

# Allergens in bee venom

## I. Separation and identification of the major allergens

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*Honeybee venom was separated into seven fractions by gel filtration on Sephadex G-75. Allergenic activities of these fractions were assessed by the paper disc radioallergosorbent test (RAST) with a panel of sera from 24 individuals who had systemic reactions to bee stings, 7 who had large local reactions, and 10 control subjects who had reactions of 5 cm or less following bee stings. Three fractions were identified by enzyme or direct hemolytic activity. Twenty-nine of 31 sera from patients having either systemic or large local reactions to bee stings were positive when radioallergosorbent tested with whole bee venom; 22 were positive to phospholipase A, and 28 were positive to both fractions 1 and 2. Thirteen sera combined most strongly with fraction 1, 12 sera most strongly with fraction 2, hyaluronidase, and three sera about equally with fractions 1, 2, and 3. Reactions with other fractions were much weaker. Fractions 1 and 2 were potent inhibitors of RAST with whole venom in the sera reacting most strongly with fractions 1 or 2, respectively. Fraction 3, phospholipase A, and commercial bee venom phospholipase A were significantly less potent inhibitors with the sera tested. In the cases in which IgE antibody bindings to fractions 1, 2, and 3 were of similar magnitude, inhibitions of RAST using the various fractions both on the discs and as inhibitors demonstrated substantial cross-reactivity between the fractions. These results strongly indicate that by using RAST with human sera from bee sting-sensitive individuals, fraction 1, the high molecular materials, and fraction 2, hyaluronidase, are the major allergens in honeybee venom. Phospholipase A appears to be of secondary importance.*

There have been over 400 cases of death due to Hymenoptera stings in the United States during the last decade.<sup>1</sup> Treatment of stinging insect allergy by injections of whole body extracts has been reported to be highly effective in preventing severe reactions to future stings.<sup>2, 3</sup> However, occasional treatment failures have been reported,<sup>1, 4-6</sup> and recent studies have questioned the efficacy of whole body extract treatment.<sup>5</sup> In order to investigate the importance of immunologic variables in immunotherapy, it is necessary to isolate, identify, and characterize the important allergens. Studies using histamine release from human leukocytes have led to the proposal of phospholipase A as the major allergen of honeybee venom.<sup>7</sup>

In this study, we have fractionated fresh whole venom from the Italian

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Presented in part at the Thirty-first Annual Meeting of the American Academy of Allergy, San Diego, Calif., Feb. 15-19, 1975.

Received for publication Jan. 5, 1976.

Accepted for publication March 18, 1976.

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honeybee, *Apis mellifera ligustica*, and studied the allergenicity of the various fractions by means of the radioallergosorbent test (RAST) with sera from 31 patients clinically allergic to honeybee stings. The presence of multiple allergens is demonstrated. The major allergens appear to be the high molecular weight materials and hyaluronidase. Phospholipase A appears to be less important. In several sera, the various allergens show substantial cross-reactivity which is not the result of cross-contamination.

## MATERIALS AND METHODS

### Sera

Sera were obtained from 24 individuals with recent histories of systemic reactions from honeybee stings and from 7 individuals with large local reactions crossing at least one major joint. A group of 10 sera from individuals who had smaller local reactions of less than 5 cm in the longest dimension following bee stings, normal adult sera, and newborn sera all served as controls. All samples from patients were obtained before beginning any Hymenoptera immunotherapy. Almost all samples were obtained between 2 wk and 4 mo following the reactions.

Approximately 85% of individuals from southern California with severe reactions to Hymenoptera stings are stung by honeybees. All sera were RAST-tested with yellow jacket venom sac extract and wasp whole body extract. All of those reacting strongly with either yellow jacket or wasp, who did not give histories clearly implicating bees, were deleted from this study.

### Bee venom

Fresh whole Italian honeybee venom was purchased from Charles Mraz, Middlebury, Vt., and was obtained by the electric shock method. The fresh venom had a hyaluronidase activity of 252 N.F. U/mg dry weight, comparable to that reported by Franklin and Baer.<sup>8</sup> Hyaluronidase is the most labile of the bee venom enzymes known.<sup>8</sup> Three different lots of bee venom phospholipase A were purchased from Sigma Chemical Company, St. Louis, Mo.

