin were found to be 0.82%, 0.95%, 0.80%, and 0.92%, respectively. Radioactive uptakes obtained with the raw and roasted crude preparations tested with sera of the peanut-sensitive patients were comparable with the values obtained with the commercial allergen discs (Fig. 2, A).

Results for the crude fractions arachin and conarachin from both raw and roasted peanuts revealed no consistent pattern of reactivity with the different sera tested. As with the crude whole extracts, radioactive uptakes were generally high (Fig. 2, B).

The three purified preparations, α -arachin, con-

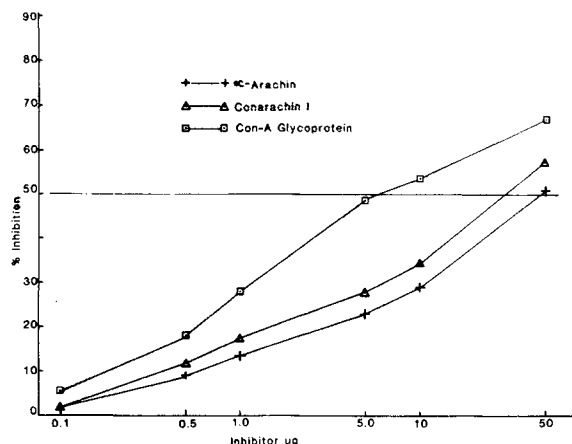


FIG. 3. RAST inhibition results with discs coupled with CPE-raw (100 μ g/disc). A mixed serum from the 10 peanut-sensitive patients was preincubated with quantities of α -arachin, conarachin I, and Con A-reactive glycoprotein. The graph indicates the extent of inhibition of serum IgE binding to the CPE-raw discs after incubation.

arachin I, and Con A-reactive glycoprotein, each reacted strongly with the sera tested, but again no obvious pattern emerged for the different preparations (Fig. 2, C).

IgE binding to discs coupled to the commercial peanut proteins, phospholipase D and peanut agglutinin, was weak with only the two most potent sera, L. R. and P. J., showing uptakes in the region of, or greater than 10% (Fig. 2, C). This result also indicated that competitive binding of IgE by the peanut lectin would appear not to be a problem in the other RAST experiments.

RAST inhibition with purified peanut proteins

The Con A-reactive glycoprotein proved to be the most potent of the three purified preparations in inhibiting the reaction between CPE-raw-treated discs and IgE antibodies in the allergic sera tested (Fig. 3). The weights per RAST disc of glycoprotein, conarachin I, and α -arachin giving 50% inhibition were 6, 30, and 47 μ g, respectively.

Crossed immunoelectrophoresis

Typical patterns obtained with the crude extracts are displayed in Fig. 4, A and B, where the antigenic complexity of the extracts is revealed. Twenty-two peaks were distinguishable on the original plate in the case of CPE-raw, but in CPE-roast, only about 10 remained. The apparent loss of some antigens was accompanied by changes in relative peak heights. Tentative assignment of the peaks, 1 for α -arachin and 2 for conarachin I as marked on Fig. 4, A, was made by preliminary spiking experiments and by ref-

