

The allergic response to stinging insects

I. Preparation of extracts and their biochemical characterization

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Allergic reactions to stings from insects are quite common, and they may be fatal.¹ The clinical importance of allergic and anaphylactoid reactions to Hymenoptera stings and the relative paucity of information regarding the immunologic response to such stings prompted these investigations. We have studied rabbits immunized with, and patients sensitive to, the following stinging Hymenoptera: bee (*Apis mellifera*), wasp (*Polistes exclamans*), and yellow jacket (*Vespula pennsylvanicus*).

Three sources of sensitizing antigen from Hymenoptera have been reported previously: pollen,² insect body protein,³⁻⁶ and venom.⁷ Ellis and Ahrens⁸ clearly demonstrated by skin tests and passive transfer tests that the sensitivity was not caused by pollen carried by these insects. The same authors prepared extracts of the insect body minus the venom sac, and reported that these extracts gave reactions on intracutaneous tests equal to those obtained by an equivalent dilution of the venom itself. Other investigators also demonstrated that the sensitizing antigen was located throughout the whole insect and not

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simply in the venom.^{3, 4} Fackler and Loveless⁷ examined the venom sac antigens and the antigens of whole insect extracts on the basis of acid-precipitated nitrogen, and reported the venom preparation to be three and one-half to five times more active by skin test.

Several potent pharmacologic substances have been identified in the venom of Hymenoptera. Histamine, serotonin, and bradykinin were identified in wasp venom by Jaques and Schaeter^{9, 10} and by Mathias and Schaeter.¹¹ Bee venom, however, was found to lack serotonin and bradykinin. Several enzymes were also demonstrated in the venom of Hymenoptera; hyaluronidase¹²⁻¹⁴ and lecithinase¹⁴⁻¹⁶ were demonstrated in the venom of all Hymenoptera, and cholinesterase¹⁴ in wasp venom.

Our investigation was directed toward the preparation of potent allergenic and antigenic extracts and the localization, purification, and identification of the antigen or antigens responsible for the reactions mentioned. Extracts of different parts of stinging insect bodies, such as venom sac, whole insect, and sacless insect, were prepared and investigated, and the immunologic cross-reactions between these different types of extracts were studied. We were also interested in studying the cross-reactivity between the different insects investigated. Various fractionated products were obtained, and further studies have been made on these fractions.*

MATERIALS AND METHODS

Insects

Three different types of stinging insects were purchased.† These were bee, wasp, and yellow jacket. They were received frozen and kept at -20° C. in our laboratory until ready for extraction or for collection of venom sacs.

Collection of venom sacs

The venom sac of each insect was obtained as follows: Under a dissecting microscope the stinger was pulled out, along with the stinging apparatus, by a pair of fine forceps. The stinging apparatus was quickly frozen on a glass slide set upon dry ice. The venom sac was removed from the stinging apparatus; contamination from other parts of the stinging apparatus was avoided, as far as possible. The venom sacs were kept in a freezer until a sufficiently large number were collected and then processed for extraction.

Procedure of extraction

A. Extracts of whole insects. A volume of 5 ml. of precooled saline (0.15M sodium chloride) was used for each gram of insect bodies. Such a mixture was homogenized with a Servall Omni-Mixer in the cold room for three 1 minute intervals, the mixture being allowed to cool between intervals. This suspension was centrifuged at 12,000 to 16,000 r.p.m. for 45 minutes in the refrigerated centrifuge to give a clear supernatant liquid. The rather large residue could be resuspended with the initial volume of fresh saline and homogenized and

*A preliminary report of these studies has been published.¹⁷

†Greer Drug and Chemical Corporation, Lenoir, N. C.

centrifuged to yield a second extract of this preparation. Sometimes a third successive extract was obtained before the residue was discarded. Every new preparation of material was labeled with a letter in alphabetical sequence, and each of the consecutive extractions was numbered as 1, 2, or 3.

B. Extracts of venom sacs. The venom sacs were crushed by a glass rod in precooled saline (0.15M sodium chloride); a volume of 3 ml. of saline was used for 50 venom sacs. The suspension thus obtained was centrifuged at 12,000 to 16,000 r.p.m. for 45 minutes in the refrigerated centrifuge to give a clear supernatant liquid. Again, a rather large residue was discarded. These preparations were all designated V, for venom, and they were numbered in sequence of preparation.

C. Extracts of sacless insects. After the venom sacs were removed, the insect bodies were extracted by the same procedure as described for whole insect extracts. These extracts were labeled as extracts of sacless insects. All extracts were kept at -20° C. until ready for use.

Commercial extracts

Commercial extracts of whole insects of bee, wasp, and yellow jacket were purchased* and used for skin testing.

Allergenic activity of extracts

In order to determine whether or not our extracts contained the allergen responsible for allergic manifestations in man, and also in order to ascertain the proper dilution that would not give a false positive wheal-and-flare reaction, several nonallergic individuals and insect-sensitive patients were skin-tested with our extracts. Evaluation of the allergenicity of our extracts was performed by direct intradermal skin tests and passive transfer studies. All extracts were Seitz-filtered and then checked for sterility prior to skin testing.

