

Antigens and allergens from the common house dust mite *Dermatophagoides pteronyssinus*

Part I. Demonstration of multiple allergens by immunochemical and biologic analyses

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The complexity of a highly allergenic water-soluble extract of the common house dust mite Dermatophagoides pteronyssinus has been investigated by use of the preparative fractionation techniques of ultrafiltration, gel permeation chromatography, and isoelectric focusing, and the allergenic properties of the resultant fractions are assessed by skin prick tests, RAST, and a histamine-release assay. In addition, two analytical approaches were used to identify allergens—radioimmuno-electrophoresis and a new technique for detecting IgE-binding proteins separated by isoelectric focusing and transferred onto a nitrocellulose membrane by transverse electrophoresis. These various techniques for allergen detection revealed differences in the number and distribution of allergens in the crude mite extract. The data obtained are consistent with the conclusion that allergenic activity of D. pteronyssinus extracts is due to the existence of a number of antigenically distinct allergens and not due to a single, major allergen. (J ALLERGY CLIN IMMUNOL 74:132-41, 1984.)

It is now well-established that the common house dust mites of the genus *Dermatophagoides* are a source of some of the most important and ubiquitous allergens involved in allergic disease.¹⁻⁸ Despite this, progress in the identification of mite allergens has been slow. The first pure house dust-mite allergen isolated was prepared by direct reaction of crude mite extracts with the lectin tridacnin.⁹ Although the isolated material reacted strongly with IgE antibodies in

Abbreviation used

MW: Molecular weight

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the RAST, it was only weakly active in skin prick test studies.¹⁰ Isolation of an allergen, designated antigen P₁ from *D. pteronyssinus*, was recently reported by Chapman and Platts-Mills¹¹ who claim that antigen P₁ is the major allergen from this species. Allergen P₁, a glycoprotein of MW 24,000, was found to exist in at least two isoallergenic forms and may be identical with allergen Dpt 12, recently identified.^{12, 13} From the limited clinical evaluation of antigen P₁ undertaken in only eleven patients,¹¹ the lack of data identifying clinically important mite allergens, and the absence of comparative studies with other purified mite allergens, the claim for the isolation of the major house dust-mite allergen must, for the present, be treated with caution.

TABLE 1. RAST-inhibition results by use of *D. pteronyssinus* Amicon filtration fractions together with serum from a patient allergic to the house dust mite and discs coupled with *D. pteronyssinus* crude extract

Amicon filtration fractions (MW)	Quantity of fraction (μ g)* needed to produce 50% inhibition of the uptake of IgE antibodies from allergic serum†
50,000 to 100,000	2
>100,000	4
20,000 to 50,000	8
10,000 to 20,000	50
5000 to 10,000	60
500 to 5000	88
>500	‡

*Total weight.

†Detected as 50% inhibition of the uptake of ^{125} I-labeled anti-IgE.

‡No inhibition detected up to 200 μ g.

Our detailed studies of house dust-mite allergens, described in this and the following articles, reveal the antigenic and allergenic complexity of this species and clearly establish that a number of "major" or clinically important allergens exist. These allergens must be taken into account when the role of *D. pteronyssinus* is assessed in provoking immediate allergic reactions in man.

MATERIAL AND METHODS

House dust mite extracts

Dead, "media-free" mites and a sample of spent medium used to grow the mites were obtained from Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Aqueous extracts were prepared as described.¹⁴

Human sera. Sera from individuals allergic to *D. pteronyssinus* were collected at the Royal Prince Alfred Hospital Allergy Clinic, Sydney, Australia, after clinical diagnosis had been confirmed by case history, skin prick test, and spirometry. Cord serum samples were supplied by St. John of God Hospital, Perth, Western Australia. Sera were stored at -20° C.

Ultrafiltration. Ultrafiltration of the aqueous *D. pteronyssinus* extract was carried out in an Amicon model 52 Diaflo filtration (Amicon Corp., Danvers, Mass.) unit with separate reservoir and membrane diameter of 43 mm. The filters used, with MW cut-offs demonstrated in parentheses, were: UM 2 (1000), DM 5 (5000), UM 10 (10,000), UM 20 (20,000), XM 50 (50,000), and XM 100A (100,000). Retentates were freeze-dried before examined further.

Gel permeation chromatography. This was performed by use of a 3 by 40 cm column of Sephacryl S-300 Super Fine (Pharmacia, North Ryde, Australia) in 0.1M Tris-HCl, pH 8. The void volume of the column was determined with Blue Dextran (Pharmacia). MW standards, supplied by Phar-