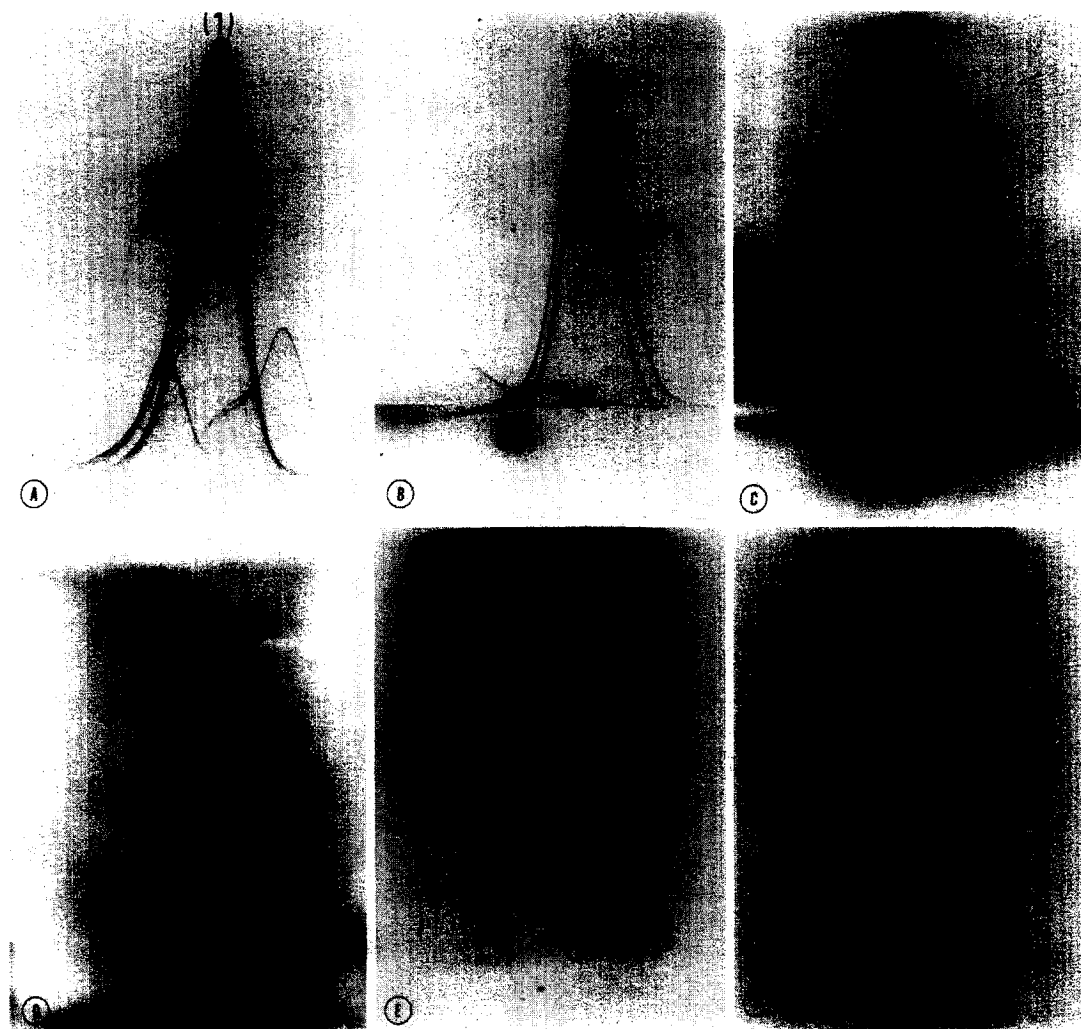


FIG. 4. CIE and CRIE of peanut extracts. The illustrations show the anodically migrating antigens in the first dimension left to right (anode to right) and second dimension (anode at top). **A** and **B**, CIE, showing the precipitated, stained antigen-rabbit antibody complexes produced with CPE-raw (**A**) and CPE-roast extracts (**B**). Incubation of plates **A** and **B** with a mixed serum and subsequent labeling with ^{125}I -anti-IgE enabled the CRIE autoradiographs (**C** and **D**) to show specific IgE binding to antigen peaks. Incubation with weakly RAST-reactive individual sera (radioactive uptake <10%) gave two patterns of antigen binding seen in the typical autoradiographs, **E** and **F**. Peaks (1) and (2) marked on (**A**) were tentatively identified as α -arachin and conarachin I, respectively.

erence to the immunoelectrophoretic patterns of Daussant et al.³⁰

With a mixed serum sample, CPE-raw and CPE-roast showed 16 and seven allergens, respectively, in CRIE experiments (Fig. 4, **C** and **D**). Individual sera having RAST radioactivity uptakes greater than 15% (three) gave autoradiograms on CPE-raw CIE plates similar to that in Fig. 4, **C**. The remaining sera appeared to fall into two groups: four principally binding conarachin I together with arachin (Fig. 4, **E**) and three binding arachin and a clearly defined peak on the anode side of arachin (Fig. 4, **F**). No labeling of

peaks was apparent when the mixed nonatopic serum was used as a control.

These results refer to anodically migrating allergens only. In preliminary experiments, four weakly precipitating, apparently minor antigens were found to migrate cathodically but were not investigated further.

DISCUSSION

The results obtained in this study indicate that the allergenicity of peanut, in terms of specific IgE binding, is spread through both crude arachin and con-

arachin fractions and that roasting of peanuts produces little overall change in allergenicity as measured by the RAST. It is common practice to use raw peanut extracts for skin prick tests, but most patients, particularly children, may have been sensitized to roasted material in products such as peanut butter.³¹ The RAST does not seem to distinguish between the extracts of raw and roasted peanut, but recent extensive skin prick tests^{31a} using the extracts described in this paper, indicate that a smaller number of patients give a positive reaction to the roast extract than to raw extract. The reason for the apparent difference between the results of RAST and skin prick tests is unclear at present but may be related to changes in allergen solubilities, as indicated in our CIE results. Nordlee et al.⁷ reported the results of RAST inhibition studies using a variety of peanut products, which had been extensively heat processed. It was shown that only hydrolyzed peanut protein and peanut oil⁶ failed to inhibit the RAST. The remarkable thermal stability of peanut allergens demonstrated in that study and also that of Heiner and Neucere¹² is largely confirmed by the results reported here.

