

Shared and specific allergenic and antigenic components in the two sexes of American cockroach— *Periplaneta americana*

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Summary

The whole body extracts (WBEs) of female and male cockroaches (*Periplaneta americana*; *Pa*) were prepared separately to study the specific and/or shared allergenic and antigenic components in the two sexes. These two extracts were skin-tested on 170 respiratory allergy patients and 52 (30.6%) of them elicited a markedly positive cutaneous reaction (2+ to 4+) to any one or both the WBEs. Of these 52 patients, 32 (61.5%) produced a 2+ to 4+ response to only one, and the remaining 20 (38.5%) to both the extracts. In female and male RASTs to *Pa* both the extracts produced dose-related inhibition. Using rabbit anti-*Pa* female serum: (1) immuno-diffusion experiments resulted in lines of identity with the two extracts, and (2) the two-dimensional immunoelectrophoresis of *Pa* female and *Pa* male WBEs elicited 12 and nine precipitin peaks, respectively. In partial purification studies, only fraction *Pa*(F)III (~50 kD) of *Pa* female WBE and *Pa*(M)I (≥600 kD) and *Pa*(M)III (~50 kD) fractions of *Pa* male WBE revealed significant allergen activity both on skin testing and also in RAST inhibition studies. These results provide evidence for the presence of shared as well as specific allergenic and antigenic components in the two sexes of American cockroach.

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Introduction

The role of different species of cockroach in the aetiology of respiratory allergic disorders is well established [1–7]. Using an immunochemical technique, Swanson *et al.* [8] demonstrated the presence of cockroach-derived allergens in the air giving further evidence about the role of cockroach in producing inhalant allergens. Based on the purification studies, Twarog *et al.* [9] suggested the occurrence of some shared allergenic components between the whole body extracts (WBEs) of German and American cockroaches and found three major allergens Cr I (25.5 kD), Cr II (63–65 kD) and Cr III (10 kD) in

these extracts. However, Richman *et al.* [10] reported that allergens of these two species of cockroach demonstrated little cross-reactivity. Recently, Stankus and O'Neil [11] suggested the occurrence of shared as well as specific allergens between these two species.

Whether the allergen profile of the WBEs of the two sexes of cockroach also vary and the two extracts contain some shared and some unique allergenic and antigenic components is not known. We investigated this aspect, as the relative density of the two sexes of cockroach is likely to differ from place to place and may lead to varying concentrations of airborne male and female cockroach-derived allergens. The results of the presence of shared and specific allergenic and antigenic components in the extracts of different sexes of American cockroach—*Periplaneta americana* (*Pa*) are reported here.

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Materials and methods

Subjects

A group of 170 consecutive patients with respiratory allergic disorders (bronchial asthma and/or allergic rhinitis) attending the out-patient department of the VP Chest Institute were included in the present study. The diagnosis of the subjects was established on the basis of personal and family history, physical examination and various routine laboratory investigations. Those subjects having any other associated pulmonary or systemic disease were excluded from the study. Before performing skin tests the subjects were instructed to stop antihistamines and tranquilizers for 48 hr and other sympathomimetic drugs for at least 12 hr.

Allergens

The WBEs of *Pa* female and *Pa* male were prepared separately by the method earlier described [7] and also by a modified method. In the later method, insects were killed by freezing at -20°C and the crude WBEs were prepared by lyophilization, pulverization, defatting with solvent ether, and extraction in 0.1 M ammonium bicarbonate at 4°C for 72 hr (1:50, w/v) with periodic shaking. The extracts obtained were thoroughly dialysed against 0.5 M NH_4HCO_3 , the last change was given with double-distilled water. The extracts were then freeze-dried and stored at -20°C until used.