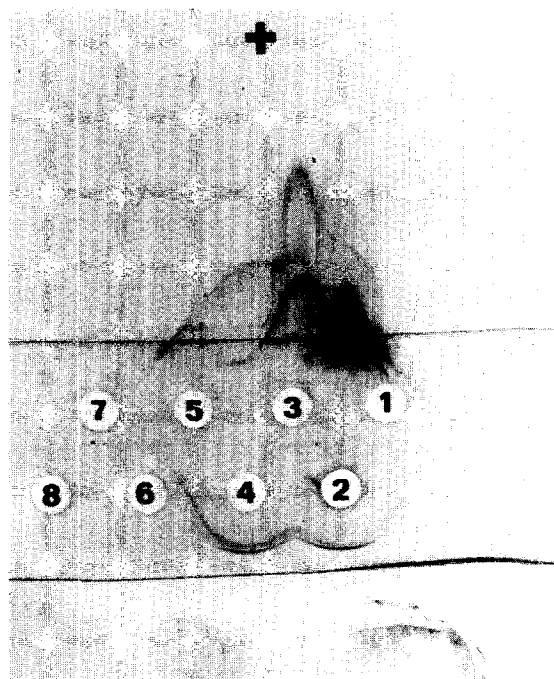


FIG. 1. Fused-rocket immunoelectrophoresis pattern of Amicon filtration fractions of an extract from the house dust mite *D. pteronyssinus*. Samples (15 μ l, 10 mg/ml) of each fraction were added to wells in a 2.5 cm strip of 1% agarose placed on a 10 by 10 cm glass plate. Diffusion was allowed to occur for 1 hr before electrophoresis into a 1% agarose gel containing 3 ml of hyperimmune rabbit anti-*D. pteronyssinus* anti-serum in 12 ml of agarose. Electrophoresis was carried out by use of a constant current at 10 mA for 20 to 24 hr at 9° C. Immunoprecipitin peaks were visualized using Coomassie brilliant blue. Wells, Amicon filtration fractions: 1, MW > 100,000; 2, MW 50,000 to 100,000; 3, MW 20,000 to 50,000; 4, MW 10,000 to 20,000; 5, MW 5000 to 10,000; 6, MW 500 to 5000; and 7 and 8, empty.

macia, were bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), and chymotrypsinogen A (25,000 daltons). Fractions were monitored for protein by measuring the absorbance at 280 nm.

Rabbit antisera. A number of antisera to the numerous components of *D. pteronyssinus* water-soluble extract were prepared by immunizing a number of New Zealand white rabbits. One rabbit produced a particularly potent antiserum, and this antiserum was used in all the subsequent studies involving serologic examination of *D. pteronyssinus* extract.

Immunoelectrophoretic methods. Fused-rocket immunoelectrophoresis was performed as described by Svendsen.¹⁵ Protein solutions (15 μ l) were added to 2.5 mm wells punched at 5 mm intervals in 1% agarose gel (Sigma Chemical Co., St. Louis, Mo., low EEO), and allowed to diffuse for 1 hr before being electrophoresed into a 1% agarose gel (12 ml) containing 3 ml of rabbit anti-*D. pteronyssinus* serum. Electrophoresis was performed by use of a constant current of 10 mA for 20 to 24 hr at 9° C. Gels were pressed, washed, dried, and stained as described by Weeke.¹⁶

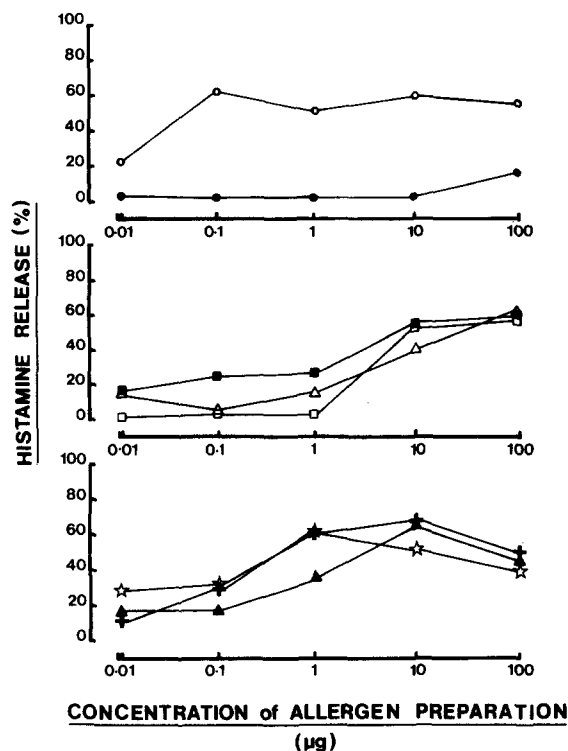


FIG. 2. Allergic histamine release after challenge of aliquots of whole human blood (200 μ l) from a house dust-mite allergic subject. (○) Unfractionated *D. pteronyssinus* extract. Fractions of different MWs: (●), <500 daltons; (□), 500 to 5000 daltons; (■), 5000 to 10,000 daltons; (△), 10,000 to 20,000 daltons; (▲), 20,000 to 50,000 daltons; (+), 50,000 to 100,000 daltons; and (*), >100,000 daltons. Histamine was estimated by the enzymatic-isotopic microassay (see Material and Methods).