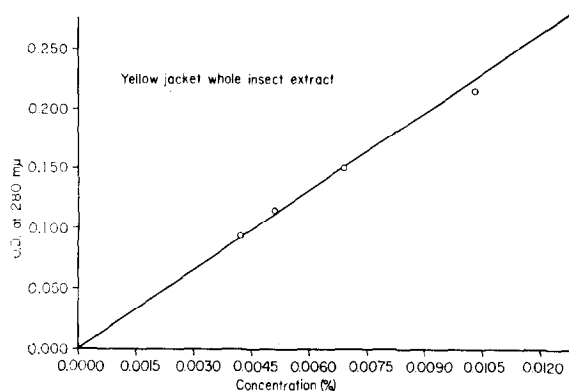
Protein determination

Two different light absorption methods were used. All measurements were made with the Beckman DU spectrophotometer. The methods were (a) absorption in ultraviolet light and (b) the reaction with the biuret reagent, according to the method of Gornall, Bardawill, and David.¹⁸⁻¹⁹ Each of these methods detects a different protein group, and they differ in their degree of sensitivity. Extinction coefficients were determined for each method and for each insect, as a consequence of comparison of light absorption with dry weight measurement on well-dialyzed samples. This calibration procedure will be described in detail.

Determination of extinction coefficient

Extinction coefficients were determined for two preparations of each stinging insect (whole insect extract and venom sac extract). The samples were dialyzed at 4° C. against a large volume of 0.15M sodium chloride with several changes of the external solution in order to obtain a known concentration of

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**Fig. 1**

Optical density at 280 $m\mu$ plotted against concentration of a yellow jacket whole insect extract.

the salt in the extract solutions. Portions of the dialyzed samples of extract, along with samples of the final dialysis bath, were dried at 105° C. for 48 hours to allow calculation of concentration of the salt-free protein. The concentration in these particular whole insect preparations was 0.415 per cent for yellow jacket, 0.407 per cent for wasp, and 0.459 per cent for bee. The venom sac extract concentration was 0.035 per cent for yellow jacket, 0.080 per cent for wasp, and 0.040 per cent for bee.

A. Ultraviolet absorption. The optical density (O.D.) was determined at 280 $m\mu$ for a series of dilutions of the dialyzed samples of bee, wasp, and yellow jacket whole body extracts and venom sac extracts; these values were plotted against the concentration (c), to give a straight line passing through the origin. From the slope and from the known concentration (as shown in Fig. 1) the extinction coefficient, $E_{1\%}^{1\text{cm.}}$, was determined. The results are illustrated in

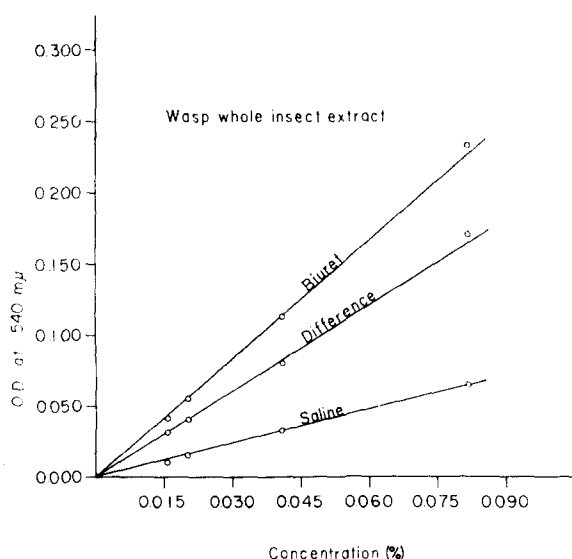
Table I. This is in accordance with the equation $c = \frac{\text{O.D.}}{E}$.

B. Biuret absorption. A series of dilutions of whole extracts of the three stinging insects was also prepared for use with the biuret reagent. A volume of 0.50 ml. of each dilution was mixed with 2.00 ml. of the reagent, and after 30 minutes the readings (O.D._b) against a suitable blank of diluted biuret reagent were taken at 540 $m\mu$. As the original extract was colored by the pigments from the insect, measurements of mixtures of these solutions with saline (O.D._s) in place of the biuret reagent were also made.

Each type of measurement, when plotted against the concentration, gave a

Table I. Extinction coefficients of insect extracts for ultraviolet absorption at 280 $m\mu$

Insect	$E_{1\%}^{1\text{cm.}}$ of extracts	
	Whole body	Venom sac
Bee	11.6	16.6
Wasp	13.9	11.3
Yellow jacket	22.9	32.3

**Fig. 2**

Optical density at 540 $m\mu$ plotted against concentration of a wasp whole insect extract in the biuret reaction. The upper line represents the reaction with biuret reagent, the lower line that with saline, and the middle line is the difference in the pair of readings.

straight line passing through the origin, as shown in Fig. 2. A plot was also constructed for the differences between the pairs of readings. The slopes for these three lines were 3.27, 1.30, and 1.97 for yellow jacket; 2.52, 1.98, and 0.54 for bee; and 2.77, 1.99, and 0.78 for wasp. In order to calculate concentrations by means of the biuret reaction it was then necessary to obtain two readings, one with the reagent and the other with the salt solution being used. The difference between these must be divided by the difference in the extinction coefficients. This is summarized in the following equation, and the extinction coefficients for the whole insect extracts are illustrated in Table II.

$$\begin{aligned}
 c &= \frac{O.D._b - O.D._s}{E_b - E_s} = \frac{O.D._b - O.D._s}{1.30} \text{ for yellow jacket} \\
 &= \frac{O.D._b - O.D._s}{1.98} \text{ for bee} \\
 &= \frac{O.D._b - O.D._s}{1.99} \text{ for wasp}
 \end{aligned}$$

Electrophoresis

Two different methods of zone electrophoresis were used for the tentative separation of our extracts.

A. Paper electrophoresis was conducted in the Spinco Model R instrument with different buffer preparations at various pH levels. Bee, wasp, and yellow jacket whole insect extracts were submitted to electrophoresis on filter paper strips with barbital buffer, pH 8.6, of ionic strength 0.05. Phosphate buffers (ionic strength 0.05, pH 7.5, 7.0, 6.5, and 6.0) and acetate buffers (ionic strength 0.05, pH 5.5, 5.0, and 4.5) were also evaluated to determine if they might pos-

sibly provide a better separation. The strips were stained with bromphenol blue and examined in the Analytrol unit.