For RAST and RAST-inhibition experiments, solid-phase allergen discs were prepared from *Pa* female and *Pa* male WBEs as for the method of Adolphson *et al.* [12]. In brief, 50 mg of each allergen extract was dissolved in 1 ml of borate saline (0.2 M H_3BO_3 , 0.04 M NaOH, 0.16 M NaCl, pH 8.0) and allowed to react with cyanogen bromide-activated 6-mm diameter cellulose paper discs (Whatman No. 50 filter paper) at 4°C for 72 hr on a horizontal shaker (100 r.p.m.). After incubation the discs were repeatedly washed and suspended in 5 ml of 1 M ethanolamine for 24 hr to block uncoupled sites. The discs were finally washed and stored in RAST diluent at 4°C .

Skin tests

Intradermal skin tests with the WBEs of *Pa* female and *Pa* male (1:500, w/v in PBS) were performed on 170 subjects to compare the immediate weal-and-flare reactions elicited by the two extracts. In addition, buffered saline and histamine (100 $\mu\text{g}/\text{ml}$) were also tested as negative and positive controls, respectively. As a double control these extracts were also tested on a group of 50 healthy non-allergic volunteers. The tests were performed and graded according to the method and criteria of Shivpuri and Agarwal [13]. Only markedly positive reactions (2+ to 4+) were considered significant.

Radioallergosorbent test

Pa female and *Pa* male specific IgE levels were estimated in the sera of 35 subjects (20 *Pa* female and 15 *Pa* male) eliciting different grades of skin reactions in paper disc RASTs [14].

RAST inhibition studies

To study the presence of shared or specific allergens in two different sexes of cockroach, crossed inhibition experiments of *Pa* female and *Pa* male RASTs with the two WBEs, as well as purified fractions, were conducted [15]. For *Pa* female RAST inhibition studies the IgE serum pool used was from eight non-immunized subjects with positive skin tests and high RAST values to *Pa* female extract. Similarly, in *Pa* male RAST inhibition studies a serum pool from seven subjects showing highly positive skin and RAST values was used. Of the various sera used, four showed high binding in both *Pa* female as well as *Pa* male RASTs and were included in both the serum pools.

Antibodies

Hyperimmune rabbit antiserum was raised in three adult rabbits against *Pa* female WBE [16]. Briefly, 1 mg (dry weight) of the WBE, in Freund's complete adjuvant (CFA), was administered subcutaneously in the foot pads and neck region of the rabbits, followed by two more subcutaneous injections of 2.85 mg each in CFA at an interval of 2 weeks. After another 2 weeks, the fourth injection (2.85 mg in 0.15 M saline) was given intravenously. Thereafter several monthly booster injections of 10 mg extract in CFA were given to the animals subcutaneously. The blood was obtained after every booster injection and tested by immunodiffusion for the presence of precipitating antibodies. Finally, the positive serum samples were pooled and stored at -20°C .

Immunodiffusion

Double immunodiffusion [17] in agarose gel was performed with various allergen extracts and rabbit anti-*Pa* female antibodies.

Two-dimensional immunoelectrophoresis

Two-dimensional immunoelectrophoresis (TDIEP) was performed [18] to study the number of shared and specific antigenic components present in both the cockroach WBEs. A pool of rabbit antisera raised against *Pa* female WBE was used for these experiments.

TDIEP gels were prepared from 1% (w/v) agarose (Sigma Chemical Co., St Louis, U.S.A.) in barbital buffer (0.024 M barbital, 0.073 M Tris, 0.04 M calcium lactate and

0.003 M sodium azide, pH 8.6). The first and second dimension electrophoresis were performed in 1.5 mm thick gel at 10 V/cm for 35 min and 2 V/cm for 16 hr, respectively. The plates were washed, pressed, dried and stained with Coomassie brilliant blue stain R 250 (Sigma Chemical Co.).

Purification of allergenic components

Salt precipitation. WBEs of both *Pa* females and *Pa* males were subjected to 70% $(\text{NH}_4)_2\text{SO}_4$ precipitation at 4°C. The precipitates were washed and redissolved in 0.1 M ammonium bicarbonate. Both the supernatants and the redissolved precipitates were dialysed and freeze-dried. The allergenic activity of the different fractions was checked by performing intradermal skin tests on three subjects known to be hypersensitive to the respective WBEs. The major allergenic activity was located in 70% $(\text{NH}_4)_2\text{SO}_4$ precipitate in both the extracts.