Fused-rocket radioimmuno-electrophoresis was performed as described for crossed radioimmuno-electrophoresis, Baldo et al.¹⁴ and Weeke and Lowenstein.¹⁷

Preparative isoelectric focusing. An aqueous extract of *D. pteronyssinus* was subjected to electrofocusing in Ultradex (LKB, Bromma, Sweden) granulated gel in the presence of Ampholine (LKB), pH range 3.5 to 10, as described by Winter et al.¹⁸ Electrofocusing was carried out at 9° C for 20 to 24 hr with 8 W constant power. Fifteen fractions were cut by use of the LKB fractioning grid, and 2 ml of water was added to each fraction before removal of the Ultradex on sintered glass filters. After estimation of pH and protein content (by measuring absorption at 280 nm), individual fractions were dialyzed against frequent changes of water and freeze-dried.

Skin prick tests. Prick testing was performed on human subjects as described previously¹⁹ with histamine acid phosphate (David Bull Laboratories) (1 mg/ml) being used as a positive reference.²⁰ Wheal sizes were recorded 15 min after

testing as the average diameter $\frac{D + d}{2}$, where D is the

largest diameter of the wheal and d the diameter perpendicu-

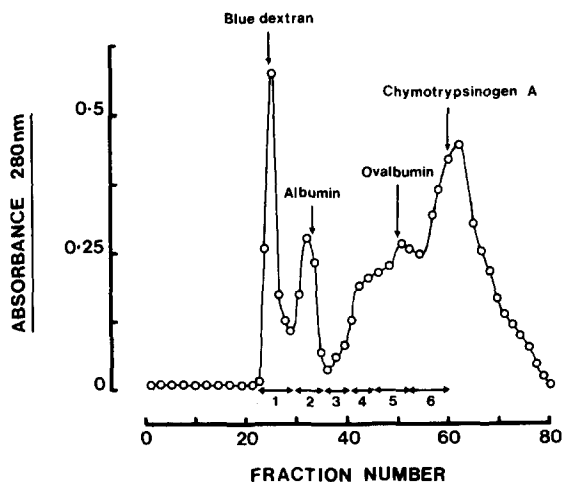


FIG. 3. Gel filtration on Sephacryl S-300 of crude *D. pteronyssinus* extract. Crude *D. pteronyssinus* extract (100 mg) was applied to a 3 by 40 cm column of Sephacryl S-300. Each fraction (4 ml) was analyzed for protein by measuring the absorbance at 280 nm. Arrows indicate the fractions that were pooled, dialyzed against distilled water, lyophilized, and designated Sephacryl pools 1 to 6. The Sephacryl S-300 column was calibrated with protein MW standards (see Material and Methods).

lar to this. Relative potencies of allergen extracts to the histamine standard were assessed by comparing the average diameters of wheals and by allotting the histamine solution a potency of 1.²¹

RAST. Mite extracts were reacted with cyanogen bromide-activated Whatman grade 44, 6-millimeter paper disks as described by Ceska et al.²² RAST²³ examinations were carried out as described by Baldo and Uhlenbruck¹⁰ by use of 50 μ l quantities of sera. Cord serum was used as an "IgE-free" control.

Histamine-release studies. Histamine released from human leukocytes after allergen challenge was determined with the enzymatic-isotopic microassay^{24, 25} with the use of whole human blood, S-adenosyl-L-[methyl-³H]-methionine, 500 mCi/mmol (Radiochemical Center, Amersham, England), as the methyl donor and mouse brain as a source of histamine methyltransferase. Assays with heparinized blood were carried out within 4 hr of collection, and release of histamine was determined in duplicate on 100 μ l aliquots in 400 μ l Beckmann microfuge tubes. After addition of allergen extracts, tubes were incubated at 37° C for 60 min and centrifuged at 2000 $\times$ g for 10 min before removing 20 μ l aliquots of plasma for estimation of the liberated histamine. With each assay, blanks and internal and external histamine standards were included.²⁵

Electrophoretic transfer of mite proteins from isoelectric focusing gels and detection of IgE-binding zones. Isoelectric focusing in the pH range of 3.5 to 10 with 2% carrier ampholytes (Pharmalyte, Pharmacia) was carried out in 7.5% polyacrylamide gel slabs (90 by 120 by 1.5 mm) as described previously.²⁶ After electrophoretically transferring proteins from the separating gel to a nitrocellulose mem-

