

# A high molecular weight allergenic fraction of honeybee venom

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*Chromatography of honeybee venom on Sephadex G-150 super fine revealed a high molecular weight (HMW) fraction that elutes prior to hyaluronidase (HYAL) and comprises 2% to 4% of the venom weight. HMW appears to exist in polymeric form, and the polymer which is present in greatest concentration has an estimated molecular weight of 105,000 D. The 12% nitrogen content of HMW suggests it may not be all protein. HMW is antigenically and enzymatically distinct from HYAL and phospholipase A<sub>2</sub> (PHOS A). The acid phosphatase activity known to be present in honeybee venom was found in the HMW fraction. Since it reacts by RAST with the sera of most individuals known to be sensitive to honeybee venom, and releases histamine from the peripheral leukocytes of such individuals, its role as an allergen is confirmed. Since individuals react to different degrees to HMW, HYAL, and PHOS A, there does not appear to be a single principal allergen in honeybee venom.*

Honeybee (*Apis mellifera*) venom contains hyaluronidase (HYAL), phospholipase A<sub>2</sub> (PHOS A), melittin, and a number of smaller molecular weight peptides. HYAL and PHOS A are allergens for most individuals sensitive to this venom, and melittin is an allergen for some.<sup>1-6</sup>

Earlier studies in this and other laboratories demonstrated an additional high molecular weight antigen.<sup>3, 4, 7, 8</sup> The purpose of the present study was to further elucidate the biochemical and allergenic properties of this high molecular weight fraction of honeybee venom.

## MATERIALS AND METHODS

Dried bee venom grade I (WBV), lots 14C-1040, 89B-0080, 122C-2170, 66C-345 (Sigma Chemical Co., St. Louis, Mo.), and whole liquid bee venom (LBV), obtained shortly after collection by Mr. Charles Mraz, Champlain Valley Apiaries, Middlebury, Vt., were used. All venoms were stored at -30° C. LBV contained 46.7 mg nitrogen per milliliter, and, based on 15.1% nitrogen for whole bee venom,<sup>7</sup> this contained 309 mg protein per milliliter. The moisture content of LBV, determined by thermogravimetric analysis at 50° C, was 56% and at 100° C was 64% (Ther-

mogravimetric Analyzer Model 442, Fisher Scientific Co., Silver Spring, Md). The total solids obtained after drying at 40° C in a vacuum oven were 750 mg per ml.

## Gel filtration

Sephadex G-150 super fine (SF) (Pharmacia, Piscataway, N. J.) was used in a column measuring 1.6 × 100 cm (K 16/100 Pharmacia) equilibrated with 0.05 M ammonium acetate adjusted to pH 4.6 with glacial acetic acid and 0.1% chlorobutanol (Fisher Scientific Co., Silver Spring, Md.) added as preservative. WBV 0.5 to 1.0 gm in 2 ml of buffer, or 0.5 ml of LBV in 1.5 ml of buffer, was added to the column and eluted at a flow rate of 4.4 to 7.2 ml per hour. The effluent was monitored at 280 nm with an LKB Uvicord III (LKB Instruments, Inc., Rockville, Md.), and fractions of 5.8 or 1.9 ml were collected.

The molecular weight of the effluent fractions was estimated by using human immunoglobulin G, 153,000 D; bovine serum albumin, 66,400 D; ovalbumin, 45,000 D; chymotrypsinogen, 25,000 D; and cytochrome C, 12,400 D as markers. Pools prepared from peaks 1 and 2 (Fig. 1) were further purified by rechromatography on the same column, but for the pool from peak 3, the column was packed with G-50 fine. In addition, a 3 × 1000 mm column (MB-3-1000, Cheminert, Laboratory Data Control, Riviera Beach, Fla.) packed with Sephadex G-150 SF was used and was loaded with 10 µl of buffer containing 2.27 mg WBV and eluted at a flow rate of 0.6 ml per hour. Fractions of 0.15 ml were collected on a Gilson Micro Fractionator (Gilson Medical Electronics, Inc., Middletown, Wis.) and optical density at 280 nm determined on individual fractions using a Beckman microcuvette in a Beckman Model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