B. Starch block electrophoresis with barbital buffer, pH 8.6, ionic strength 0.05, was performed according to the method described by Shulman and colleagues.²⁰ Fractionation by starch block electrophoresis was also attempted at several pH levels.

Ultracentrifugal analysis

The ultracentrifugal studies were made in the Spinco Model E instrument. A conventional cell and a synthetic-boundary cell were used. Each type of cell had advantages for different studies. The running time was about 2 to 3 hours, and the speed was approximately 60,000 r.p.m. The sedimentation coefficient (*s*) was calculated by plotting the logarithm of the distance (*x*) from the axis of rotation, against the time (*t*). Temperature readings were taken during the run with the RTIC unit. The constants have been corrected to the values at 20° C., *s*_{20, w}, and were recorded in Svedberg units (*S*). A value of 0.75 was assumed for the partial specific volume. For determination of the relative proportions of the several constituents, area measurements were made by planimetry of the enlarged tracings. These were corrected in the usual way to account for the radial dilution effect.

RESULTS

Table III shows the protein concentration of whole insect extracts by the biuret and ultraviolet methods, after dialysis.

Both methods of measurement compared favorably unless the material was not dialyzed. When dialysis was not performed, the ultraviolet readings were considerably higher than the biuret, probably because of low-molecular weight ultraviolet-absorbing materials present in the original extract. The protein con-

Table II. *Extinction coefficients of whole insect extracts for biuret absorption at 540 mμ*

<i>Insect</i>	<i>E</i> $\frac{1\%}{1\text{ cm.}}$
Bee	1.98
Wasp	1.99
Yellow jacket	1.30

Table III. *Protein concentration of dialyzed whole insect extracts*

<i>Extract</i>	<i>Method</i>	
	<i>Biuret (%)</i>	<i>Ultraviolet (%)</i>
Bee A-1	0.47	0.43
Yellow jacket A-1	0.42	0.42
Wasp A-1	0.41	0.43

Extracts are labeled A-1 because A designates this particular preparation for each insect and because each solution shown here was the first of several successive extractions.

Table IV. *Protein concentration of nondialyzed whole insect extracts*

Extract	Method	
	Biuret (%)	Ultraviolet (%)
<i>Laboratory</i>		
Bee D-1	0.40	1.65
Wasp D-1	0.55	1.88
Yellow jacket D-1	0.63	1.10
<i>Commercial</i>		
Bee	0.44	1.39
Wasp	0.59	1.12
Yellow jacket	0.61	1.10

Table V. *Protein concentration determined by the biuret method on successive extracts of whole insects*

Extract	Concentration (%)		
	Bee	Wasp	Yellow jacket
A-1	0.45	0.41	0.41
A-2	0.16	0.15	0.23
A-3	0.00	0.08	0.04

Table VI. *Protein concentration determined by the ultraviolet method on nondialyzed and dialyzed venom sac extracts*

Extract	Concentration (%)	
	Nondialyzed	Dialyzed
Bee V-1	0.73	0.30
Wasp V-1	4.00	0.42
Yellow jacket V-1	1.14	0.42

Extracts are labeled V-1 because V designates a venom sac preparation and because each solution shown here was the first preparation for this insect.

centration measurements of commercial extracts were similar to those of the extracts prepared in our laboratory. These comparisons are illustrated in Table IV.

Successive extractions of the residue encountered in whole insect preparations resulted in a steady decrease in protein concentration, as illustrated in Table V. The volumes of the extracting saline solution were kept constant.

The venom sac extracts were also examined for their levels of protein, as determined by the ultraviolet method on the nondialyzed extracts. These nondialyzed extracts exhibited much higher values than the corresponding dialyzed extracts, as was also true for the whole insect extracts. This is illustrated in Table VI. The comparison of these concentrations with those of the whole body extracts is not really meaningful, since the number of venom sacs per volume of extracting fluid was chosen arbitrarily.

Removal of venom sacs from insect bodies did not appreciably alter the protein concentration, and these sacless body extracts had the same protein concentrations as did whole insect extracts.

Allergenicity of the extracts

The allergenicity of our laboratory whole insect extracts was determined by direct skin tests in patients sensitive to stinging insects. These reactions were compared to results obtained with similar tests by commercial extracts.

The results with our laboratory extracts are illustrated in Table VII. Of eighteen untreated insect-sensitive patients, five had a positive skin test to only one of the three stinging insects studied. Nine had positive skin tests to more than one type of stinging insect, and four had delayed skin reactions. These results indicated a wide range of cross-reactivity. Of sixteen patients treated by hyposensitization injections of stinging insect extracts, five reacted to only

Table VII. *Direct skin tests with whole insect extracts in untreated and treated patients*