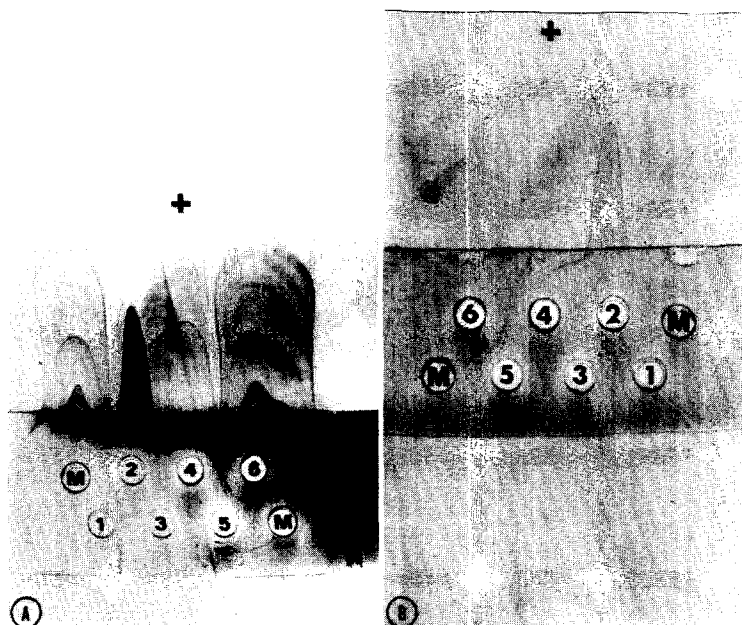


FIG. 4. Fused-rocket radioimmuno-electrophoresis of *D. pteronyssinus* Sephadryl S-300 fractions and spent medium components. After fused-rocket immuno-electrophoresis (see Material and Methods and Fig. 1), gels were incubated with 2 ml of serum from a house dust-mite allergic patient (who was skin test positive and RAST positive to *D. pteronyssinus*), in 12 ml of phosphate buffer. After frequent washes in saline with 1% Tween 20, ^{125}I -labeled anti-human IgE (300,000 cpm) in 14 ml of phosphate buffer was added, and the gels were left for 24 hr at room temperature. The gels were then washed repeatedly with saline for 2 days before carrying out autoradiography. Wells: pools/through 6; M, spent mite medium (20 mg/ml). A, Gel stained with Coomassie brilliant blue. B, Autoradiograph obtained from gel after the use of serum from a house dust-mite allergic patient and ^{125}I -labeled anti-human IgE.

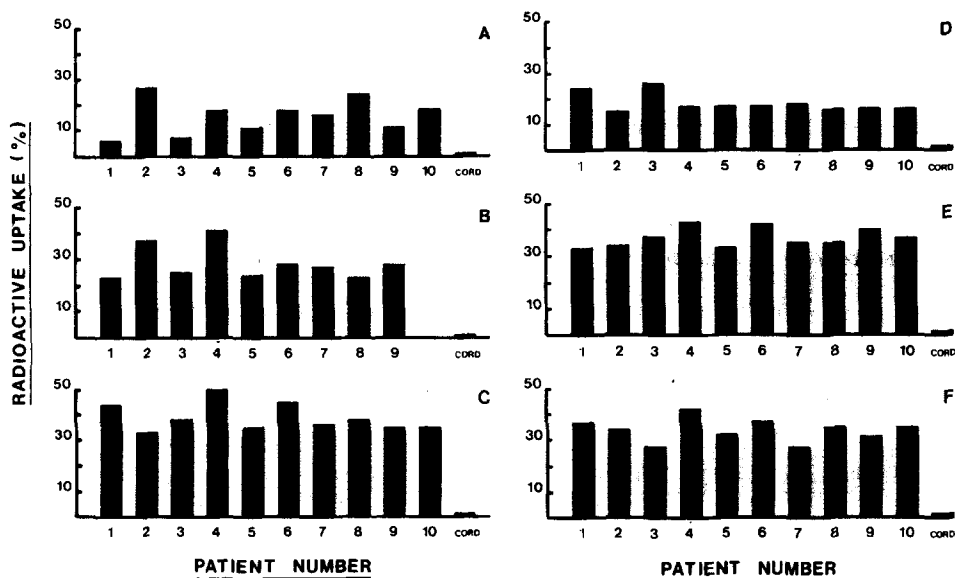


FIG. 5. Estimation by RAST of the presence, in serum, of IgE antibodies for house dust-mite allergens. Ten sera from subjects allergic to the house dust mite and one serum from umbilical cord (IgE-"free" control) (50 μl) were used with cyanogen bromide-activated discs coupled to the different Sephadryl S-300 pools (see Fig. 3). A, pool 1; B, pool 2; C, pool 3; D, pool 4; E, pool 5; F, pool 6.