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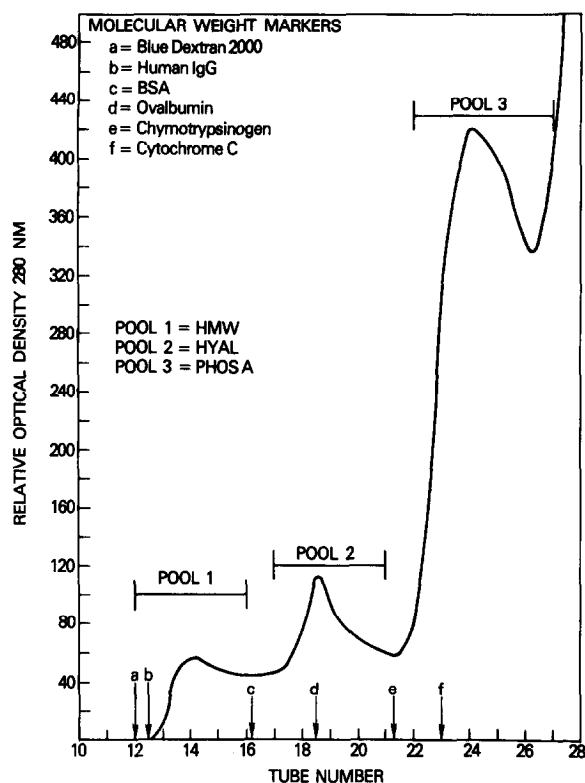


FIG. 1. Chromatography of honeybee venom on G-150 SF.

### Immunodiffusion

Immunodiffusion was carried out on microscope slides as described.<sup>7</sup> Antibody wells were filled and diffusion allowed to proceed for 1 hr; then antigen wells were filled and the slides developed for 18 to 24 hr at approximately 26° C in moist chambers.

Antisera to the fractions were prepared in rabbits, using an initial injection of 200 to 300  $\mu$ g in complete Freund's adjuvant. Animals were given booster injections in incomplete Freund's adjuvant or aluminum hydroxide gel.

### Protein determination

The protein was hydrolyzed with NaOH<sup>9</sup>; then the *o*-phthalaldehyde procedure was carried out, with minor modifications, as described.<sup>10</sup> Protein values were calculated relative to a solution of HMW used as a standard which was prepared by dialysis against distilled water and had 0.034 mg nitrogen per milliliter. A sample of the same batch of HMW was freeze-dried directly from the chromatography buffer which contains only volatile materials; the residue contained 11.9% nitrogen, suggesting that HMW may not be all protein. A conversion factor was derived:  $\frac{100}{11.9} = 8.4$ , and the standard calculated to contain

286  $\mu$ g protein per milliliter. Protein concentrations were adjusted on the basis of this test for use in the RAST.

### Enzyme analyses

Hyaluronidase was determined using hyaluronic acid agarose plates (Worthington Biochem Corp., Freehold, N. J.), amylase activity using starch agar,<sup>11</sup> and proteinase ac-

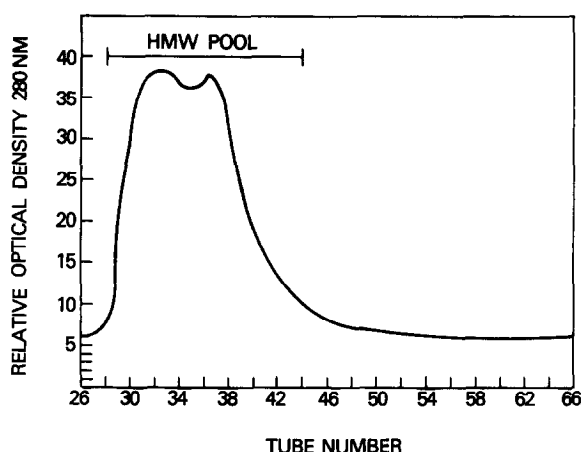


FIG. 2. Chromatography of HMW fraction pool on G-150 SF.

