

The allergic response to stinging insects

V. Fractionation of whole body and venom sac extracts of bee

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*Extracts of whole body and of venom sac of bees (*Apis mellifera*) were fractionated by DEAE-cellulose chromatography. Nine components were separated from the whole body and three from the venom sac preparations. By direct skin testing it was seen that fraction 1 of each type of extract contained the major portion of the allergenic activity. A fraction 6 of whole body extract also had allergenic activity, and in addition showed a specific antigen, among the group in this fraction, which seemed to be responsible for the cross-reaction with wasp preparations. This fraction 6 developed precipitation with antiwasp rabbit serum as well as with anti-bee rabbit serum. The antigenic heterogeneity of each of the fractionated products was examined by means of appropriate rabbit antisera to bee extracts. Six of the nine body fractions gave precipitation, as did one of the three venom sac fractions. Patterns ranged between one and four components in these various fractions. The one antigen detected in venom sac fraction 1 was proved to be identical with one of the four seen in whole body fraction 1. A final discussion for these three papers, comparing the findings for bee, wasp, and yellow jacket, is elaborated in the third paper of this series.⁶*

The complex antigenic structure of stinging insect extracts was demonstrated in previous publications¹⁻⁴ by several methods: paper electrophoresis, starch block electrophoresis, analytical ultracentrifugation, gel diffusion precipitation, and immunoelectrophoresis. These methods revealed the presence of several components in yellow jacket, wasp, and bee whole body and venom sac

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extracts. Immunological and clinical investigations² showed that the antigens eliciting allergic reactions in man were not solely localized in the extracts prepared from venom sacs, but were also present in the body of the insects. Other studies^{3, 4} showed that species-specific antigens are probably localized in the venom sac extracts, whereas antigens common to several stinging insects are localized in the insect bodies. This antigenic distribution could explain why certain patients are sensitive to only one type of insect (allergy to venom) and other patients are sensitive to many types of insects (allergy to both venom and body constituents).

It was felt that by fractionation of our extract and by immunological and clinical studies of isolated fractions from venom sac extracts in comparison with fractions from whole body extracts, additional data could be obtained to support the above theory. This report deals with such fractionations and analyses of extracts of bee. The following papers^{5, 6} present similar information on extracts of wasp and yellow jacket.

MATERIALS AND METHODS

Anion exchange chromatography and insect extracts

Venom sac and whole body extracts of bee (*Apis mellifera*) were prepared as previously described.¹ The insects were purchased*; they were received frozen and were kept at -20° C. in our laboratory until ready for extraction or for collection of venom sacs. The concentrated venom sac and whole body extracts were dialyzed against the starting buffer (buffer I), with three changes of the dialysis bath, before applying the samples to the columns. A modification of the technique of Peterson and Sober⁷ was employed. Twenty grams of DEAE-cellulose† was mixed overnight with 0.5N sodium hydroxide (NaOH), then filtered, and washed thoroughly with distilled water. This procedure was repeated twice. The cellulose was then mixed with the starting buffer (I), filtered, and resuspended in the same buffer (Table I). The cellulose was packed in glass columns, 1 cm. by 68 cm., for trial runs with venom sac extract and 2.5 cm. by 60 cm. for whole body extract. After the column was packed, the starting buffer (buffer I) was passed through the column until a regular flow was obtained, and at appropriate intervals, judged from the decreasing values of optical density, the series of buffers shown in Table I were put through the column. Rates of flow varied from 2 to 5 ml. per hour for small columns (1 cm. by 68 cm.) to 12 ml. per hour for the larger columns (2.5 cm. by 60 cm.). A sample of 2 ml. of concentrated venom sac extract was applied to the small columns, and a sample of 6 ml. of concentrated whole body extract was applied to the large columns. The eluates were collected hourly in the cold room at 4° C., using stepwise elution. After the last buffer, 100 ml. of 0.5N NaOH was used to remove all the material that might be retained on the column.