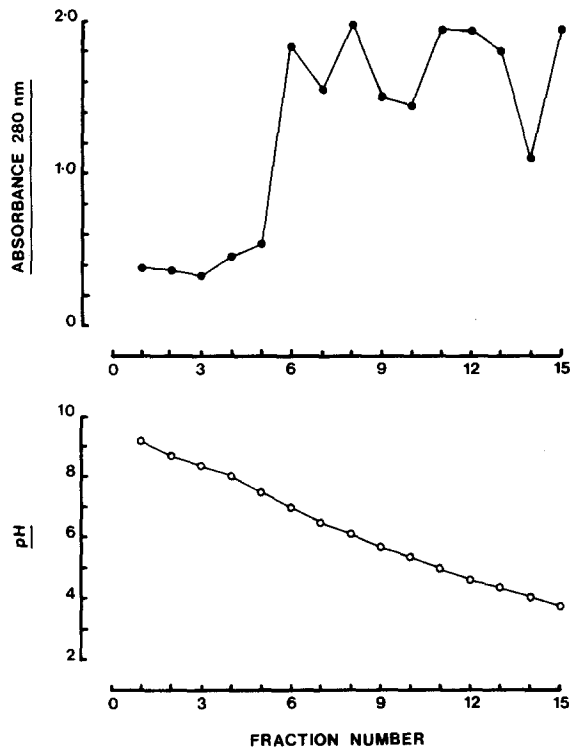


FIG. 6. Isoelectric fractionation of crude *D. pteronyssinus* extract. Flat-bed isoelectric focusing was carried out in Ultradex (LKB) at 9° C for 24 hr as described in Material and Methods. Lyophilized crude *D. pteronyssinus* extract (93 mg) was dissolved in 5 ml of Ampholine (pH range 3.5 to 10) before isoelectric focusing. Fifteen fractions were obtained, diluted with 2 ml of distilled water, and filtered through a sintered glass filter to remove the Ultradex before reading the absorbance (*top*) at 280 nm and the pH values (*bottom*).

brane (Type GWSP pore size 0.22 μ m, Millipore Corp., Bedford, Mass.),²⁷ IgE-binding zones on the membrane were identified by use of sera from six different mite-allergic patients and ¹²⁵I-labeled rabbit anti-human IgE (Pharmacia).²⁸

RESULTS

Mite extract fractions obtained by ultrafiltration. A preparation of whole mites (2 gm) was extracted with water and subjected to successive fractionation by ultrafiltration. The following MW cuts were obtained: <500, 500 to 5000, 5000 to 10,000, 10,000 to 20,000, 20,000 to 50,000, 50,000 to 100,000, and >100,000. Examination of the fractions by fused-rocket immunoelectrophoresis (Fig. 1) demonstrated that many components in the low MW range were not precipitated, and overlap of precipitin lines originating from antigens in wells 3 to 6 (i.e., wells containing components of MW 10,000 to 20,000 to >100,000), oc-

curred. From these same wells components migrating to the cathode were also visible (Fig. 1).

Examination of ultrafiltration fractions for allergenicity. Individual fractions were examined for allergenic properties by skin prick tests, direct RAST, RAST inhibition, and in histamine-release studies with blood from house dust-mite allergic patients. Skin test and direct RAST examinations demonstrated a spread of allergenic activity throughout the MW range. These results have been presented in detail^{14, 29} and will not be considered further here.

RAST-inhibition findings revealed a range of potencies with high MW (50,000 to >100,000) fractions causing the greatest inhibition. Table I illustrates results that are typical of those obtained with individual sera from mite-allergic subjects.

Histamine-release results also suggest that higher MW fractions may be more active (Fig. 2). Comparable results were obtained with blood from three other mite-allergic subjects.

Fractionation by gel permeation. Because of the antigenic overlap obtained with fractions prepared by ultrafiltration, gel filtration of the crude *D. pteronyssinus* extract was investigated as an initial antigen-separation procedure. Passage of the extract through Sephacryl S-300 yielded multiple peaks, some of which were overlapping (Fig. 3). Electrophoretic and immunologic examinations of the resultant six pools revealed a far more complex pattern than was obtained with the ultrafiltration fractions. Fused rocket immunoelectrophoresis for example (Fig. 4, A), demonstrated clear cross-reactivity between mite components from wells 2 to 4 and wells 5 and 6. Fraction 2 contained the same deeply staining band, antigen number 36,^{14, 30} observed in line with well 5 in Fig. 1. Autoradiographs obtained from the fused-rocket patterns revealed at least two peaks, including antigen number 36, which bound human IgE. In addition, a component of the spent mite medium and cathode-migrating medium components were observed in the autoradiograph studies with ¹²⁵I-labeled anti-human IgE (Fig. 4, B).

Allergenicity of gel-filtration fractions. Direct RAST experiments with the six Sephacryl fractions and sera from ten house dust-mite allergic patients demonstrated a spread of IgE-binding components. Fractions 3 (Fig. 5, C) and 5 (Fig. 5, E), however, were slightly more potent than the other fractions (Fig. 5, A, B, D, F).

Fractionation of mite extract by isoelectric focusing. Flat-bed electrofocusing in granulated gel and in the pH range of 3.5 to 10 resolved the mite extract into four main peaks when the protein distribution