TABLE I. Peak area ratio at 280 nm of honeybee venoms chromatographed on G-150 SF

| Run number | Venom lot number | Peak area ratio |      |         |
|------------|------------------|-----------------|------|---------|
|            |                  | HMW             | HYAL | PHOS A* |
| 1†         | 14C-1040         | 0.50            | 1    | 3.8     |
| 2          | 122C-2170        | 0.94            | 1    | 7.3     |
| 3          | 89B-0080         | 0.75            | 1    | 6.7     |
| 4          | LBV <sup>B</sup> | 0.85            | 1    | 11.0    |
| 5          | 66C- 345         | 1.1             | 1    | 5.0     |
| 6          | 66C- 345         | 2.1             | 1    | 5.2     |
| 7          | 66C- 345         | 2.3             | 1    | 5.8     |

\*Abbreviations used: LBV, liquid bee venom; HMW, high molecular weight fraction; HYAL, hyaluronidase; PHOS A, phospholipase A<sub>2</sub>.

†Runs 1 to 5 were passed through the same gel bed; for runs 6 and 7 the bed was repoured using the gel from the previous runs.

tivity using gelatin agar.<sup>12</sup> Acid and alkaline phosphatase were determined using a *p*-nitrophenylphosphate reagent (Sigma Chemical Co.). Acid phosphatase levels were determined by the serum total acid phosphatase test procedure (Sigma Technical Bulletin No. 104). Phospholipase activity was measured using egg yolk agar.<sup>13</sup>

### Nitrogen analysis

Determination of nitrogen by the micro-Kjeldahl or Nessler method was performed by the section of Microanalytical Services and Instrumentation, National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), National Institutes of Health.

### Histamine release studies

Sensitivity to HMW was assessed using leukocytes of honeybee allergic and nonallergic patients.<sup>14, 15</sup>

### Radioallergosorbent test (RAST)

The direct RAST was performed according to Wide, Bennich, and Johansson<sup>16</sup> using venom or venom fractions

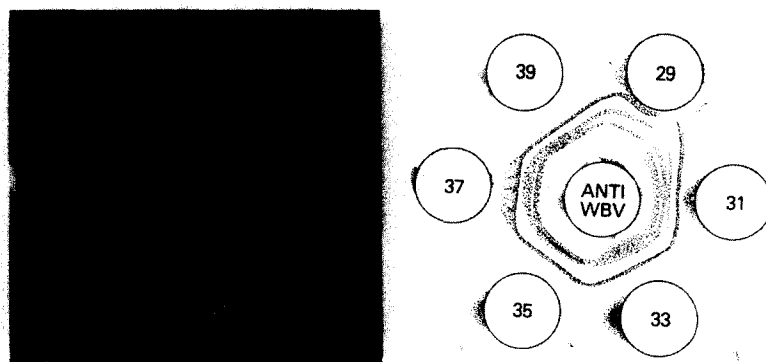


**FIG. 3.** Gel diffusion of fractions from chromatography of HMW pool against anti-whole bee venom. Numbers refer to tube numbers of Fig. 2.

coupled to cyanogen bromide-activated paper discs (Schleicher & Schuell, Keene, N. H., grade 595,  $\frac{1}{4}$  inch diameter). Sixteen sera from honeybee-allergic individuals were used for the RAST. Two of these have been described. Serum M contained IgE mainly directed to HYAL, while that in serum V was mainly directed to PHOS A.<sup>17</sup> These correspond to sera numbers 9 and 5, respectively, of Table II.

### Acrylamide gel electrophoresis

The method of Weber and Osborn<sup>18</sup> for SDS acrylamide gels was used. Protein markers were: reduced fibrinogen  $\alpha$  chain = 70,900 D;  $\beta$  chain = 60,400 D;  $\gamma$  chain = 49,400 D; human IgG = 153,000 D; BSA = 66,400 D; cytochrome C = 12,400 D. Alkaline polyacrylamide gel electrophoresis was done by the method of Davis and Ornstein.<sup>19</sup>

### RESULTS

One gram of WBV was applied to G-150 SF; everything eluting after peak 3 was discarded. Peak 1, called HMW, was the peak previously described but uncharacterized;<sup>7</sup> peak 2 was HYAL, and peak 3, PHOS A (Fig. 1); each peak was pooled (Fig. 1). All 3 peaks were found in each of the 5 lots tested, although the ratios of the peak areas differed for each lot (Table I). It may be significant that the largest peak area for PHOS A was in LBV, which was also the freshest and least altered preparation.