### Hyaluronidase assay

Hyaluronidase activity was measured turbidimetrically with acid albumin solution.<sup>9, 10</sup> Hyaluronic acid from human umbilical cord was obtained from Sigma Chemical Company. Results are expressed in National Formulary (N.F.) units.

### Phospholipase A assay

Phospholipase A activity was measured colorimetrically by following the decrease in ester bonds during the hydrolysis of hen's egg lecithin to lysolecithin.<sup>11</sup> The color was produced by the reaction of hydroxamates with ferric chloride. Hen's egg lecithin type III-E was obtained in hexane solution from Sigma Chemical Company. The preparation used showed a maximum 49% decrease in ester bonds when incubated with excess phospholipase A. One unit hydrolyzes one micromole of phosphatidyl choline per minute at 37° and pH 8.5.

### Direct hemolytic assay

Direct hemolytic activity of the fractions was measured with washed human type O erythrocytes in Ca<sup>++</sup>-free phosphate-buffered saline, pH 7.4.

### Polyacrylamide gel electrophoresis (PAGE)

Whole bee venom and the various fractions were electrophoresed in 4%, 7%, and 12% polyacrylamide gels at pH 3.6 in 0.188 M glycine acetate buffer and stained with amido black in 7% acetic acid.

TABLE I. RAST for the diagnosis of honeybee sting hypersensitivity

| Type of reaction | No. of sera | Bee venom —positive (%) | Fr. 1 —positive (%) | Fr. 2 —positive (%) | Fr. 3 or phospholipase A —positive (%) |
|------------------|-------------|-------------------------|---------------------|---------------------|----------------------------------------|
| Systemic         | 24          | 92                      | 92                  | 92                  | 75                                     |
| Large local      | 7           | 100                     | 86                  | 86                  | 57                                     |
| Local <5 cm      | 9           | 0                       | 0                   | 0                   | 0                                      |
| Control          | 103         | 0                       | 0                   | 0                   | 0                                      |

### Dry weight determinations

Filtered samples of fresh whole bee venom were lyophilized and desiccated over  $P_2O_5$ . The total dry weight of 1 ml of bee venom was  $441 \pm 80$  mg.

### Fractionation of whole bee venom

A sample of 6 ml of fresh whole bee venom was filtered through a Millipore filter, and the supernatant fluid was applied to a 2 by 300 cm column of Sephadex G-75 (Pharmacia Fine Chemicals). The separation was carried out in 0.1 M ammonium formate, pH 4.5, at a flow rate of 0.03 ml min<sup>-1</sup> cm<sup>-2</sup>. Then 4-ml fractions were collected. The effluent was monitored at 280 nm. Optical densities at 230 nm gave a curve almost parallel to that obtained at 280 nm. The high molecular weight exclusion peak, about 20 mg, was concentrated by ultrafiltration with an Amicon PM 20 membrane and further separated by gel filtration on an 0.9 by 60 cm column of Sepharose 6-B at a flow rate of 5 ml/hr. The fourth fraction was further separated by chromatography on carboxymethyl Cellex (Bio-Rad Laboratories) using a gradient of 0.01 to 2.0 M ammonium formate, pH 4.5. The low molecular weight peak, 7, could be separated into many substances by a combination of gel filtration on Sephadex G-25 and ion-exchange chromatography.<sup>15, 16</sup>

### Radioallergosorbent test (RAST)

The method used in our laboratories for specific IgE antibody determinations has been described previously, and the evidence for its specificity has been presented.<sup>12, 13</sup> Paper discs of 6 mm diameter punched from Schleicher and Schuell No. 595 filter paper were activated with cyanogen bromide.<sup>14</sup> Bee venom was coupled at a ratio of 10 mg dry weight per 200 activated discs in borate buffer, pH 8.0. The various fractions were coupled at a ratio of 1 mg per 200 discs; increasing the amount of antigen to 10 mg did not significantly increase specific IgE binding. Remaining reactive sites were blocked with ethanolamine, and the discs were extensively washed.

The Fc-specific anti-IgE was of horse origin raised against PS myeloma and specifically purified with ND myeloma.<sup>12</sup> The anti-IgE was labeled with <sup>125</sup>I using 0.2 mCi per 5  $\mu$ g. One nanogram of radioactive anti-IgE was used for each assay tube.