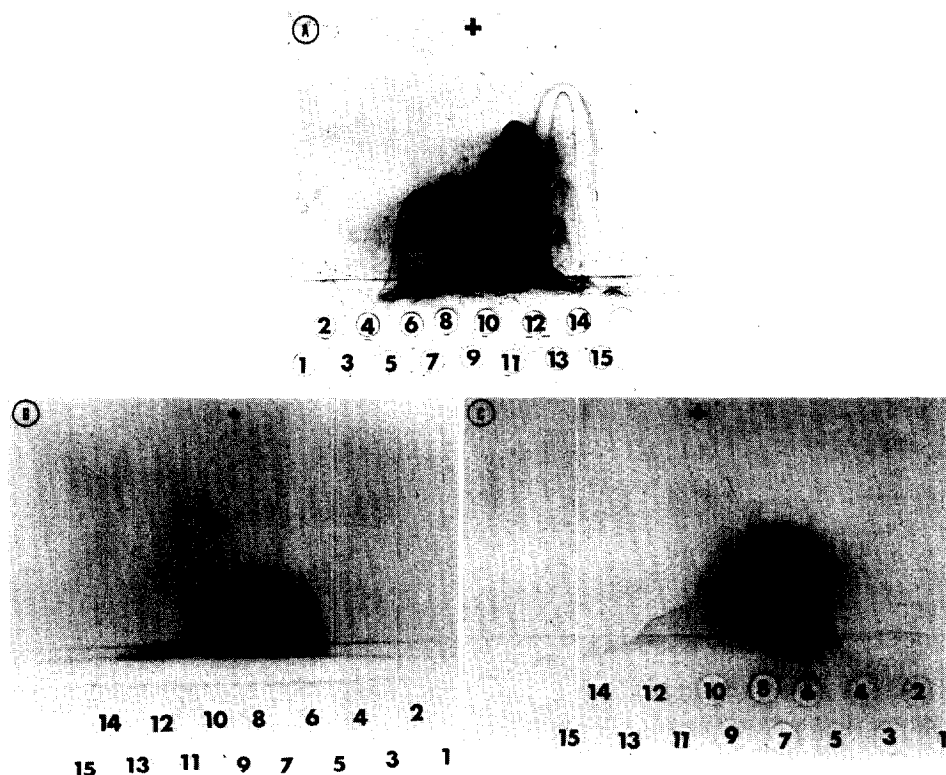


FIG. 7. A, Fused-rocket immunoelectrophoresis of isoelectric-focusing fractions of *D. pteronyssinus* extract. Fractions were obtained by flat-bed isoelectric focusing. For experimental details see Material and Methods. Wells 1 to 15 contained flat-bed isoelectric focusing fractions 1 to 15. Gel stained with Coomassie brilliant blue. B and C, Autoradiographs with sera from house dust-mite allergic patients and ^{125}I -labeled anti-human IgE. After fused-rocket immunoelectrophoresis, gels were incubated with 2 ml of serum with different house dust-mite allergic patients (who were skin test positive and RAST positive to *D. pteronyssinus*), in 2 ml of phosphate buffer. IgE binding was revealed by ^{125}I -labeled anti-human IgE (300,000 cpm) and autoradiography was obtained as described in Material and Methods and Fig. 4.

was monitored (Fig. 6). When each of the 15 fractions was examined by fused-rocket immunoelectrophoresis, a complex pattern of more than 26 precipitin arcs was obtained and marked; cross-reactivity was observed between antigens in the pI range of 4 to 7.5 (fractions 5 to 12). The rabbit antiserum used failed to react with mite proteins with a pI >7.5 (fractions 1 to 5) (Fig. 7, A).

Heterogeneity of some of the mite components was also apparent. For example, proteins focusing at pH 4.4, 4.6, 5.4, and 6 and at pH 5.4, 6.3, 6.6, 7, and 7.2 each demonstrated single lines of identity when these were used in fused rocket experiments with rabbit anti-*D. pteronyssinus* serum (Fig. 7, A). Fused-rocket radioimmunoelectrophoresis demonstrated that the latter antigen bound IgE from the sera of mite-allergic patients. At least three of the precipitin peaks were visible on the autoradiographs (Fig. 7, B and C).

Allergenicity of isoelectric-focusing fractions. Dif-

ferences in the patterns of reactivity of the 15 different pools were observed, both between these two diagnostic methods and between the individual patient responses, when these were examined in skin prick test and direct RAST studies. Positive prick test reactions were obtained to mite components with pIs in the range of 4 to 8.5 with three groups most prominent, namely, pI 4 to 5, 5 to 5.7, and approximately 8.5 (Fig. 8). The RAST results by use of sera from mite-allergic subjects demonstrated an obvious difference when the results were compared to the skin test findings. Radioactive uptakes were clearly highest to fractions with pIs at the higher end of the range, namely, 6.5 to 9 (Fig. 9). Such a clear difference in the relative reactivities to mite components with high and low pIs was not obtained in the skin prick test results.

Detection of IgE-binding zones in mite extract after electrophoretic transfer. Proteins from *D. pteronyssinus*