Patients	Titers		
	Bee	Wasp	Yellow jacket
<i>Untreated</i>			
Bac.	0	Delayed	Delayed
Bea.	10 ³	10 ⁴	10 ³
Bla.	10 ³	0	0
Boe.	10 ²	10 ²	10 ⁴
Bro.	0	0	10 ³
Fre.	10 ²	10 ²	0
Hya.	10 ³	10 ⁴	10 ³
Mey. B.	Delayed	Delayed	0
Mey. D.	Delayed	Delayed	0
Pea.	10 ²	10 ²	10 ²
Phi.	10 ²	0	0
Reg.	10 ²	0	0
Rub.	10 ²	10 ²	10 ²
Sec.	0	10 ²	10 ³
Sha.	10 ²	0	10 ²
Smi.	0	Delayed	Delayed
Swe.	10 ²	10 ⁴	0
Ver.	0	0	10 ²
Total = 18			
<i>Treated</i>			
Bae.	0	0	10 ²
Broc.	0	10 ²	10 ³
Cle.	10 ⁵	10 ⁴	10 ³
Col.	10 ³	10 ²	10 ²
Cur.	10 ²	0	0
Ett.	10 ³	10 ⁴	0
Gar.	10 ²	10 ²	10 ²
Hya.	10 ³	10 ⁴	10 ³
Lop.	10 ³	10 ²	10 ²
Mor.	0	0	10 ²
Pal.	10 ⁴	10 ³	10 ²
Pren.	10 ²	10 ²	10 ²
Pres.	10 ³	10 ³	10 ³
Rot.	10 ³	0	0
Sec.	0	10 ²	10 ³
Tru.	10 ³	0	0
Total = 16			

The protein concentration of the extracts used for skin tests was bee D-1, 0.40 per cent; wasp D-1, 0.55 per cent; and yellow jacket D-1, 0.63 per cent. Each value represents the reciprocal of the greatest dilution of antigen still producing a positive wheal-and-flare reaction.

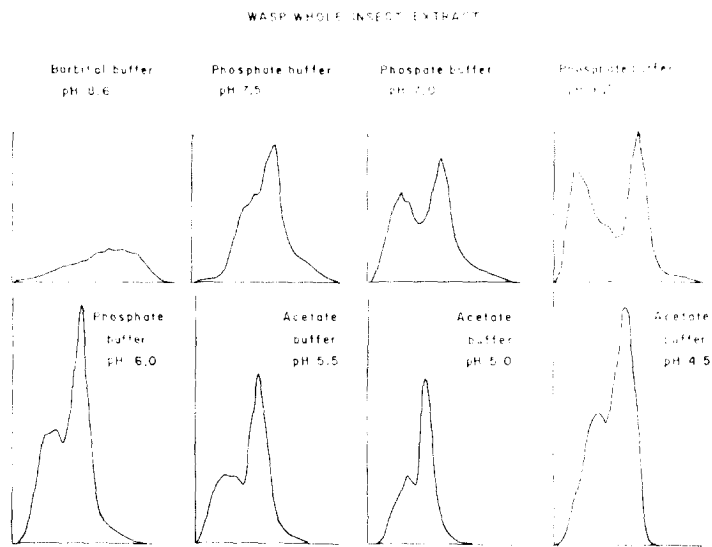


Fig. 3
Electrophoretic distributions in wasp whole insect extract, as studied at various pH levels. Shown are the densitometer tracings for the stained filter paper strips.

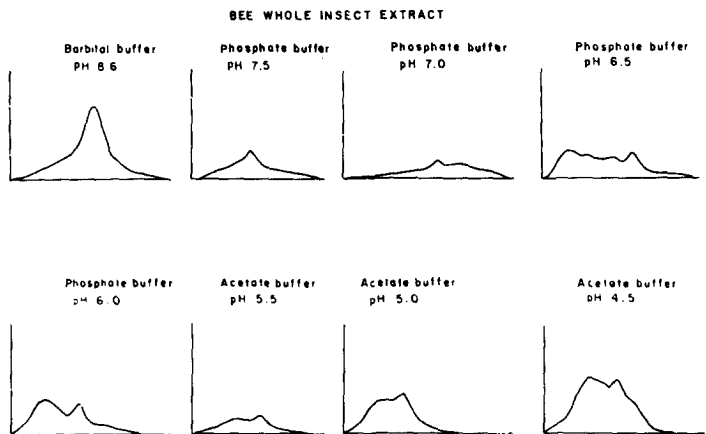


Fig. 4
Electrophoretic distributions in bee whole insect extract, as studied at various pH levels. Shown are the densitometer tracings for the stained filter paper strips.

one type of insect, and the other eleven reacted to two or three different insects. One of the three insects studied caused a higher titer than the other two in twenty-three out of the total of thirty-four patients. Commercially prepared extracts gave the same distribution of results. A 1:10 dilution (protein concentration of 0.04 to 0.06 per cent) of the stock solutions of commercial* and laboratory extracts gave a nonspecific wheal-and-flare reaction in nonallergic

*Labeled 1 to 10 by the manufacturer.

individuals. However, dilution of 1:100 or greater did not produce these reactions.

Zone electrophoresis

Paper electrophoretic analysis was performed on each type of whole insect extract. In order to find the conditions providing the best separation, several buffers were used. The resolution obtained in the customary barbital buffer, pH 8.6, was very poor. At successively lower pH levels, the patterns for the wasp extract showed an increasing degree of resolution into two major components. The best separation was obtained with a phosphate buffer of pH 6.5, ionic strength 0.05. A whole range of separations with these different buffers is

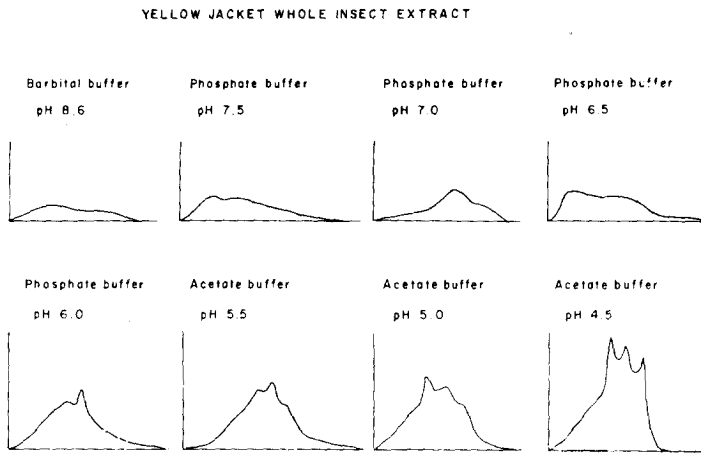


Fig. 5
Electrophoretic distributions in yellow jacket whole insect extract, as studied at various pH levels. Shown are the densitometer tracings for the stained filter paper strips.