Size exclusion chromatography

The 70% $(\text{NH}_4)_2\text{SO}_4$ precipitate of each extract was purified further over a Sephadex G-200 column (2 × 90 cm). Elution was performed at a flow rate of 15 ml/hr with 0.05 M ammonium bicarbonate. Absorbance of eluates was read at 280 nm. The eluates of the three major peaks were pooled separately, lyophilized and checked for allergenic activity. Ferritin (440 kD), catalase (232 kD), aldolase (158 kD) and albumin (67 kD) were used as molecular weight markers.

Results

Skin tests

Initially two sets of the cockroach WBEs were prepared separately following our earlier method and the present modified method. The recovery of biological activity with the present method was higher as shown by a higher percentage (35.7% with *Pa* female and 32.1% with *Pa* male) of markedly positive intradermal reactions in 28 subjects as compared to the results obtained with the extracts prepared by our earlier method (17.1% with *Pa* female and 10.7% with *Pa* male). Of the 15 healthy controls tested with these extracts, 6.7% showed a markedly positive reaction to *Pa* female and *Pa* male WBEs prepared by the new method, as against 6.7% and 0% with the WBEs prepared by the earlier method. Hence, for subsequent studies the allergen extracts were prepared by the new method only.

The results of intradermal tests performed on 170 subjects with both *Pa* female and *Pa* male extracts (1:500, w/v) are presented and compared in Tables 1 and 2. While 24.7% and 17.6% of the subjects gave markedly positive reactions with *Pa* female and *Pa* male extracts respec-

tively, only 2% of the healthy controls showed a 2+ reaction to both the WBEs.

Of the 170 subjects, 52 gave markedly positive reactions to either one or both the extracts tested (Table 2). Of these 52 subjects, 20 (38.5%) showed a positive reaction to both the extracts and 32 (61.5%) to only one of them; 22 to *Pa* female and 10 to *Pa* male alone. Although 20 subjects showed 2+ to 4+ reactions to both the extracts, only 13 showed identical grades of intradermal reactions, while the remaining seven produced varying degrees of skin reactivity.

RAST

A total of 32 (91.4%) of the 35 sera from the subjects with 1+ to 4+ skin reaction to *Pa* female or *Pa* male WBE and 28 (96.6%) of the 29 with 2+ to 4+ skin reactions were RAST positive. These results established the specificity of the intradermal response (results not shown).

Table 1. Skin test results with *Periplaneta americana* female and male WBEs on 170 subjects with respiratory allergy and 50 healthy non-allergic controls

	Subjects (n = 170)				Controls (n = 50)*			
	1+ to 4+		2+ to 4+		1+ and 2+		2+	
	n	%	n	%	n	%	n	%
Insect extract (1:500, w/v)								
<i>Pa</i> female WBE	85	50	42	24.7	5	10	1	2
<i>Pa</i> male WBE	69	40.6	30	17.6	8	16	1	2

* No 3+ or 4+ reactions were obtained in controls.

Table 2. Analysis of the intradermal test results of 96 subjects giving a positive cutaneous response to any one or both of the cockroach WBEs

Cockroach extract(s) showing positive intradermal response	1+ to 4+ response (n = 96)		2+ to 4+ response (n = 52)	
	n	%	n	%
To only one extract	38	39.6	32	61.5
<i>Periplaneta americana</i> , female alone	27	28.1	22	42.3
<i>Periplaneta americana</i> male alone	11	11.5	10	19.2
To both the extracts	58	60.4	20	38.5
Identical intradermal reactions	34	35.4	13	25.0
Varying intradermal reactions	24	25.0	7	13.5
Total	96		52	

Purification of allergenic components

The results of skin tests with various purified fractions of *Pa* female and *Pa* male extracts are presented in Tables 3 and 4 respectively. In both cases 70% (NH₄)₂SO₄ precipitable fractions elicited major allergenic activity on skin testing.