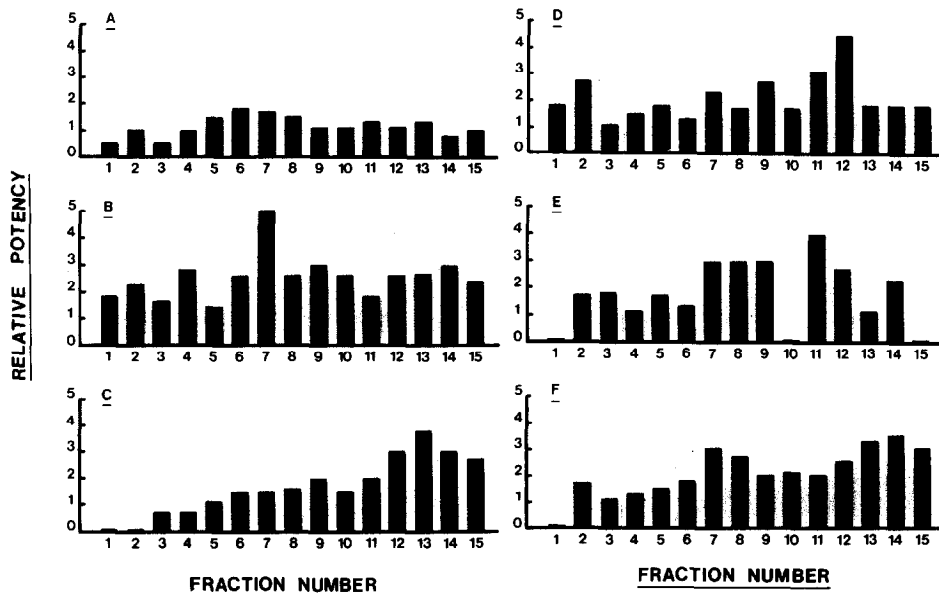


FIG. 8. Skin prick test responses in six house dust-mite allergic patients to the 15 *D. pteronyssinus* fractions obtained by flat-bed isoelectric focusing (see Figs. 6 and 7). Histamine acid phosphate 1 mg/ml was used as a positive reference. Relative potencies of fractions to the histamine standard were assessed as described in Material and Methods.

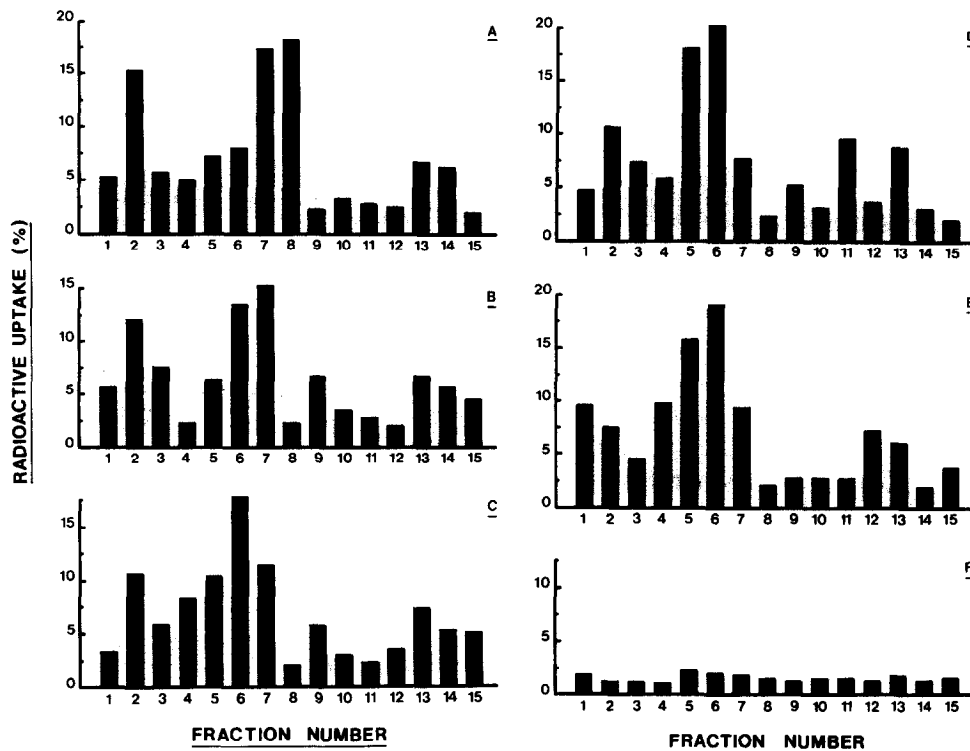


FIG. 9. Estimation by RAST of the presence of IgE antibodies reactive with *D. pteronyssinus* flat-bed isoelectric-focusing fractions (see Figs. 6 and 7). A through E, Sera from five mite-allergic subjects and F, serum from cord blood (IgE-“free” control) (50 μ l) were used with cyanogen bromide-activated paper discs coupled to the 15 different isoelectric-focusing fractions.

sinus water-soluble extract were separated by gel isoelectric focusing and subsequently transferred electrophoretically to a nitrocellulose membrane. IgE-binding components were then detected by incubating membrane strips, first with sera from six mite-allergic patients and then with ^{125}I -labeled rabbit anti-human IgE. Autoradiography revealed up to 14 different bands spread throughout the pH range (Fig. 10).

DISCUSSION

From this study two important conclusions emerge. First, a water-soluble extract of the common house dust mite *D. pteronyssinus* contains a number of allergens; allergenic activity is not confined to a single, discrete protein. This was demonstrated when mite extracts were subjected to different fractionation methods. Second, it is clear that the number and nature of the allergens detected depends not only on the separation techniques applied but also on the test methods used to detect biologic (allergenic) activity.