Eluates were read at $280\text{ m}\mu$ with the Beckman DU spectrophotometer against a blank, corresponding to the buffer used for elution. The eluates were collected into appropriate pools, lyophilized, then redissolved in 20 ml. of

*Greer Drug and Chemical Corp., Lenoir, N. C.

†DEAE-cellulose was obtained from Brown Co., Berlin, N. H.

Table I. *Buffers used for column chromatography*

<i>Buffer</i>	<i>pH</i>	<i>Total molarity of phosphate</i>	<i>Molarity of chloride</i>
I	7.5	0.02	—
II	7.0	0.04	—
III	6.5	0.08	0.05
IV	6.0	0.10	0.50
V	5.5	0.20	1.00
VI	5.0	0.40	1.50
VII	4.0	0.40	2.00

All buffers were mixtures of NaH_2PO_4 , NaOH , and (if needed) NaCl .

0.15M NaCl , and dialyzed against buffered 0.15M NaCl , pH 7.0. These fractions were kept at -20°C . until used for gel diffusion, immunoelectrophoresis, and skin testing.

Skin tests

All fractions used for skin testing were Henning filtered and then checked for sterility before use. Using serial tenfold dilutions of the fractions, intradermal skin tests were performed on the deltoid region of patients sensitive to stinging insects.

Rabbit antisera

Rabbits were immunized with either whole body extracts or venom sac extracts of each type of insect. The immunization procedure was described in a previous publication.³

Agar gel diffusion

The agar gel double diffusion method was used as described by Ouchterlony.⁸

RESULTS

Chromatographic fractionation

Fig. 1 illustrates the fractionation of bee whole body extract by column chromatography. Nine peaks were obtained at various pH levels: two peaks at pH 7.5, three at 6.5, two at 6.0, and one each at 5.5 and 4.0. None had been obtained at pH 7.0, and this buffer was omitted from the experiment shown. The highest peak, peak 7, was eluted at pH 6.0. Thus, this provides the conditions for obtaining the major component(s) of the extract. Each peak was considered as a fraction, and the tubes were appropriately pooled together, as were also the samples between peaks. There was no activity, as judged either by immunological or clinical methods, in the samples located between the peaks. In contrast, the pooled peaks were found to have various degrees of activity, as described below. Fig. 2 illustrates the fractionation of an extract of venom sac. Only three peaks were eluted, one each at pH 7.5, 7.0, and 6.0. The highest peak, peak 1, was obtained at pH 7.5.

Skin reactivity in humans

Skin tests were performed on six nonimmunized patients with bee whole body and venom sac fractions. The type of insect sensitivity is shown at the bottom of Table II. Tests were also done on 10 individuals, including atopic (but not sensitive to insects) and nonatopic people, using all these fractions in the highest concentrations available, and they were consistently negative.

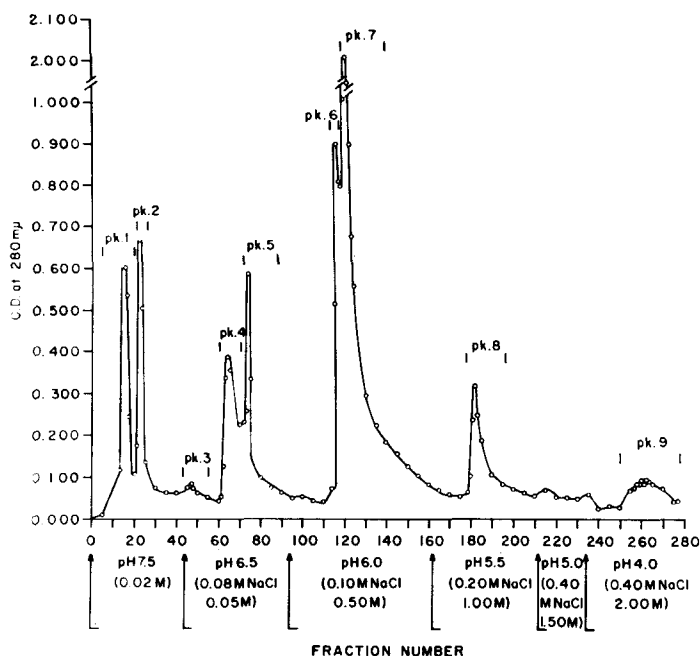


Fig. 1

Column chromatography of bee whole body extract on DEAE-cellulose, 2.5 cm. by 60 cm. column. Buffer II (0.04M sodium phosphate, pH 7.0) was omitted (*pk.* = peak). The pairs of vertical lines show the groups of samples that were pooled to form each peak.