The RAST was run in quadruplicate for each sample with one disc, 10  $\mu$ l of serum, and 100  $\mu$ l of buffer in each 12 by 75 mm plastic tube. After 3 to 6 hr, the discs were washed three times with 3 ml of 1% Tween-20 in saline. Radioactive anti-IgE was then added in 100  $\mu$ l of RAST buffer containing 6% normal horse serum. After 18 hr incubation, the discs were washed four times with Tween saline and counted. Positive and negative controls were run for each antigen and batch of discs included in a run. Newborn serum and negative serum controls were comparable. Background binding for each antigen was calculated for each run by averaging the negative controls. The background was subtracted from the total binding for each sample. Background binding was a function of antigen and batch as well as of radioactive antibody. Since the controls bound an average of  $1.3 \pm 0.1\%$  (SD) of counts added to bee venom discs, positive was defined as greater than 2.0% binding or 1.5 times background binding for this system. A further group of 91 controls bound  $0.8 \pm 0.2\%$  (range) using a second batch of bee venom discs.

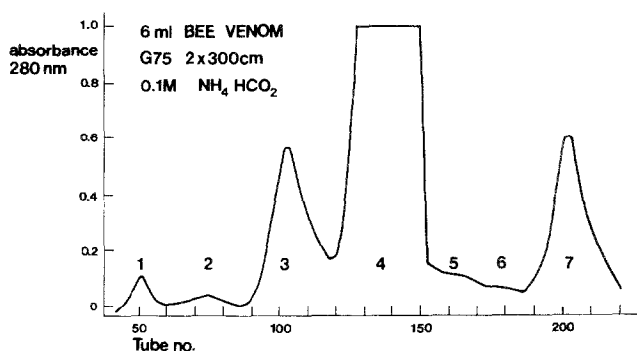


FIG. 1. Gel filtration of 6 ml of whole bee venom on Sephadex G-75 showing labeling of fractions. Fractions were 4 ml.

TABLE II. RAST to whole bee venom and type of reaction to bee sting for 10 individuals with reactions and the control sera

| Serum       | % of total counts bound* | Reaction to bee sting             |
|-------------|--------------------------|-----------------------------------|
| Newborn     | 1.3                      | None                              |
| 12 controls | 1.3 $\pm$ 0.1 (SD)       | Local, less than 5 cm             |
| CC          | 8.6                      | Anaphylaxis                       |
| 514         | 4.9                      | Systemic†                         |
| 545         | 4.9                      | Large local                       |
| 546         | 6.6                      | Urticaria and large local         |
| 598         | 4.0                      | Systemic†                         |
| 599         | 5.5                      | Systemic†                         |
| 645         | 10.1                     | Anaphylaxis                       |
| 662         | 2.3                      | Systemic and generalized erythema |
| 809         | 8.0                      | Large local                       |
| 935         | 13.8                     | Systemic†                         |

\*There were 57,500 cpm added per tube; 47% could be bound to ND myeloma discs. Sera binding greater than 2.0% of counts added were scored positive. All samples were run in at least quadruplicate.

†Including urticaria-angioedema.

### Inhibition of RAST

All inhibition assays were performed in duplicate and those for one serum all at the same time. Ten microliters of serum and 100  $\mu$ l of buffer were mixed with 20  $\mu$ l of varying concentrations of inhibitors in buffer. The appropriate antigen disc was added, and the assay was performed similarly to a RAST assay. Backgrounds were run with each antigen system each time, as well as 100% tubes with no inhibitor. Values were averaged, backgrounds subtracted, and percent inhibition calculated and plotted on semilogarithmic paper. The relative binding constant, Krel, was calculated as the ratio of concentrations giving 50% inhibition.