In relation to the first of these conclusions, gel permeation chromatography and isoelectric focusing each revealed a wide spread of those mite components that were subsequently identified as allergens. With ultrafiltration, marked overlap of components occurred between different MW cuts. For example, immunoelectrophoretic methods revealed the presence of shared antigens in some fractions. This sort of overlap that may occur with ultrafiltration³¹ has led us to interpret our results with caution when the method is used in allergen separation studies.^{14, 32}

Separation of allergens by gel-permeation chromatography on Sephacryl S-300 was more encouraging and yielded better resolution of components as demonstrated by fused-rocket immunoelectrophoresis. Again, however, a spread of allergenic activity was found, although the most potent RAST-positive fraction from the Sephacryl column eluted in the MW range of 25,000 to 70,000 daltons. Previous estimates of the MWs of the most active house dust-mite allergens fall within the MW range of 10,000 to 69,000 daltons.^{12, 33-39} Recently, however, both high MW (>100,000 daltons)^{9, 10, 30, 40-42} and dialyzable²⁹ allergens have been identified or isolated from *D. pteronyssinus* and *D. farinae*. These studies, and our results described here, point to a multiplicity of allergens in the common house dust mites.

Isoelectric focusing, the third separation method we used, has already been applied to the study of house dust-mite allergens.³⁶⁻³⁸ Although the previous studies demonstrated allergenic activity throughout the pH range of 3.5 to 10 and allergenic cross-reactivity between mite components with different pI



FIG. 10. Detection of *D. pteronyssinus* allergens by crossed radioisoelectric focusing. Autoradiograph demonstrating IgE-binding bands of *D. pteronyssinus* extract after transfer of separated zones to a nitrocellulose membrane and incubation with sera from six patients allergic to the house dust mite. For details see Material and Methods.

values, there are some objections associated with these studies. First, allergenic activity was assessed only by RAST. Second, no attempt was made to examine antigenic relationships between fractions by use of antisera to mite extracts. Allergenicity of mite fractions that we obtained was assessed by skin prick testing, RAST, histamine release, radioimmuno-electrophoresis, and a new electrophoretic transfer method involving binding of allergen-specific IgE to proteins immobilized on a nitrocellulose membrane.

Findings with these different methods lead to our second main conclusion, namely, that different techniques for demonstrating the presence of allergenic

activity may reveal discrepancies in the detection and distribution of allergens in a crude extract. This was demonstrated, for example, by comparing skin prick test and RAST results obtained with the mite fractions separated by isoelectric focusing. Likewise, radio-immunoelectrophoresis in agarose by use of fractions obtained by ultrafiltration and isoelectric focusing demonstrated a small number of IgE-binding mite components distributed over a relatively narrow pH range. By contrast, the isoelectric focusing-membrane transfer method detected many more IgE-binding bands over a wider pH range.

Some investigators have suggested that house dust-mite extracts contain one major allergen existing in different MW forms and exhibiting marked heterogeneity.^{11, 36} Although such heterogeneity of mite allergens does occur, our findings and those of other workers^{42, 43} suggest that a number of antigenically distinct and important allergens exist, and one major allergen does not account for the total allergenic activity of *D. pteronyssinus* extracts. This conclusion is supported by results of crossed radio-immunoelectrophoresis studies reported in the following paper.

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Erratum

In the article by Delehunt et al., "Inhibition of antigen-induced bronchoconstriction by methylprednisolone succinate," published in the April 1984 issue of *THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY*, p. 481, data in Table I were transposed. The corrected data are shown in Table I.

TABLE I. Effect of glucocorticoid pretreatment (3 hr) on antigen-induced bronchoconstriction

	R_L (cm H ₂ O · L ⁻¹ ·sec)	V_{tg} (L)	SR_L (cm H ₂ O · sec)	Cdyn (L · cm H ₂ O ⁻¹)	PaO ₂ (mm Hg)
Baseline	1.01 ± 0.31	1.07 ± 0.14	1.17 ± 0.19	0.08 ± 0.04	90.8 ± 6.9
Post-antigen	4.60 ± 3.83*	1.26 ± 0.23*	7.78 ± 4.83*	0.05 ± 0.02*	57.6 ± 17.1*
Baseline	1.10 ± 0.27	1.06 ± 0.05	1.10 ± 0.30	0.08 ± 0.02	91.6 ± 4.6
Post-glucocorticoid	1.01 ± 0.31	1.06 ± 0.08	1.12 ± 0.35	0.07 ± 0.02	86.0 ± 3.3
Post-antigen	0.84 ± 0.29†	1.08 ± 0.07†	0.99 ± 0.32†	0.08 ± 0.02†	78.8 ± 10.7

Values are means ± SD of the same six sheep for mean R_L , V_{tg} , SR_L , Cdyn and PaO₂. There were no significant differences in baseline values between the control and the steroid studies.

*Significantly different from baseline $p < 0.05$.

†Significantly different from antigen alone $p < 0.05$.