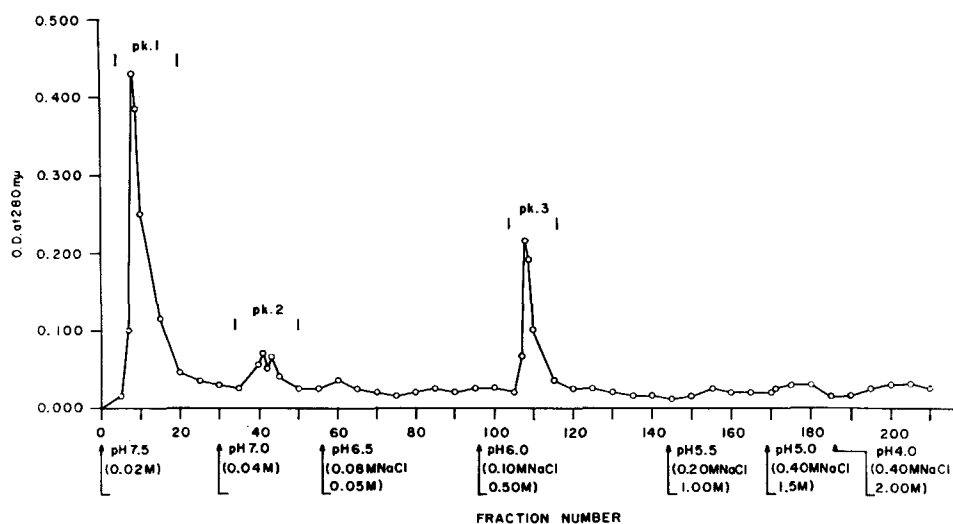


Fig. 2

Column chromatography of bee venom sac extract on DEAE-cellulose, 1 cm. by 68 cm. column (*pk.* = peak). The pairs of vertical lines show the groups of samples that were pooled to form each peak.

Table II. *Skin tests with bee venom sac, bee whole body fractions, and with whole insect extracts*

	Optical density	Patient					
		Hya.	Pal.	Pre.	Col.	Cle.	Bla.
Bee venom sac fractions							
Fract. 1	0.26	10 ²	10 ²	10 ³	10 ²	10 ²	10 ³
Fract. 2	0.16	nr	nr	nr	nr	nr	nr
Fract. 3	0.34	nr	nr	nr	nr	nr	nr
Bee whole body fractions							
Fract. 1	1.01	10 ²	10 ²	10 ³	10	10 ²	10 ³
Fract. 2	0.25	nr	nr	nr	nr	nr	nr
Fract. 3	0.16	nr	nr	nr	nr	nr	nr
Fract. 4	0.66	nr	nr	nr	nr	nr	nr
Fract. 5	0.56	nr	nr	nr	nr	nr	nr
Fract. 6	0.61	10	10 ²	10 ²	10	10	nr
Fract. 7	1.26	nr	nr	nr	nr	10	nr
Fract. 8	0.38	nr	nr	nr	nr	10	nr
Fract. 9	0.23	nr	nr	nr	nr	nr	nr
Whole insect extracts							
Bee		10 ⁴	10 ⁴	10 ³	10 ³	10 ⁵	10 ³
Wasp		10 ³	10 ³	10 ³	10 ²	10 ⁴	nr
Yellow Jacket		10 ²	10 ²	10 ³	10 ²	10 ³	nr

The optical density readings are essentially proportional to the protein concentrations of the samples.