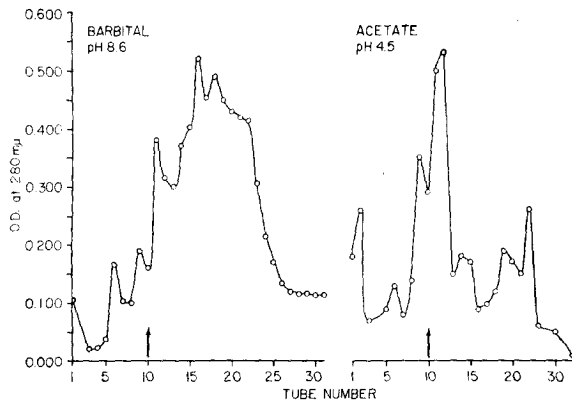


Fig. 6
Starch block electrophoresis of yellow jacket whole insect extract with barbital buffer, pH 8.6, and acetate buffer, pH 4.5.

depicted in Fig. 3, which shows the densitometer tracings from the stained strips. Similar results were obtained with bee whole insect extract. Two major components can be seen in Fig. 4. For yellow jacket whole insect extract, acetate buffer of pH 4.5, ionic strength 0.05, gave the best results, showing a resolution into three components, as depicted in Fig. 5.

Starch block electrophoresis gave, in general, a poor resolution of whole insect preparations. The bulk of the extract migrated slowly toward the anode, and poor fractionation resulted. A typical result is shown in Fig. 6. In Fig. 6, yellow jacket whole insect extract was fractionated with the conventional barbital buffer of pH 8.6, ionic strength 0.05, and with acetate buffer of the same ionic strength, pH 4.5. A slightly better separation occurred at pH 4.5, as was also demonstrated on paper electrophoresis. Although starch block electrophoresis could have been attempted for extensive fractionation of extracts, column chromatography on DEAE-cellulose proved to be more useful. This will be reported in a subsequent article.

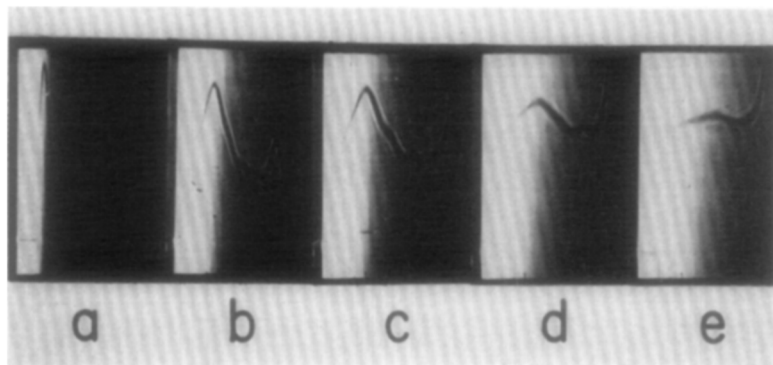


Fig. 7

Ultracentrifugal sedimentation pattern of a yellow jacket whole insect extract. Concentration of protein = 2.61 per cent. Photographs (left to right) at 8, 30, 46, 78, and 110 minutes after rotor reached speed of 59,800 r.p.m. Schlieren angles were 55, 40, 35, 30, and 30 degrees, respectively. Direction of sedimentation in each picture is to the right. Peak I is best seen in photographs *a* and *b*; peak II in *b*, *c*, and *d*; peak III in *c*; and peaks IV and V (resolved) in *e*.

Ultracentrifugation

Ultracentrifugal studies of the extracts of whole insects, of yellow jacket, bee, and wasp, revealed fairly similar patterns consisting essentially of five components. Since the sedimentation coefficients for the different preparations seemed to correspond to each other, a single set of designations was introduced. The sedimenting boundaries were termed I, II, III, IV, and V, in the order of decreasing sedimentation rates. The proportions of the various components varied appreciably, however, in the preparations from different insects. A typical set of pictures from a concentrated extract of yellow jacket whole insect is shown in Fig. 7. Peak I is seen as the sharp, well-separated, and fastest con-