Column chromatography (Sephadex G-200) of 70% (NH₄)₂SO₄ precipitable fractions of the two extracts yielded three major peaks. Appropriate eluates were pooled to obtain fractions designated as *Pa*(F)I, *Pa*(F)II, *Pa*(F)III and *Pa*(M)I, *Pa*(M)II and *Pa*(M)III, respectively. The molecular weight of fraction III of both the extracts was about 50 kD, whereas *Pa*(M)I had a molecular weight of ≥ 600 kD (Fig. 1). On gel filtration, the major allergenic activity was associated with fractions *Pa*(F)III and *Pa*(M)III, though *Pa*(M)I also showed some allergenic activity.

RAST inhibition studies

The results of the inhibition of *Pa* female and *Pa* male RASTs with increasing amount of both the cockroach extracts are presented in Fig. 2. Both the extracts produced linear dose-related inhibition of *Pa* female as well as *Pa* male RASTs. In the case of *Pa* female RAST,

Table 3. Skin test results with *Periplaneta americana* female WBE and its fractions

Allergen extract	Concentration (mg/ml)	Skin test response on				
		SJ	DC	RK	SB	AK
WBE	1:500*	2+	2+	2+	3+	2+
70% (NH ₄) ₂ SO ₄ precipitate	0.2	2+	2+	ND	ND	2+
	0.02	2+	2+	ND	ND	2+
	0.002	2+	2+	2+	3+	+
70% (NH ₄) ₂ SO ₄ supernatant	0.2	+	—	ND	ND	—
	0.02	+	—	ND	ND	—
	0.002	—	—	ND	ND	—
<i>Pa</i> (F)I	0.2	—	+	—	—	ND
	0.02	—	—	—	—	ND
	0.002	—	—	—	—	ND
<i>Pa</i> (F)II	0.2	—	—	—	—	ND
	0.02	—	—	—	—	ND
	0.002	—	—	—	—	ND
<i>Pa</i> (F)III	0.2	2+	3+	2+	2+	ND
	0.02	2+	2+	2+	2+	2+
	0.002	2+	2+	+	2+	2+

ND = not done; — = negative.

* Dilution (w/v).

Table 4. Skin test results with *Periplaneta americana* male WBE and its fractions

Allergen extract	Concentration (mg/ml)	Skin test response on			
		RK	SDB	SK	KK
WBE	1:500*	3+	3+	3+	3+
70% (NH ₄) ₂ SO ₄ precipitate	0.2	3+	2+	ND	2+
	0.02	2+	2+	ND	2+
	0.002	2+	2+	3+	+
70% (NH ₄) ₂ SO ₄ supernatant	0.2	+	—	ND	—
	0.02	—	—	ND	—
	0.002	—	—	ND	—
<i>Pa</i> (M)I	0.2	2+	2+	3+	ND
	0.02	+	2+	2+	ND
	0.002	+	—	+	ND
<i>Pa</i> (M)II	0.2	—	+	+	ND
	0.02	—	—	—	ND
	0.002	—	—	—	ND
<i>Pa</i> (M)III	0.2	3+	3+	3+	ND
	0.02	2+	2+	3+	ND
	0.002	2+	2+	3+	ND

ND = not done; — = negative.

* Dilution(w/v).

however, only 1 µg of the homologous allergen extract in comparison to 2.6 µg of the *Pa* male extract was required to produce a 50% inhibition. It is worth mentioning that in the *Pa* male RAST only 1.4 µg of *Pa* female allergen was required, compared to 2.4 µg of the homologous allergen for a 50% inhibition.

Results of RAST inhibition with various fractions of cockroach extracts are presented in Table 5. It is evident that in both the cases only the (NH₄)₂SO₄ precipitate showed significant inhibition while the supernatant did not. Of the three fractions obtained after gel filtration of *Pa* female, 4 µg of *Pa*(F)III produced the maximum inhibition (50.2%). In the case of *Pa* male, 4 µg of both *Pa*(M)I and *Pa*(M)III produced 49.8% and 59.8% inhibition respectively.