Each skin-test value represents the reciprocal of the greatest dilution of antigen still producing a positive wheal and flare reaction.

nr = no reaction.

The protein concentration of each extract and fraction was estimated by ultra-violet light absorption (optical density) by criteria and assumptions previously discussed.¹ Within reasonable limits, these values were essentially the same, and therefore the skin test titrations, using tenfold dilution steps, were significant. The results are illustrated in Table II. Only fraction 1 of venom sac gave positive skin tests, and it was positive in all six patients. Fractions 2 and 3 of venom sac were entirely negative by skin testing.

Examination of whole body products showed two major loci of activity. Fraction 1 of whole body extract gave positive skin tests in all six patients. Fraction 6 was positive in five of these, but was somewhat weaker than 1. In contrast, fractions 7 and 8 were positive in only one patient, Cle., and only very weakly. All other fractions of whole body extract were completely negative. It is noteworthy that five of these six patients had positive skin tests to bee, wasp, and yellow jacket extracts. Only one patient, Bla., had a positive skin exclusively to bee extracts. This is illustrated at the bottom of Table II. This patient, Bla., was the only one to react solely with fraction 1 of bee whole body extract and not with fraction 6. This lack of species distinctiveness for fraction 6 will be further described, by means of rabbit antisera, below.

Gel diffusion

Fraction 1 of venom sac gave a line of precipitation when tested in gel diffusion with rabbit antiserum to bee venom sac extract, and this line showed a reaction of complete identity also with the unfractionated venom sac extract.

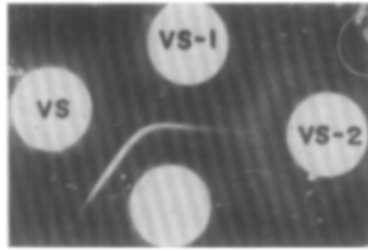


Fig. 3

Gel diffusion precipitation. Center well, anti-bee venom sac rabbit serum No. 66. Peripheral wells, *VS*, bee venom sac extract; *VS-1*, fraction 1 of venom sac extract, *VS-2*, fraction 2 of venom sac extract.

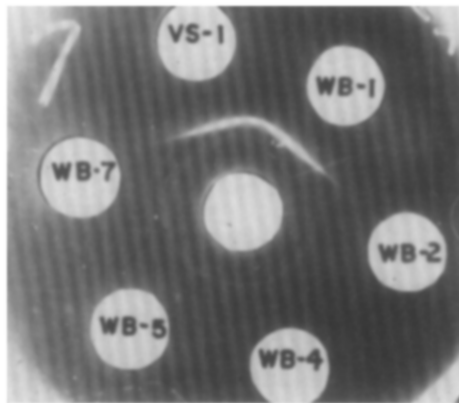


Fig. 4

Gel diffusion precipitation. Center well, anti-bee venom sac rabbit serum No. 66. Peripheral wells: *WB-1* to *WB-7*, fractions of corresponding number, of bee whole body extract; *VS-1*, fraction 1 of bee venom sac extract.

This is illustrated in Fig. 3. Fraction 2 failed to precipitate with the rabbit antibee venom sac serum, as did also fraction 3 in other experiments. Fraction 1 of venom sac extract gave a reaction of complete identity also with fraction 1 of whole body extract when they were both precipitated by an antivenom sac serum, as illustrated in Fig. 4. No fraction of bee whole body, other than fraction 1, was precipitated by an antivenom sac serum.