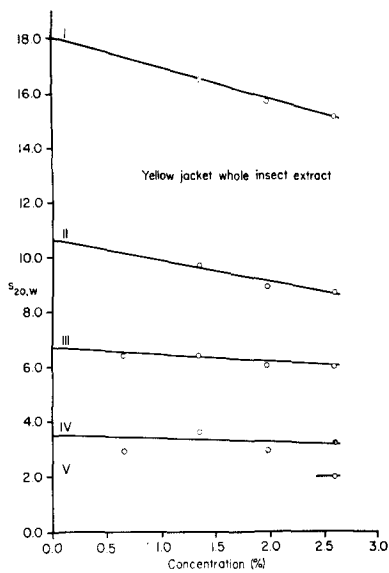


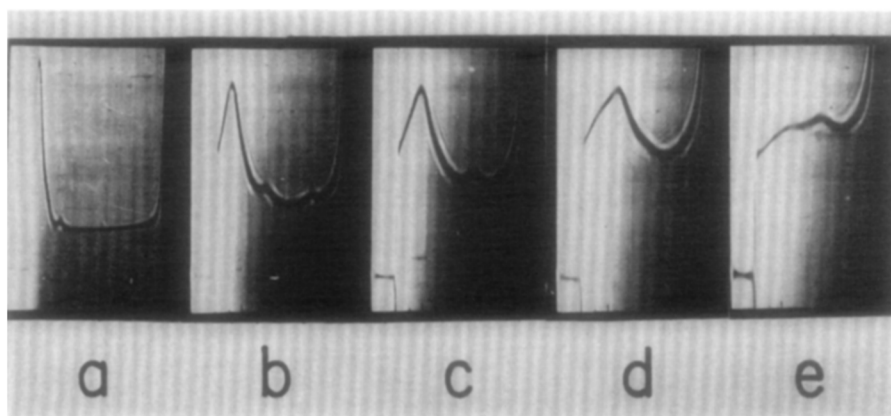
Fig. 8
Sedimentation coefficient, $S_{20,w}$, plotted versus total protein concentration per cent for a yellow jacket whole insect extract. Component V was detected only at the highest concentration. The other four components are represented by the four lines.

stituent, while II is also well separated but very small. In third place, there is an overlapping group composed of III, IV, and V. Peak IV is the predominant one, with III and V as leading shoulder and trailing shoulder, respectively. At the protein concentration of 2.61 per cent, these components had sedimentation coefficients of 15.1, 8.7, 6.0, 3.2, and 2.0 S. A series of dilutions was examined in order to obtain the extrapolated values of the sedimentation coefficient at zero concentration. The graph for this analysis is shown in Fig. 8, and the extrapolated values are shown in Table VIII. The relative proportions of these components has also been studied. Since III, IV, and V cannot be reliably measured as separate areas, and since II is so very small, it seemed that the only feasible measurement was the percentage of peak I in the total pattern. This is also shown in Table VIII. This parameter shows a striking variation in the three types of insect.

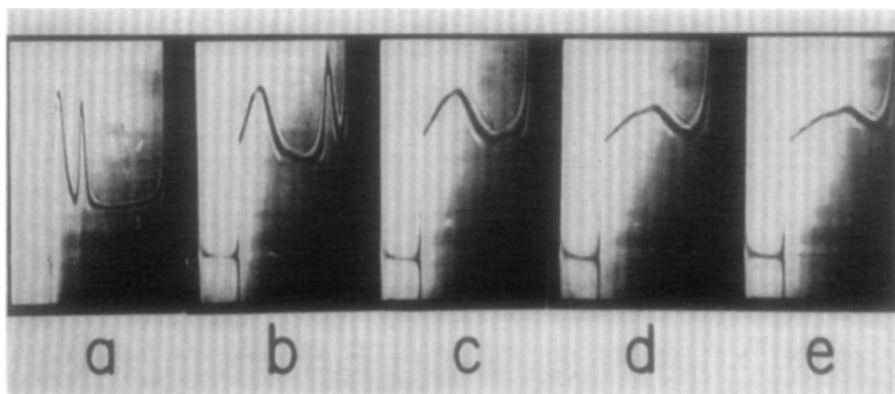
Table VIII. *Extrapolated values of sedimentation coefficient of whole insect extracts at zero protein concentration*

Components	Yellow jacket	Bee	Wasp
I	18.0	18.0	18.6
II	10.6	9.4	11.1
III	6.7	5.6	5.5
IV	3.5	3.0	3.4
V	2.0*	1.5	—
% of peak I	17.3	3.2	34.1

*Not extrapolated; too dilute.

**Fig. 9**

Ultracentrifugal sedimentation pattern of a bee whole insect extract. Concentration of protein = 1.00 per cent. Photographs (left to right) at 8, 30, 46, 70, and 118 minutes after rotor reached speed of 59,700 r.p.m. Schlieren angles were 55, 40, 35, 30, and 30 degrees, respectively. Direction of sedimentation in each picture is to the right. Peak I is best seen in photographs *a* and *b*; peak II in *b* and *c*; peak III (poorly resolved) in *c* and *d*; and peaks IV and V (resolved) in *d* and *e*.

**Fig. 10**

Ultracentrifugal sedimentation pattern of a wasp whole insect extract. Concentration of protein = 1.36 per cent. Photographs (left to right) at 8, 28, 44, 60, and 68 minutes after rotor reached speed of 59,700 r.p.m. Schlieren angles were 50, 35, 30, 30, and 30 degrees, respectively. Direction of sedimentation is to the right. Peak I is best seen in photographs *a* and *b*; peak II (very small) in *b*; and peaks III and IV (resolved) in *d* and *e*.

The pattern observed for bee extract is shown in Fig. 9 and for wasp extract in Fig. 10. The bee pattern consists of a very small peak I, followed by a distribution very similar to that of yellow jacket; there is a very small II, and a III-IV-V group with IV predominant. The extrapolated sedimentation coefficients are listed in Table VIII; they are quite similar to those for yellow jacket. The contrasting percentage of peak I is also shown. The wasp pattern, on the other hand, showed some great differences. Here, a very large peak I is