### RESULTS

The results of radioallergosorbent tests using whole bee venom and fractions 1, 2, and 3 are presented in Table I. Sera from 29 of 31 clinically sensitive patients gave positive RAST results with whole bee venom and 22 were positive with phospholipase A and fraction 3. None of the control sera or sera from individuals with less than 5 cm reactions gave a positive RAST with any of the antigens. All but one of the sera positive to bee venom were positive to fractions

TABLE III. Composition of whole honeybee venom\*

| Fraction | % of total dry weight | Contains                     |
|----------|-----------------------|------------------------------|
| 1        | 1.1 $\pm$ 0.5         | High molecular weight        |
| 2        | 0.7 $\pm$ 0.5         | Hyaluronidase                |
| 3        | 8.6 $\pm$ 2.0         | Phospholipase A <sub>2</sub> |
| 4        | 56.2 $\pm$ 12         | Melittin                     |
| 5        | 3.4 $\pm$ 1.0         | Toxin                        |
| 6        | 9.7 $\pm$ 2.0         |                              |
| 7        | 20.3 $\pm$ 4.0        |                              |

\*The variation of three different lots of bee venom is approximately  $\pm 20\%$ ; 6.4 ml of whole filtered bee venom contained an average of 2,824 mg of nonvolatile material.

TABLE IV. Enzyme activities and direct hemolytic activities of bee venom and its fractions

| Fraction                | Hyaluronidase<br>(N.F. unit/mg) | Phospholipase A<br>(unit/mg) | Direct hemolysis |
|-------------------------|---------------------------------|------------------------------|------------------|
| 1                       | <2                              | <1                           | 0                |
| 2                       | 15,300                          | 28                           | 0                |
| 3                       | 3                               | 1,750                        | +                |
| 4                       | <2                              | 24                           | 4+               |
| 5                       | <2                              | <1                           | 0                |
| Phospholipase A (Sigma) | <2                              | 1,220                        | 0                |
| Whole bee venom         | 252                             | Not determined               | 4+               |

TABLE V. Patterns of RAST reactions with bee venom fractions

| Pattern of reaction<br>with fraction number | Example (Fig. 2) | Number of sera |
|---------------------------------------------|------------------|----------------|
| 1>2 $\geq$ 3                                | 809,598          | 13             |
| 2>1=3                                       | 935              | 12             |
| 1=2=3                                       | 645              | 3              |
| Other                                       |                  | 1              |

1 and 2. This one serum was weakly positive with only fraction 5. The percent of total counts bound and description of reactions to bee stings for 10 representative patients and the control sera are presented in Table II. Positive sera bound from 2.3% to 13.8% of counts added when radioallergosorbent tested with whole bee venom. No control serum bound more than 1.5% of counts added.

Fresh whole bee venom was fractionated on Sephadex G-75 to yield 7 peaks labeled fractions 1 to 7 as shown in Fig. 1. This pattern is similar to those reported by others.<sup>15-19</sup> The fractions were lyophilized and dried, and the weights are given in Table III. The data in this table are the result of at least three determinations.

The fractions were identified and cross-contamination was measured by testing for hyaluronidase, phospholipase A, and direct hemolytic activity. The results of these experiments are presented in Table IV. It has been reported by Franklin and Baer<sup>8</sup> that the enzyme activity and antigenic activity in rabbits of bee venom hyaluronidase are parallel. Venom preparations with no remaining enzyme activity did not show the immunodiffusion band associated with hyaluronidase from active venom preparations. The hyaluronidase activity of the venom used in the present study is comparable to that found for the active preparations by Franklin

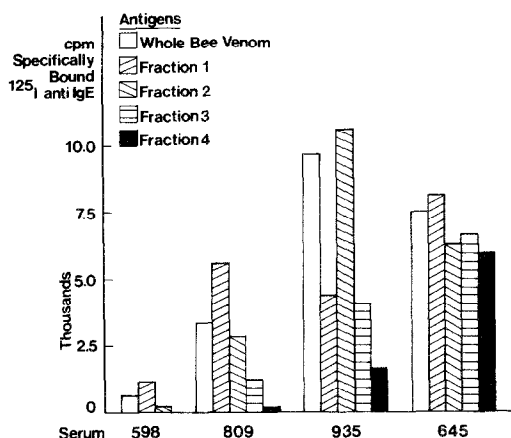


FIG. 2. Radioallergosorbent test patterns using whole bee venom and fractions 1, 2, 3, and 4 with sera from 4 patients with severe hypersensitivity to bee stings.