The increasing ratio of HMW relative to HYAL from successive runs (Table I, run 7 value was 4.6 times that of run 1) suggested adsorption of HMW to a fresh gel bed. To test this, solutions of a single lot of WBV were loaded serially on the 3-mm column; the HMW peak area increased 2.6 times from the first to the fifth run.

Following concentration of Pool 1 on a PM-10 membrane (Amicon, Lexington, Mass.), it was rechromatographed and shown to be biphasic (Fig. 2), indicating that HMW was not a homogeneous material. Bee venom solutions placed on a previously unused column did not show a biphasic HMW peak.

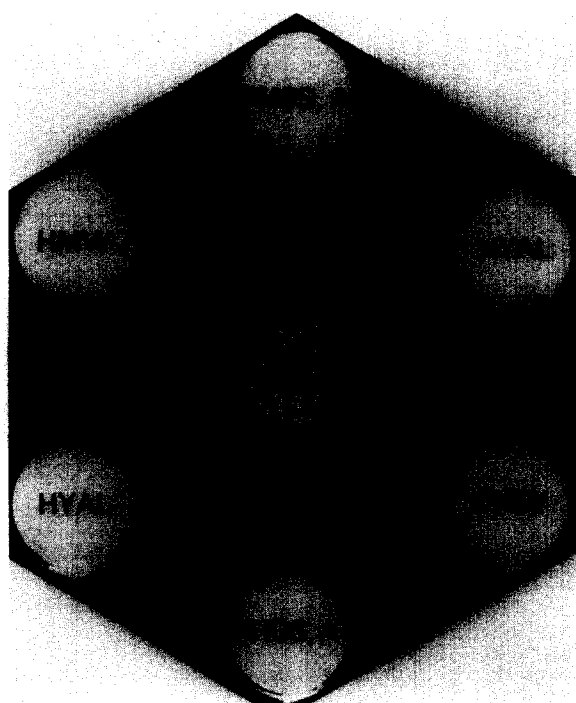


**FIG. 4.** Gel diffusion of HMW, HYAL, and PHOS A against anti-WBV.

However, repeated use of the same column eventually results in a biphasic peak. Consequently, Fig. 2, showing rechromatography of HMW on a column after four venom loads, has a biphasic character. The HMW peak of subsequent venom runs on this column also had a pronounced biphasic appearance. Gel diffusion of samples from tubes 29 to 39 (Fig. 2) against anti-WBV revealed one strong band common to all samples and 2 (tubes 35 to 39) or 3 (tube 31) additional faint bands (Fig. 3).

Gel diffusion against anti-WBV indicated nonidentity of HMW, HYAL, and PHOS A (Fig. 4), while anti-HMW produced 2 to 3 precipitin lines with HMW or LBV, and none against HYAL and PHOS A (Fig. 5). Anti-HYAL demonstrated a precipitin line with HYAL only.

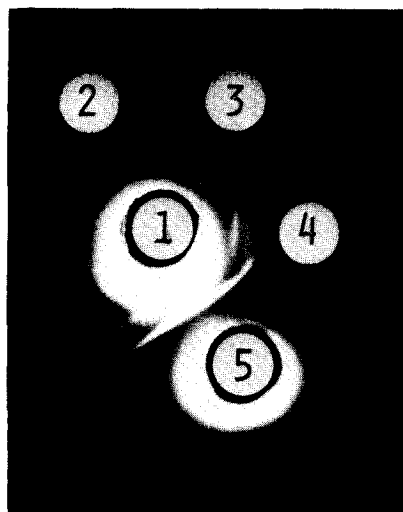


**FIG. 5.** Gel diffusion of honeybee venom and its fractions against anti-HMW. 1, anti-HMW; 2, HYAL; 3, PHOS A; 4, HMW; 5, LBV.