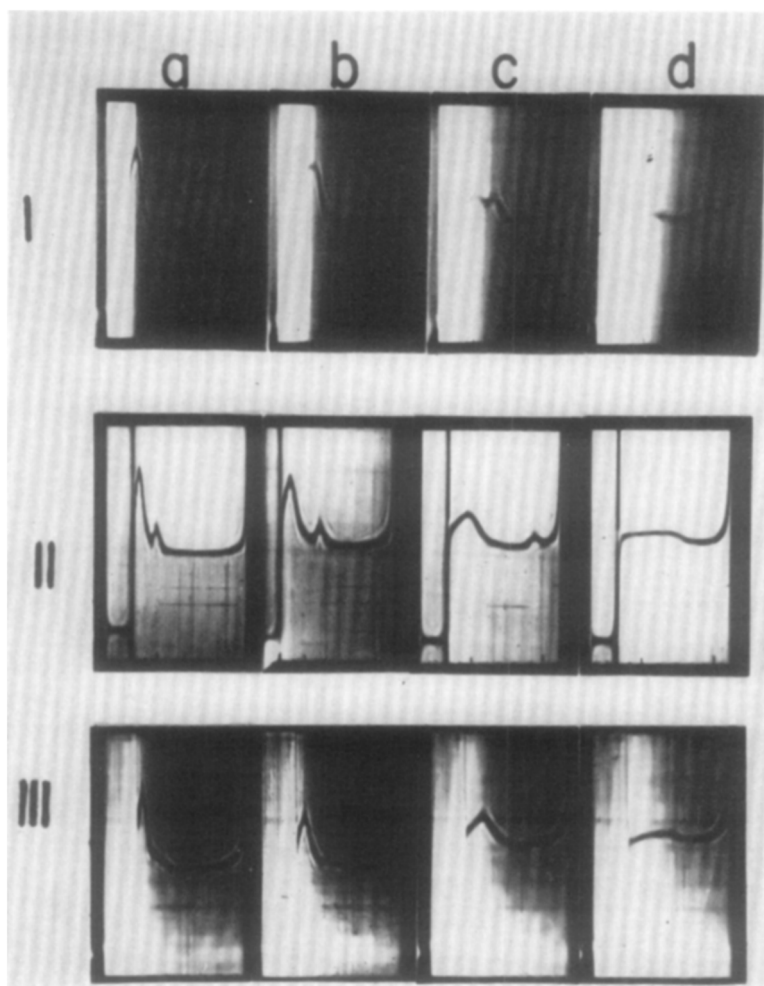


Fig. 11

Ultracentrifugal sedimentation patterns of venom sac extracts.

Row I. Sedimentation pattern of a yellow jacket venom sac preparation. Concentration of protein = 0.83 per cent. Photographs (left to right) at 6, 12, 28, and 68 minutes after rotor reached speed of 59,800 r.p.m. Schlieren angles were 35, 30, 30, and 30 degrees, respectively. Direction of sedimentation is to the right. Peak F_2 is seen as a very small leading peak in photograph *b*; peaks S_1 and S_2 are resolved in *c* and *d*.

Row II. Sedimentation pattern of a wasp venom sac preparation. Concentration of protein = 0.71 per cent. Photographs (left to right) at 6, 12, 28, and 64 minutes after rotor reached speed of 59,700 r.p.m. Schlieren angles were 35, 30, 30, and 30 degrees, respectively. Direction of sedimentation is to the right. Peak F_1 is seen as a small leading peak in photographs *a*, *b*, and *c*; the slow peak is seen in all the photographs and has formed a flat plateau by *d*.

Row III. Sedimentation pattern of a bee venom sac preparation. Protein concentration = 0.55 per cent. Photographs (left to right) at 6, 12, 30, and 62 minutes after rotor reached speed of 59,800 r.p.m. Schlieren angles were 35, 35, 30, and 30 degrees, respectively. Direction of sedimentation is to the right. Peaks F_1 and F_2 are seen as very small leading peaks in photographs *a* and *b*; the slow peak is seen in all the photographs and has partially resolved by *d*.

followed by the usual tiny II. But a leading shoulder on the predominant slow peak cannot be seen; in fact, when sedimentation coefficients are carefully compared, individually as well as by extrapolation, with those for the other insects, one is strongly tempted to believe that the major slow peak is indeed III, indicating a higher proportion in this animal, and that the trailing shoulder is IV, and that V is not seen. The extrapolated sedimentation coefficients are listed in Table VIII, as is the percentage of peak I, also.

Results of the second group of studies, dealing with the extracts of the venom sacs, were quite different from those with the whole insect. Most of these sedimentation patterns could be reconciled with a general picture of two very small fast peaks (designated F_1 and F_2) and two approximately equal-sized slow peaks (designated S_1 and S_2). Typical patterns for all three insects are shown in Fig. 11. Not enough runs have been done to provide extrapolated sedimentation coefficients, and hence the data have merely been averaged for each boundary and each insect. These values are listed in Table IX, along with the measurements of the proportions. For yellow jacket the resolution of the components was quite good, but for bee and wasp very poor separation of the slow components was observed. For wasp extract, where only a single experiment was done, the slow peak eventually stretched out into a broad plateau, and therefore only the fast edge was measured for its rate. In bee, the slow region was almost as flat late in the run, but a slower separate boundary could be reliably measured. The percentage for S_1 refers to the total slow material. Furthermore, it is not yet completely clear whether the slow components in yellow jacket really do correspond to the slow material in bee and wasp.

Table IX. *Sedimentation coefficients (s) and relative proportions (%) in venom sac extracts*

Components	Yellow jacket		Bee		Wasp	
	s	%	s	%	s	%
F_1	—	—	17.4	10.3	17.2	15.5
F_2	12.0	5.4	11.9*	—	—	—
S_1	7.2	38.2	3.5	89.7	(4.0)	84.5
S_2	2.8	56.4	—	—	—	—

*Seen in only one of four runs.

DISCUSSION

Almost all of the available protein from Hymenoptera bodies was obtained in the first two extraction stages of insect material with the described homogenization procedure. Our method of extraction produced a material equal to commercially prepared extracts as compared by skin tests. This method of preparation of extracts, in which another (nonstinging) insect type is used, had been previously demonstrated to be less tedious and yet productive of an extract as potent as those obtained by other methods.¹⁸

For routine measurements of concentration, procedures involving light absorption have been developed and utilized. Determination of the absorption of

ultraviolet light by employment of the extinction coefficient determined for each type of Hymenoptera was useful for either venom sac or whole insect extracts. It is important to keep in mind that an undialyzed extract may contain a large proportion of nonprotein light-absorbing material. This may lead to a falsely high protein concentration when the ultraviolet procedure is used.