Immunodiffusion

Immunodiffusion studies using rabbit anti-*Pa* female antibodies and *Pa* female and *Pa* male allergen extracts showed lines of identity, indicating the presence of shared antigens in the two extracts (Fig. 3).

Two-dimensional immunoelectrophoresis

The results of TDIEP experiments with *Pa* female and *Pa* male extracts using rabbit anti-*Pa* female antibodies are

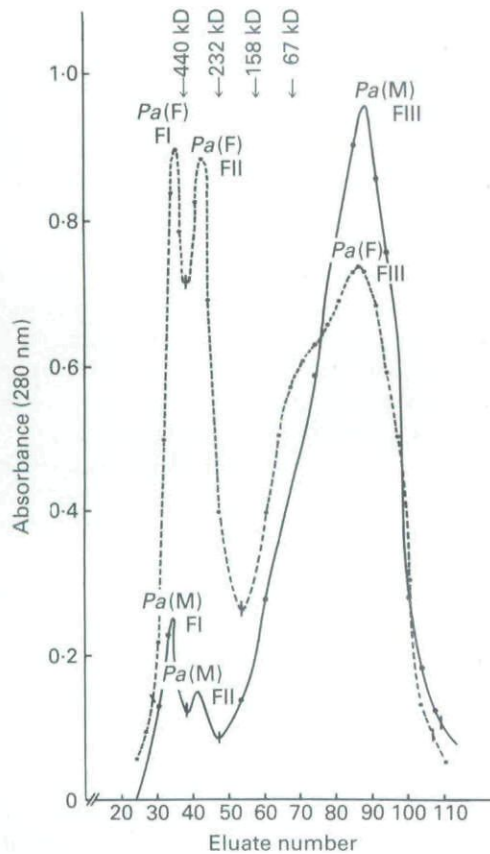


Fig. 1. Elution profile of 70% $(\text{NH}_4)_2\text{SO}_4$ precipitable fractions of *Periplaneta americana* female (---) and male (—) WBEs on a Sephadex G-200 column. Eluent: 0.05 M NH_4HCO_3 buffer (pH 7.8); column size: 2×90 cm; flow rate: 15 ml/hr; and fraction size: 3.0 ml.

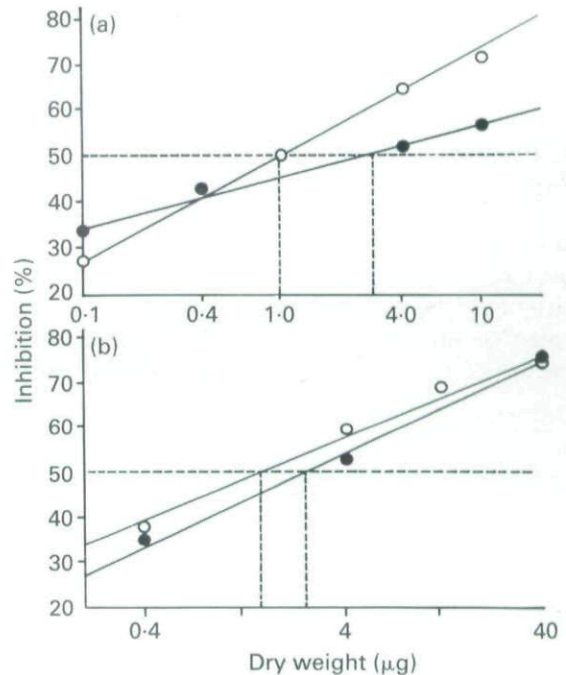


Fig. 2. Inhibition of (a) *Periplaneta americana* female and (b) *Periplaneta americana* male RASTs using *Pa* female (●—●) and *Pa* male (○—○) WBEs as inhibitors.

shown in Fig. 4. The *Pa* male extract gave a total of nine visible bands as against 12 bands produced by *Pa* female WBE.

Discussion

Of the many species of cockroach encountered in India, *Periplaneta americana* is the commonest in the Delhi area.