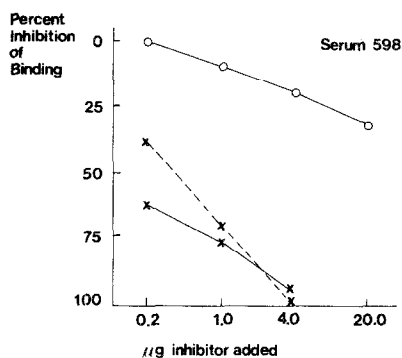


FIG. 3. Inhibition of RAST for serum 598. Solid lines, inhibition of binding to fraction 1; dashed line, inhibition of binding to whole bee venom; x, inhibition by fraction 1; O, inhibition by fraction 2.

and Baer.<sup>8</sup> The data presented in Table V show only trace contamination of fraction 3 with hyaluronidase and 1.6% contamination of fraction 2 with phospholipase A.

Each fraction was applied to 4%, 7%, and 12% polyacrylamide gels at several concentrations and run at pH 3.6. Fraction 1 gave four bands, Fraction 2 a single major band, and fraction 3 gave three bands. Fraction 1 could also be separated into three peaks by gel filtration on Sepharose 6-B, similar to the results reported by others.<sup>19</sup> Fraction 3, phospholipase A, is known to contain three materials all with enzyme activity.<sup>17, 19</sup> None of the three bands was identical to that of hyaluronidase. Fraction 4 was separated into at least four peaks on carboxymethyl cellulose. The largest peak is probably "melittin."<sup>15, 18</sup> Other substances known to be in this fraction include mast cell degranulating peptide and peptide 401.<sup>18</sup> When injected into mice, fraction 5 had an LD<sub>50</sub> of 5 mg/kg and caused symptoms similar to those reported by Habermann<sup>18</sup> for apamin poisoning.