A systematic examination of all the fractions of whole body extracts was conducted, using a rabbit antiserum to bee whole body extract. Six of the fractions did precipitate, namely, 1, 2, 4, 5, 6, and 7, whereas 3, 8, and 9 did not precipitate. Further inspection revealed that the precipitating fractions varied in their numbers of antigenic components. The comparison is shown in Fig. 5. Fraction 1 gave three lines, fraction 2 gave one line (or possibly two), fraction 4 gave one line, fraction 5, two lines, fraction 6, three lines (two of them superimposed), and fraction 7, two lines. The most striking lines are those shown by fractions 6 and 7. These and the other lines showed, in general, various extents of common antigenic identity. However, fraction 6 showed one line that did not cross-react with fraction 7 or with fraction 5. This line seemed to be specific

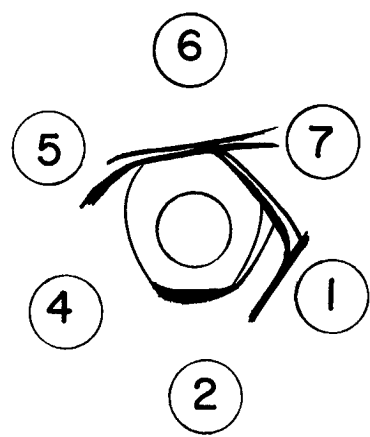


Fig. 5
Gel diffusion precipitation. Center well, anti-bee whole body rabbit serum No. 59. Peripheral wells, fractions of bee whole body extract, as numbered.

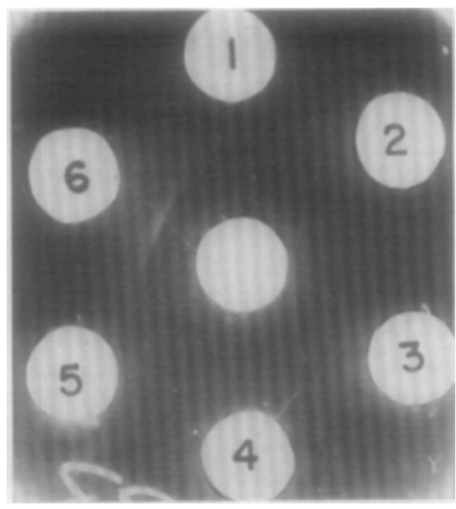


Fig. 6
Gel diffusion precipitation. Center well, antiwasp whole body rabbit serum No. 57. Peripheral wells, fraction of bee whole body extract, as numbered.

to fraction 6 and may also be of special interest in connection with common sensitization between different insects. For in fact, when fraction 6 was examined in gel diffusion for this purpose, it was the only fraction of all the bee whole body fractions to give a line of precipitation with a rabbit antiserum produced against a type of insect other than bee, in this instance, wasp (*Polistes exclamans*). This is illustrated in Fig. 6, for fractions 1 through 6. There had been no reaction between bee venom sac fractions and antiwasp rabbit serum. This specific line seen in Fig. 5 represents the common antigen between extracts of the whole body of both bee and wasp. There was an additional component in fraction 6 that also did not react with fraction 7. It is rather striking that peaks

6 and 7, although exhibiting some common antigenic composition, also show distinctive antigens, despite the fact that they were eluted so close together. Their contents of skin-sensitizing factor are also quite different, as shown in Table II. A similarly distinctive pattern is seen for peaks 1 and 2. Although their precipitation analyses are not markedly different (Fig. 5), their skin reactivities in humans are strongly different (Table II), and yet they were eluted very close together.

DISCUSSION

Previous studies³ had demonstrated the complex nature of bee whole body extracts as compared to bee venom sac extracts; by immunoelectrophoretic analysis, nine components had been identified in whole body extracts and two in venom sac extracts. Other methods have also been applied to these comparisons of heterogeneity.^{9, 10} The various components were now separated by column chromatography into well-defined fractions which permitted the study of isolated, partially purified fractions. Nine fractions were obtained in this way from whole body and three from venom sac.

The allergenic activity of whole body and venom sac extracts, as measured by skin testing, was localized chiefly in the fractions eluted with the first buffer (0.02M sodium phosphate, pH 7.5). The fraction 1 of each type of extract contained antigens which were specific to bee and did not cross-react with antisera produced against other types of stinging insects. The reaction of complete identity between fraction 1 of venom sac and one of the components of fraction 1 of whole body extract, supports previous evidence³ of a common antigen between the venom sac and the body of this stinging insect. Other studies, made subsequently, have revealed a more detailed analysis of these antigens.¹¹ By use of additional antisera, it was possible to reveal two precipitating lines for venom sac extract in gel diffusion, one of which was common to a component of body extract and one of which was unique for the venom itself.