Research directed specifically at isolating food allergens has been limited as compared to the efforts devoted to the pollen allergens.^{32, 33} With regard to legumes other than peanut, allergens from the green pea have been localized in the albumin fraction of pea proteins,^{34, 35} and recent work by Shibasaki et al.³⁶ has implicated the 2S-globulin of major soybean proteins as a most potent allergen by RAST. Peanut I,¹¹ described as a major allergen from peanut, was prepared from raw peanut extract by ion-exchange chromatography and preparative electrophoresis and was selected by a high RAST activity in fractions from the ion-exchange elution. There was, however, significant RAST activity over almost the entire chromatographic elution profile, indicating the presence of other allergens in peanut extract.

Preliminary experiments used gel filtration in an attempt to locate allergens of particular molecular weight groupings and indicated a spread of activity in eluates from approximately 300,000 to 10,000 daltons, with no particular group predominating (D. Barnett, unpublished work). This result may have been due, at least in part, to the association-dissociation behavior of the major proteins arachin and conarachin.^{13, 14} Whatever the cause, the result gave little encouragement for separation of particular allergens by such technique. The lack of localization of allergens shown by this and, to some extent, by the work of Sachs et al.¹¹ led to the testing of the purified preparations listed in this paper. This approach, of using known and partly characterized major peanut

proteins, was suggested by the work of Baldo et al.³⁷ and the findings of Moroz and Yang³⁸ in respect of the Kunitz soybean trypsin inhibitor as an allergen. The three separated and purified peanut protein preparations all showed significant RAST activity (Fig. 2, C). RAST inhibition testing with these materials revealed an interesting picture. The potency of the inhibitors is inversely related to the concentration of inhibitor found in peanut extracts, α -arachin constituting about 20% and conarachin I about 9%,²¹ whereas the Con A-reactive glycoprotein is said to form about 1%¹⁷ of the soluble protein of peanuts. The respective amounts of inhibitor required for 50% inhibition were 47, 30 and 6 μ g per RAST disc. One inference that is consistent with these figures is that each of these materials may have comparable allergenic potency and the observed clinical effect depends on a combination of allergenicity and quantity (or dose) presented to the sensitive patient's immune system.

This dose-potency hypothesis is, perhaps, also pertinent to the reasons for the severity of hypersensitivity reactions frequently seen with legumes. These plant foods are particularly rich in storage proteins. Such proteins are probably much less heterogeneous than the functional proteins of meats, for instance. Furthermore, legume protein digestibility is poor due to difficulties of hydrolysis by gastrointestinal enzymes and to the presence of specific enzyme inhibitors.³⁹ The quantity of a particular legume protein actually presented to a patient, and able to elicit an allergic response, may thus be relatively large.

The CRIE preliminary study reported here seems to support a dose-dependent sensitivity relationship, at least in terms of IgE binding. The major peak, arachin, was bound by every serum and is the major storage protein of peanuts. Although RAST and RAST-inhibition measurements indicated that arachin may not be a highly allergenic material per unit of weight, it does seem to be an important allergen for the 10 patients tested.

Neucere et al.⁴⁰ showed that α -arachin is antigenically unaltered by dry roasting of peanuts at 145° for 1 hr and is thus present in large amounts in both raw and roast peanuts. However, after roasting, the quantity of extractable total protein fell by about 60%. Changes observed in the relative peak heights of the CIEs for CPE-roast vs. CPE-raw may be due to such protein solubility changes being differential in nature.

Much further work is required to identify and characterize the other major antigens of the CIE, in particular that one reacting with Con A. The suggestive picture of serum IgE specificity also needs further exploration with larger numbers of sera.

In regard to identifying major allergens from peanut, this report does indicate that the three apparently homogeneous peanut protein preparations are all active by RAST and may be significant allergens, but do not represent the total spectrum of IgE-reactive antigens, as indicated by CRIE. Further clinical and biochemical examination of these and other peanut allergens is in progress.

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