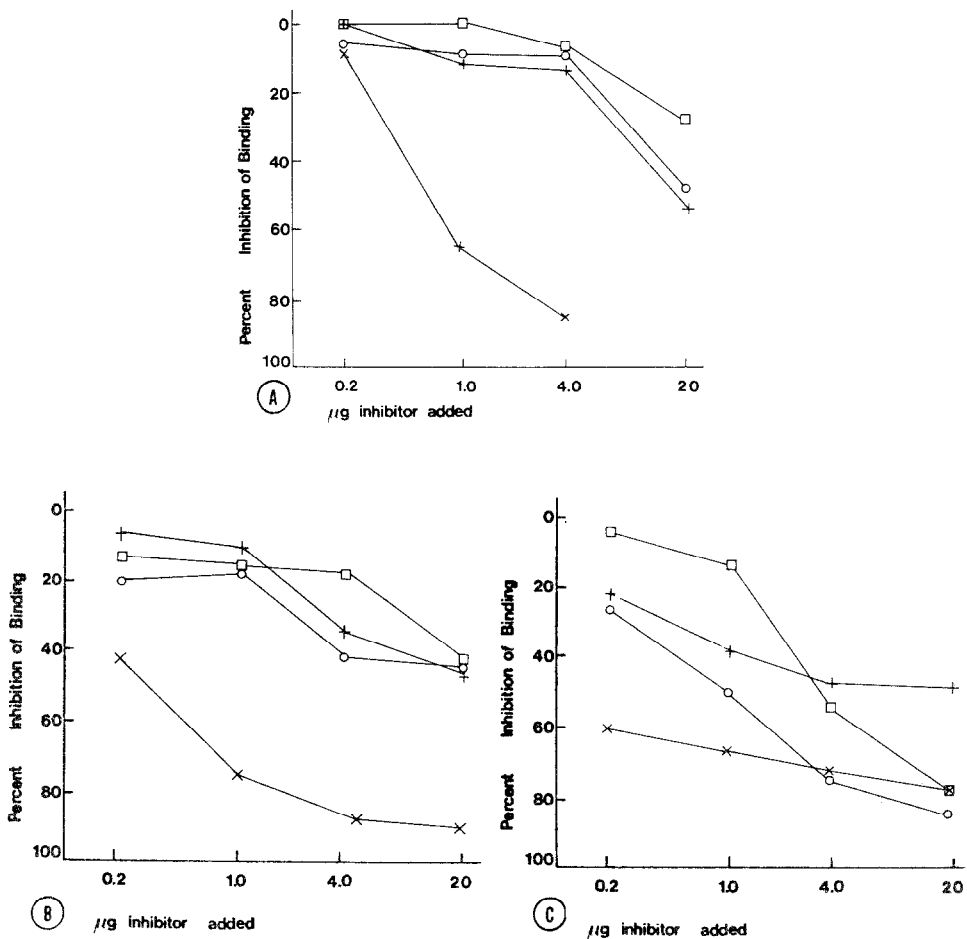


**FIG. 4.** Inhibition of RAST for serum 809. **A**, Inhibition of binding to whole bee venom. **B**, Inhibition of fraction 1 binding. **C**, Inhibition of fraction 2 binding. The inhibitors are indicated by symbols on the graphs: fraction 1, x; fraction 2, o; fraction 3, □; fraction 4, +.

Each serum was radioallergosorbent tested with fractions 1-6. Binding results were identical within experimental error when a quantity of either 1 mg or 10 mg was used to couple per 200 discs. Four representative binding patterns are shown in Fig. 2. The number of sera giving patterns similar to the prototypes are presented in Table V. No serum reacted most strongly with fraction 3 or phospholipase A, but 25 of 29 reacted most strongly with either fraction 1 or fraction 2. All reactions with fractions 5 and 6 were much less than fractions 1, 2, and 3 with the exception of one serum that reacted with fraction 5 only.

Fractions 2 and 3 were radiolabeled with  $^{125}\text{I}$ , diluted with cold antigen, and coupled to CNBr-activated discs. The average amount of fraction 2 bound was  $2.3 \pm 0.3 \mu\text{g}$  and of fraction 3,  $3.8 \pm 0.4 \mu\text{g}$  per disc. Since fraction 1 contains several substances, this type of study would not give reliable results, but studies of binding saturation indicate that less antigen is coupled than is coupled to fraction 2 discs.

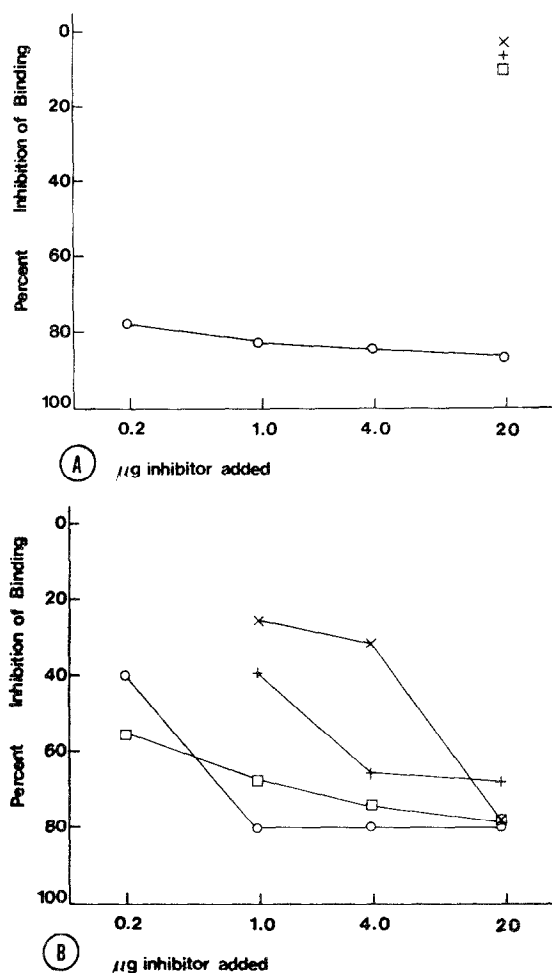


**FIG. 5.** Inhibition of RAST for serum 935. **A**, Inhibition of binding to whole bee venom. **B**, inhibition of fraction 3 binding. Symbols on the graph are the same as in Fig. 4.

In order to further evaluate the importance of factors such as low-level contamination and cross-reactivity, inhibition of RAST experiments were performed with many of the sera. Results for serum 598 are shown in Fig. 3. Fraction 1 could completely inhibit binding to whole bee venom at 4  $\mu$ g, and 94% inhibit binding to fraction 1 discs. Fraction 2 was over 1,000 times less potent an inhibitor. In the results for serum 809 shown in Fig. 4, *A*, fraction 1 could again cause 86% inhibition of binding to bee venom at 4  $\mu$ g, whereas at least 20  $\mu$ g of any of the other fractions is required for 50% inhibition. Similar results are seen with this serum and fraction 1 discs in Fig. 4, *B*, and fraction 1 is still the most potent inhibitor even with fraction 2 discs (Fig. 4, *C*), although in this case fraction 2 is closer in potency.