Hyaluronidase activity was present in the HYAL fraction and with a low level of activity in the PHOS A and none in HMW. None of the fractions showed amylase or proteinase activity. Only the PHOS A fraction had phospholipase activity. Acid phosphatase was found in HMW, and alkaline phosphatase was not present in the fractions or in WBV or LBV. There were approximately 0.56 sigma units of acid phosphatase activity per microgram of HMW protein. Storage in the refrigerator for 2.5 mo in 0.05 M ammonium acetate pH 4.6 resulted in complete loss of acid phosphatase activity. Storage at  $-30^{\circ}\text{C}$  in 50% glycerol caused no loss of activity.

From elution volumes of the molecular weight markers on the G-150 SF column, the molecular weight of the monophasic HMW peak of Fig. 1 was calculated to be 110,000; HYAL was 40,000, and PHOS A was 11,000. The values for the latter two are comparable to published data.<sup>20-24</sup>

SDS acrylamide gel electrophoresis with and without prior treatment with 2-mercaptoethanol (2-ME) gave identical results. Migration distances were measured on photographs of the gels and the mean values correlated to molecular weights. HMW showed two intense bands with two bands of intermediate migration distance varying in intensity among batches (Fig. 6). The intense bands correlated to 100,000 D and 50,000 D, and the faint bands to 80,000 D and 63,000 D. HYAL and PHOS A had broad bands correlating to 40,000 D and 20,000 D, respectively. The LBV sample displayed three distinct bands which correlated to HMW heavy component, HYAL and PHOS A and a smear in the area of the HMW light component.

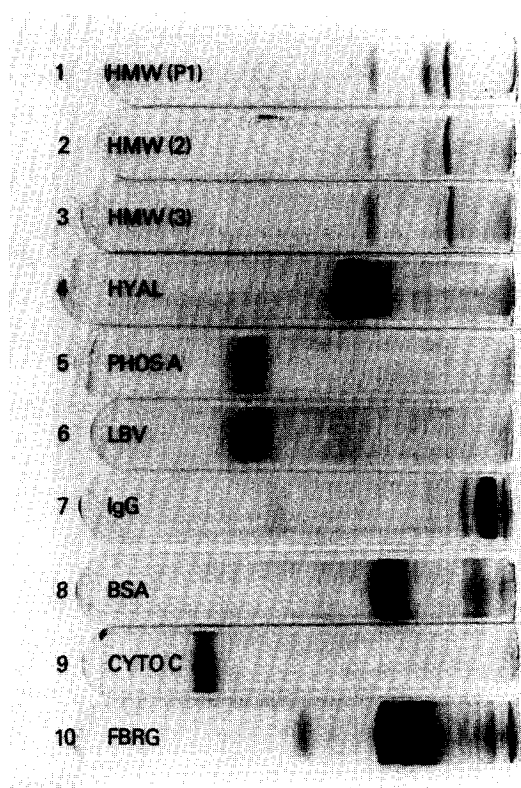


**FIG. 6.** SDS-acrylamide gel electrophoresis of honeybee venom, its fractions and molecular weight markers. Designations used: HMW (P1)(2)(3), preparations of HMW which were treated with 2-ME; IgG, human immunoglobulin G; BSA, bovine serum albumin; CYTOC, cytochrome C; FBRG, human fibrinogen treated with 2-ME.

Using the RAST with discs coupled to HMW, 16 sera from honeybee-allergic individuals showed that 15 (94%) had cpm values 1.9 to 13.8 times greater than sera from nonallergic individuals (Table II). Discs coupled to LBV had cpm values of 2.2 to 13.8 times those of nonallergic sera. In a comparison of discs coupled to HMW or LBV, four (25%) of the sera were almost equally reactive to both (Table II, sera 13 to 16).

The maximal response in direct RAST using discs coupled to LBV compared to the response of discs coupled to allergenic fractions showed serum M reacted strongly to HYAL, moderately to HMW, and was unreactive to PHOS A; serum V reacted strongly to PHOS A and moderately to HYAL and HMW (Table III).