The biuret reaction, on the other hand, detects peptide groupings, and its response is therefore more constant, although it is less sensitive. Since the extracts were heavily colored, one must also make a correction for light absorption by the pigment itself.

Our extracts, tested in untreated and treated patients who were sensitive to stinging insects, proved to be as effective in eliciting skin reactions as commercially available extracts. Titers were observed with dilutions as great as 1:10,000. These were based on dilution series from stock concentrations which were about 0.5 per cent in protein concentration. Two thirds of our patients reacted to more than one type of stinging insect; this could possibly result from the presence of a common antigen in these insects, or it could reflect multiple sensitivities to different insects in the same individual. This phenomenon was later studied in more detail, and will be reported subsequently.

Zone electrophoresis was studied as a means of separating the components of the mixture. This general procedure had been quite successful in purifying the major allergen of another insect, the Caddis fly.²⁰ In the present investigation, however, it proved to be of little value as a tool for fractionation, or even for analytic purposes, compared to other methods. It is nonetheless interesting to note that better separations were obtained at pH levels lower than 8.6, with buffers other than barbital. It is of some importance to stress that the widely used pH 8.6 procedure, while providing good resolution for blood serum, is not the best system for all other natural protein mixtures. This was well demonstrated in the filter paper electrophoresis comparisons. The extracts of bee and of wasp were best separated at pH 6.5, and yellow jacket at pH 4.5. Even so, only two or three components could be clearly resolved by this technique.

Ultracentrifugal examination was successful in revealing the number of major constituents in each type of extract and in characterizing them by their sedimentation coefficients. For the whole body extracts, the three insects show a superficial resemblance in this type of analysis. There are five sedimenting constituents, except that the wasp extract is deficient in the slowest constituent. Otherwise, these components, presumably proteins, seem to be homologous macromolecules, considering the sequence of sedimentation rate and allowing for a reasonable degree of error of measurement. Each of them may actually represent more than one protein; only later study of purified fractions can clarify this point. Interesting species differences exist in the proportions of the components. For example, the fastest component, the 18 S peak (or peak I), is only 3.2 per cent of the total protein in bee extract, whereas it is 17.3 per cent in yellow jacket extract and 34.1 per cent in wasp extract. Also, peak IV (approximately 3.0 to 3.5 S) is the predominant slow peak for yellow jacket and bee, while peak III (approximately 5.5 S) is predominant for wasp.

Venom sac extracts have a quite different pattern, which basically consists

of one or two minor fast components and two (or more) slow components. A different set of symbols has been used to avoid the implication that these are the same components seen in the whole extracts. Between the three species of venom sac preparations, the fast components, when present, seem to be corresponding entities, essentially a 17 S and a 12 S component. The slow components cannot be fully compared. Only in yellow jacket did they resolve well. In the other two, a broad distribution was seen that might contain many boundaries; only the fast edge was measured for sedimentation rate. As for comparisons between venom sac and whole body extracts from the same animal, no definitive statements can yet be made. The 17 to 18 S peak in venom might well be related to peak I of whole extract, but the 12 S component seems distinctive. The slow components seem slightly faster in bee and wasp than the similar rates in whole extract, and for yellow jacket the rates also differ somewhat.

The observed heterogeneity of the whole body extract is to be expected, but the degree of heterogeneity of the venom sac extract is perhaps surprising. It should be kept in mind, however, that one is not dealing here with pure venom, but with an extract of the crushed sacs. These sacs are separated with difficulty from the remainder of the stinging apparatus and the extracts may therefore contain contaminants from the sac wall and from adjacent muscles and other tissues. Investigations with pure venom collected from the living insect have been begun, and these will provide a basis for comparison.

None of the components detected in whole insect extracts can yet be identified with the allergenic substance. This must await fractionation studies. This purified allergen, when it is prepared, will then be identifiable in these total patterns. It can be anticipated that both the whole insect and the venom sac extracts should provide a multiplicity of antigens for study with suitably prepared antisera.

SUMMARY

Potent allergenic extracts of three different species of stinging insects were prepared by a simple procedure. The types of Hymenoptera studied were the bee (*Apis mellifera*), the wasp (*Polistes exclamans*), and the yellow jacket (*Vespula pennsylvanicus*), and extracts of each were prepared from the whole body, from the sacless body, and from the venom sac. In order to facilitate the routine determination of protein concentration, light absorption methods were developed and an extinction coefficient for each type of extract was determined for both ultraviolet absorption, at 280 $m\mu$, and for the biuret reaction at 540 $m\mu$.

The allergenicity of these preparations was determined by direct skin tests. Positive intradermal skin tests were elicited in sensitive patients with as little as a 1:10,000 dilution of the preparation of insect antigen. Most of the patients reacted to more than one type of insect. Commercial preparations gave similar results.

Zone electrophoresis, by filter paper strip and by starch block, revealed two or three separate components. It was noted that the best pH for optimal resolution, over the range of 8.6 to 4.5, was 6.5 for bee and wasp and 4.5 for yellow

jacket. This method seemed to have limited value for the possibilities of fractionation of these extracts.

The extensive heterogeneity of these extracts was better shown by analytical ultracentrifugation. All three types of whole body extracts had characteristic patterns of five homologous sedimenting boundaries, although the proportions varied for each insect. The venom sac extracts showed varying patterns of minor fast components and major slow ones. At least some of the venom constituents seemed to be absent from the whole body extracts. It will require further study to identify any of these components with the allergenic substance.

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