A quite different pattern is shown by serum 935 in Fig. 5. Only fraction 2

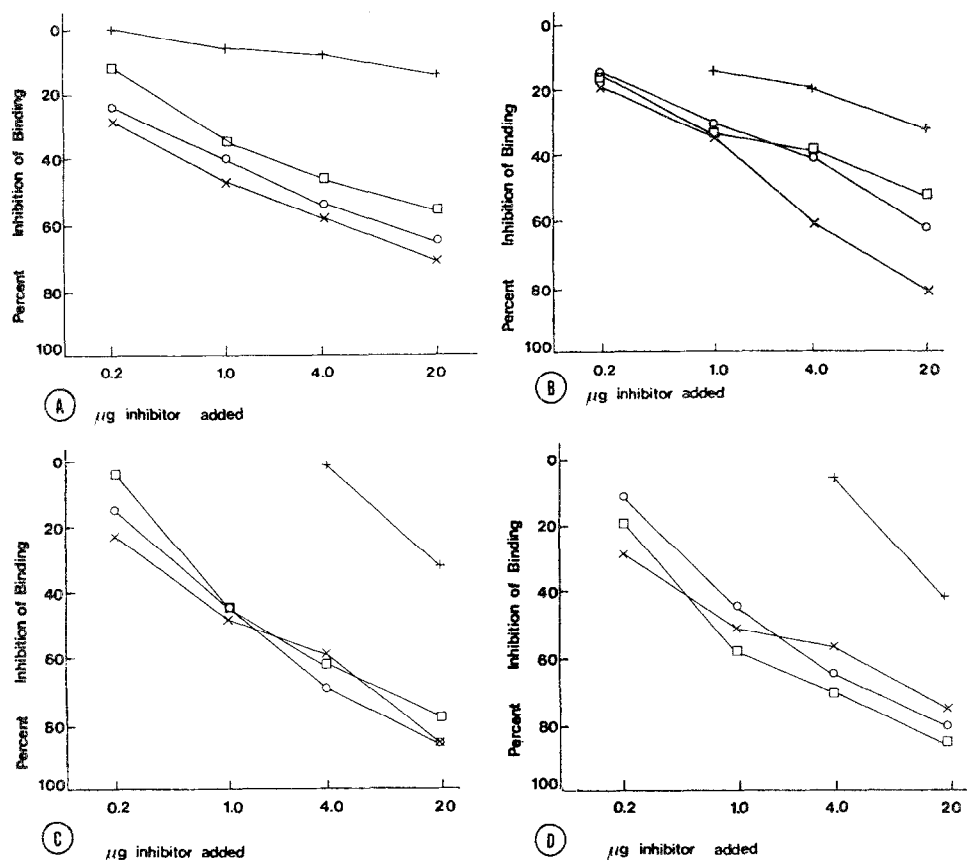


FIG. 6. Inhibition of RAST for serum 645. **A**, Inhibition of binding to whole bee venom. **B**, Inhibition of binding to fraction 1. **C**, Inhibition of binding to fraction 2. **D**, Inhibition of binding to fraction 3. Symbols on the graph are the same as in Fig. 4.

inhibits binding to whole bee venom, 87% at 20  $\mu$ g. At this concentration, the other fractions cause 11% or less inhibition. Similar results are observed with fraction 1 and fraction 2 discs. With fraction 3 discs shown in Fig. 5, *B*, fractions 2 and 3 are comparable inhibitors.

Serum 645 gives a third type of pattern, as shown in Fig. 6. In Fig. 6, *A*, it can be seen that fractions 1, 2, and 3 can all inhibit binding to whole bee venom. The inhibition is not as potent with any of the three fractions as is found for sera 598, 809, and 935. The ratio of 50% inhibition concentrations for fractions 1, 2, and 3 is 1.5 to 2.8 to 8.0. Since the fractions are in decreasing molecular weight order, the molar ratios are even higher. Fig. 6, *B* shows the results of inhibition of binding to fraction 1, similar to the results with whole venom. All three fractions give comparable inhibition on a weight basis of binding to fraction 2, as can be seen in Fig. 6, *C*. Binding to fraction 3 is best inhibited by fraction 3, then to a similar extent by both 1 and 2, as can be seen in Fig. 6, *D*.