The leukocytes of three individuals with clinical allergy to honeybee sting were assayed for ability to release histamine in the presence of HMW. Fifty percent histamine release (HR) was obtained with the following amounts of material: individual 1, 16.8 ng; individual 2, 8.4 ng; individual 3, 2.0  $\mu\text{g}$  (Table IV).

HMW was tested in 5 guinea pigs to determine its

TABLE II. RAST survey of venom-positive sera for reactivity to HMW

| Serum | Counts per minute* |       |       | Serum           | Counts per minute |       |       |
|-------|--------------------|-------|-------|-----------------|-------------------|-------|-------|
|       | HMW                | LBV   | X NHS |                 | HMW               | LBV   | X NHS |
| 1†‡   | 1,085              |       | 3.4   | 9               | 1,927             |       | 6.1   |
|       |                    | 4,568 | 11.6  |                 |                   | 3,693 | 9.4   |
| 2‡    | 874                |       | 2.8   | 10              | 707               |       | 2.2   |
|       |                    | 3,026 | 7.7   |                 |                   | 1,332 | 3.4   |
| 3     | 738                |       | 2.3   | 11              | 674               |       | 2.1   |
|       |                    | 2,573 | 6.6   |                 |                   | 1,073 | 3.0   |
| 4     | 589                |       | 1.9   | 12              | 887               |       | 2.8   |
|       |                    | 1,547 | 3.9   |                 |                   | 1,451 | 3.7   |
| 5‡    | 829                |       | 2.6   | 13 <sup>D</sup> | 1,072             |       | 3.4   |
|       |                    | 1,973 | 5.0   |                 |                   | 1,468 | 3.7   |
| 6§    | 585                |       | 1.9   | 14              | 4,358             |       | 13.8  |
|       |                    | 1,324 | 3.4   |                 | 5,408             |       | 13.8  |
| 7     | 418                |       | 1.3   | 15 <sup>D</sup> | 1,158             |       | 3.7   |
|       |                    | 877   | 2.2   |                 |                   | 1,375 | 3.5   |
| 8     | 650                |       | 2.1   | 16              | 1,458             |       | 4.6   |
|       |                    | 1,251 | 3.2   |                 |                   | 1,302 | 3.3   |

\*The cpm for NHS (normal human serum) with discs sensitized with HMW (161 µg/ml were 312, 319, 319, avg., 316; with LBV (1,500 µg/ml) they were 404, 374, 400, avg., 393. Total activity added was 24,306 cpm.  
†All individuals had a systemic reaction to honeybee sting except 12 (history not available).  
‡Received hyposensitization therapy with honeybee venom.  
§Received hyposensitization therapy with whole body extracts.

TABLE III. Comparison of HMW, HYAL, PHOS A, and LBV by RAST

| Venom or fraction | Sensitization concentration (µg/ml) | Nonsensitive serum control (cpm) | Sensitive serum M (cpm) | S*<br>C | Sensitive serum V (cpm) | S<br>C |
|-------------------|-------------------------------------|----------------------------------|-------------------------|---------|-------------------------|--------|
| HMW               | 161                                 | 868                              | 3,632                   | 4.2     | 2,548                   | 2.9    |
| HYAL              | 328                                 | 494                              | 4,658                   | 9.4     | 2,654                   | 5.4    |
| PHOS A            | 100                                 | 749                              | 779                     | 1.0     | 7,498                   | 10.0   |
| LBV               | 813                                 | 549                              | 4,910                   | 8.9     | 6,435                   | 11.7   |
| LBV               | 1,626                               | 536                              | 6,604                   | 12.3    | 7,351                   | 13.7   |

\*S  
C = Sensitive cpm / Control cpm. The total activity added = 29,760 cpm.

toxicity in skin. Samples were brought to pH 7 with 1.0 N NaOH and 0.05 ml containing either eluting buffer or 10 µg of protein was injected intradermally. No reactions were noted in either test or control sites at 2, 7, or 24 hr.