The isolation of another fraction with some allergenic activity, fraction 6, from bee whole body extracts, but not from bee venom sac, and the cross-reactivity of fraction 6 with wasp supports our theory that antigens common to several types of stinging insects are localized primarily in the bodies of the insects, instead of the venom. Furthermore, the fact that five patients who showed positive skin tests to other types of stinging insects besides bee, reacted to fraction 6, and the fact that only a patient sensitive to bee alone did not react with fraction 6, further substantiates our opinion that patients sensitive to many stinging insects may become so, by being sensitive to an allergen common to many species of stinging insects.

Some of the fractions failed to precipitate with the appropriate sera. Although they were concentrated after preliminary studies, these fractions probably failed to elicit antibody formation in the rabbit when the extracts were inoculated into the rabbits. Our study also demonstrated that with a mixture of several antigens, only one or two participate in allergic reactions.

Additional studies have been conducted more recently, utilizing pure extruded venom, along with the other three types of preparation. It was shown in several ways that venom does in fact contain only one antigen in significant

amount, and that this antigen is specific for bee venom.¹¹ One can, therefore, refer to a venom-specific antigen. The allergenic properties of venom and body constituents could thus be better differentiated.

REFERENCES

1. Shulman, S., Langlois, C., and Arbesman, C. E.: The Allergic Response to Stinging Insects. I. Preparation of Extracts and Their Biochemical Characterization, *J. ALLERGY* **35**: 446, 1964.
2. Langlois, C., Shulman, S., and Arbesman, C. E.: The Allergic Response to Stinging Insects. II. Immunologic Studies of Human Sera From Allergic Individuals, *J. ALLERGY* **36**: 12, 1965.
3. Langlois, C., Shulman, S., and Arbesman, C. E.: The Allergic Response to Stinging Insects III. The Specificity of Venom Sac Antigens, *J. ALLERGY* **36**: 109, 1965.
4. Langlois, C., Shulman, S., and Arbesman, C. E.: The Allergic Response to Stinging Insects. IV. Cross-Reactions Between Bee, Wasp, and Yellow Jacket, *J. ALLERGY* **36**: 147, 1965.
5. Langlois, C., Shulman, S., Kozmycz, S., and Arbesman, C. E.: The Allergic Response to Stinging Insects VI. Fractionation of Whole Body and Venom Sac Extracts of Wasp, *J. ALLERGY* **37**: 359, 1966.
6. Arbesman, C. E., Langlois, C., Bronson, P., and Shulman, S.: The Allergic Response to Stinging Insects. VII. Fractionation of Whole Body and Venom Sac Extracts of Yellow Jacket, *J. ALLERGY*. In press.
7. Peterson, E. A., and Sober, H. A.: Chromatography of Proteins. I. Cellulose Ion-exchange Absorbents, *J. Am. Chem. Soc.* **78**: 756, 1956.
8. Ouchterlony, O.: Diffusion in Gel Methods for Immunological Analysis, *Progr. Allergy* **5**: 1, 1958.
9. O'Connor, R., Rosenbrook, W., Jr., and Erickson, R.: Disc Electrophoresis of Hymenoptera Venoms and Body Proteins, *Science* **145**: 1320, 1964.
10. O'Connor, R., and Erickson, R.: Hymenoptera Antigens: An Immunological Comparison of Venoms, Venom Sac Extracts, and Whole Insect Extracts, *Ann. Allergy* **23**: 151, 1965.
11. Shulman, S., Bigelsen, F., Lang, R., and Arbesman, C. E.: The Allergic Response to Stinging Insects: Biochemical and Immunological Studies on Bee Venom and Other Bee Body Preparations, *J. Immunol.* In press.