## DISCUSSION

The evidence presented in this study clearly shows that the RAST is a useful and reliable method for the diagnosis of IgE-mediated bee sting hypersensitivity, and for determining which nongeneralized reactions (large local reactions) are IgE-mediated.

Seven major fractions were isolated from whole bee venom by Sephadex G-75 gel filtration. Fraction 1 consisted of 3 materials separable by agarose gel filtration, all of molecular weight greater than 75,000. Fraction 2, a single major band on PAGE, was identified as hyaluronidase by its enzyme activity; the very high specific activity of this preparation suggests a relatively high degree of purity. Fraction 3 was identified as phospholipase A by its enzyme activity and contained three bands in PAGE similar to those previously reported by Shipolini and associates.<sup>17</sup> Fraction 4, with its strong direct hemolytic activity, contained melittin as well as at least four other peptides. Fraction 5 is an impure preparation of apamin, a neurotoxin, and fractions 6 and 7 are complex mixtures of lower molecular weight materials, many with pharmacologic actions.<sup>16, 18</sup>

The primary allergen activities as measured by direct RAST with the various fractions were found in fractions 1, 2, and 3 (Table I). Much less activity was found in the other fractions. Phospholipase A, purchased from Sigma Chemical Company, was interchangeable with fraction 3 in RAST experiments. Determinations of enzyme activities (Table IV) and inhibition experiments clearly showed that the RAST results were not due to cross-contamination of the various fractions.

The results of this study suggest that there are several major allergens in bee venom<sup>20-22</sup> and that the most important allergen is not phospholipase A, as has been proposed,<sup>7</sup> but fraction 1 and hyaluronidase. Five lines of evidence are presented to support this contention:

1. Of the 29 patients' sera RAST-positive to bee venom, 28 were positive to both fraction 1 and fraction 2 and seven were negative to both phospholipase A and fraction 3.

2. Of these 29 sera, 13 gave the strongest reaction with fraction 1, 12 with fraction 2, and three about equally with fractions 1, 2, and 3 (Table V). None of the sera reacted most strongly with fraction 3. Substitution of commercial bee venom phospholipase A or using more fraction 3 in the coupling step did not change these results.

3. Phospholipase A was a poor inhibitor of RAST to whole bee venom in many of the cases studied. For example, fraction 3 did not measurably inhibit binding of sera 598 and 935 and was less than 0.03 times as potent as fraction 1 with serum 809. In some cases, such as serum 645, inhibition by fraction 3 was comparable to fractions 1 and 2;  $K_{rel_1} = 0.19$ ;  $K_{rel_2} = 0.34$ .

4. With some sera, fraction 1, with no measurable contamination by fractions 2 or 3, can almost completely inhibit binding of whole bee venom at concentrations of 33 to 167  $\mu\text{g/ml}$ . Serum 935 binding is 87% inhibited by 20  $\mu\text{g}$  of fraction 1, and serum 598 is 100% inhibited by 4  $\mu\text{g}$ . Serum 809 binding to whole bee venom is 86% inhibited by 4  $\mu\text{g}$  of fraction 2.

5. In the cases in which binding to fraction 3 is comparable to that to fractions 1 and 2, there appears to be a substantial cross-reactivity, with each of the 3 fractions being able to inhibit binding to any of the fractions. This would suggest at least one common allergenic determinant among these proteins that can be recognized by some individuals, although results with serum 598 clearly show that a highly specific determinant can also be recognized by some individuals.

Since the studies claiming phospholipase A as major allergen have reported results with only dialyzed bee venom and phospholipase A,<sup>7, 23, 24</sup> further experiments are clearly necessary using both skin-testing of human volunteers and histamine release from human lung or basophil leukocytes with whole venom and the various fractions.

We would like to thank Drs. Sherwin Gillman, Bernard Geller, Franklin Yamamoto, and C. Thomas Hartman, Jr., for contributing many of the sera used in this study. Drs. Zack H. Haddad and H. Stewart Nielsen, Jr., contributed helpful discussions.

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