Table 5. Inhibition of *Pa* female and *Pa* male RASTs with their fractions

<i>Pa</i> female RAST		<i>Pa</i> male RAST	
Inhibitor*	Inhibition (%)	Inhibitor*	Inhibition (%)
WBE	45.8	WBE	70.8
70% $(\text{NH}_4)_2\text{SO}_4$ precipitate	46.9	70% $(\text{NH}_4)_2\text{SO}_4$ precipitate	72.8
70% $(\text{NH}_4)_2\text{SO}_4$ supernatant	13.6	70% $(\text{NH}_4)_2\text{SO}_4$ supernatant	37.8
<i>Pa</i> (F)I	22.6	<i>Pa</i> (M)I	49.8
<i>Pa</i> (F)II	22.6	<i>Pa</i> (M)II	29.1
<i>Pa</i> (F)III	50.2	<i>Pa</i> (M)III	59.8

* The amount of inhibitors used = 4 μg .

The aetiological significance of this insect has been established as an important inhalant allergen in various parts of the world [1–7]. The thermostable nature of the cockroach allergen and the presence of this insect in large numbers in the environment prompted various scientists to study cockroach allergens in detail. Recently, Kang *et al.* [19] have demonstrated the therapeutic efficacy of immunotherapy with cockroach antigen in subjects with bronchial asthma with cockroach hypersensitivity. Twarog *et al.* [9] demonstrated that both American and German cockroaches are responsible for symptoms in subjects with respiratory allergy. Subsequently, the pres-

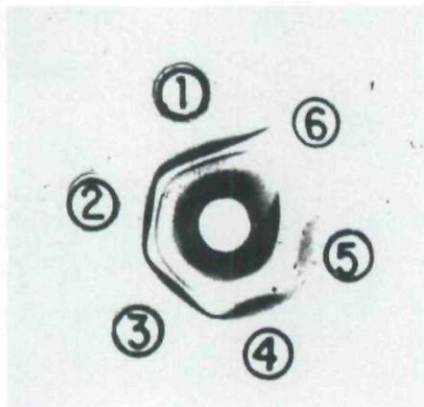


Fig. 3. Results of the immunodiffusion test using: (central well) rabbit anti *Pa* female antibodies (20 μ l); and (1) *Pa* female (1 mg/ml); (2) *Pa* female (0.5 mg/ml); (3) *Pa* male (1 mg/ml); (4) *Pa* male (0.5 mg/ml), (5) *Pa* male (0.25 mg/ml); and (6) normal saline.

ence of shared and specific allergens in different species of cockroach was documented. Based on the results of intradermal reactions the presence of sex-specific allergens was suggested for the first time in an earlier report [6]. The present study gave evidence for the presence of both crossreacting and unique allergenic/antigenic components in the two cockroach extracts (*Pa* female and *Pa* male).

Of the 170 subjects tested with *Pa* female and *Pa* male WBEs, 24.7% and 17.6% respectively, showed 2+ to 4+ skin reactions as against only 2% each in controls (Table 1). The variation in positivity to the two WBEs in subjects may have been due to differences of potencies of the two extracts, as in the RAST inhibition experiments *Pa* female extract showed better inhibition than *Pa* male extract (Fig. 2). However, of the 52 subjects showing 2+ to 4+ reactions to only one of the two extracts, 10 (19.2%) showed positivity to *Pa* male alone and 22 (42.3%) to *Pa* female alone (Table 2). Hence the differences in skin tests may have been due to differences in composition of the two allergen extracts. Further, dose-related RAST-inhibition lines obtained in the *Pa* female and *Pa* male RASTs, with both homologous as well as heterologous extracts, revealed the presence of some shared allergens between the two extracts. However, the slopes and the position of the inhibition lines were different in the *Pa* female RAST (Fig. 2a). This deviation from parallelism could have been due to the presence of some shared and some specific allergens in each extract and also due to differences in relative concentration of the shared allergens in the two extracts. This was supported further by

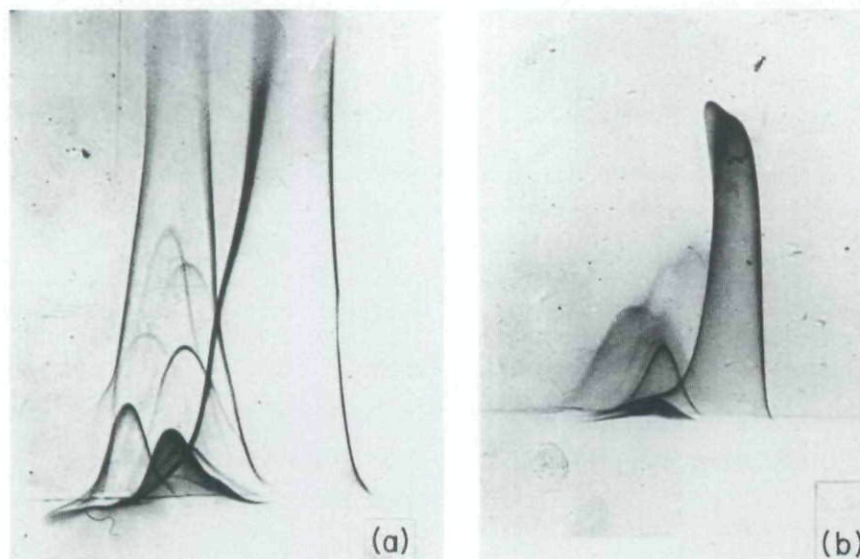


Fig. 4. Two dimensional immunoelectrophoretic analysis of WBEs of (a) *Periplaneta americana* female (100 μ g) and (b) *Periplaneta americana* male (100 μ g) using anti-*Pa* female antibodies (400 μ l) raised in rabbits. The anode is to the right in the first dimension electrophoresis and to the top in the second dimension electrophoresis.

the observation that only 1.0 µg of *Pa* female, compared to 2.6 µg of *Pa* male extract, was required to produce 50% inhibition in the *Pa* female RAST. Interestingly, in the *Pa* male RAST, the heterologous allergen (*Pa* female) produced a better inhibition line in comparison to the homologous allergen; the dose required for 50% inhibition being 1.4 µg of *Pa* female and 2.4 µg of *Pa* male extracts, respectively (Fig. 2b). The results suggested that the concentration of shared allergens in the two extracts may be more in the *Pa* female extract.

Double immunodiffusion studies revealed the presence of shared antigens in the two extracts. Two-dimensional immunoelectrophoresis of the two extracts in the gel using rabbit anti-*Pa* female antibodies, however, revealed the presence of at least nine shared antigenic components in the two extracts, while three antigenic components were specific for *Pa* female extract only. Similar TDIEP experiments using *Pa* male antisera might also have revealed the presence of some *Pa* male specific antigenic components. However, this aspect could not be investigated in the present study due to the lack of sufficient quantities of *Pa* male WBE for immunization.

Intradermal tests and RAST inhibition experiments showed that only the fraction of *Pa*(F)III contained allergenic activity of the three fractions of *Pa* female WBE, whereas in *Pa* male both fractions *Pa*(M)I and *Pa*(M)III showed significant allergenic activity. The peak of *Pa*(F)III and *Pa*(M)III of our study falls under the molecular weight level of 50 kD which may represent the allergenic components reported by Twarog *et al.* [9] and Stankus and O'Neil [11]. Further, our results suggest that the fractions *Pa*(F)III and *Pa*(M)III may contain some shared allergenic components while fraction *Pa*(M)I may have specific components of the male extract. Thus, our results of skin tests and RAST inhibition experiments on one hand and double immunodiffusion and TDIEP on the other provided evidence that the *Pa* female and *Pa* male WBEs contain crossreacting as well as specific allergenic and antigenic components. In the light of these findings we suggest that a mixed extract should be used, with individual extracts introduced if the first test is positive, and further investigation is necessary.

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