DISCUSSION

Chromatography of bee venom on Sephadex G-50 had demonstrated an antigenic fraction eluting at the void volume and overlapping the HYAL peak;<sup>7</sup> G-150 SF allowed resolution of this material, HMW (Fig. 1). It appears to be strongly adsorbed to Sephadex, so that elution from a fresh column results in low yields. Continued use of the same column results in increasing yields of HMW but does not seem to affect the quantities of PHOS A and HYAL eluted. Over the 7 column runs listed in Table I, no

regular change was seen in the absolute peak area of HYAL or PHOS A. Disappearance of HYAL, therefore, cannot account for the increasing HMW:HYAL peak area ratios. Aggregation of HYAL or PHOS A cannot have contributed to the HMW peak size since they were not shown to diminish. Consequently, adsorption of HMW to Sephadex and progressive saturation of adsorptive sites are the probable mechanisms of increasing ratios.

Rechromatography of HMW demonstrated that it is not a homogenous substance (Fig. 2). While gel diffusion analysis using anti-WBV serum confirms the fact that there are at least 2 and possibly 4 gel diffusion bands, the use of this antiserum as well as anti-HMW, anti-HYAL, and anti-PHOS A establishes that HMW is antigenically distinct from HYAL and PHOS A. Enzymatic activity also shows the unique-

**TABLE IV.** Histamine release induced by HMW from leukocytes of honeybee-allergic individuals

| Individual | Quantities of HMW for 50% release* |                                      |                                      |
|------------|------------------------------------|--------------------------------------|--------------------------------------|
|            | $\mu\text{g/ml}$                   | Molar equivalent†<br>heavy component | Molar equivalent†<br>light component |
| M.         | $1.7 \times 10^{-2}$               | $1.6 \times 10^{-13}$                | $3.5 \times 10^{-13}$                |
| V.         | $8.4 \times 10^{-3}$               | $8.0 \times 10^{-14}$                | $1.8 \times 10^{-13}$                |
| P.‡        | 2.0                                | $4.6 \times 10^{-11}$                | $1.0 \times 10^{-10}$                |

\*Leukocytes of a nonallergic control did not release histamine at HMW concentrations of  $6 \times 10^{-3}$  to  $6 \times 10^1 \mu\text{g/ml}$ .

†Molar equivalents are based on weights of the heaviest component (105,000 D) and lightest component (49,000 D) of HMW.

‡Individual P. had a highly positive skin test to bee venom 6 months prior to HR.

ness of each fraction. Hyaluronidase activity is found in the HYAL fraction and phospholipase A activity in the PHOS A fraction.

Although it has been claimed that honeybee venom contains acid<sup>25, 26</sup> and alkaline phosphatase,<sup>25</sup> no previous studies have identified a venom fraction with these activities. We confirmed the finding that honeybee venom contains acid phosphatase, but neither the fresh liquid nor the dried venom or venom fractions contained alkaline phosphatase. Only HMW has acid phosphatase activity. Furthermore, the acid phosphatase is labile since the activity is lost when stored in the elution buffer (ammonium acetate, pH 4.6) for 2.5 mo.

No amylase or proteinase could be found in whole venom or its fractions.

The HMW fraction comprises 1% to 4% of the honeybee venom weight. This figure is derived by comparing the peak area of HMW for each run with the peak area of HYAL, which is 2% of the venom weight.<sup>20</sup> The values in Table I indicate that the lowest ratio of HMW to HYAL was 0.5:1 which correlates to 1% of WBV being HMW. Run 7 shows a leveling off of value compared to run 6, indicating saturation of adsorptive sites on Sephadex. The ratio of run 7, therefore, represents the best approximation and correlates to WBV containing 4% HMW. This computation is based on the assumption that the extinction coefficients of these fractions are similar. Comparable findings were reported by Shepherd, Elliott, and Arbesman<sup>8</sup> and by Hoffman and Shipman<sup>4</sup> for a similar honeybee venom fraction.

The ratios of HYAL to PHOS A from Table I range from 1:4 to 1:11, which are in good agreement with values previously obtained.<sup>7</sup> The highest value for PHOS A was obtained for liquid venom which was chromatographed shortly after collection and had not been subjected to drying. One venom lot gave values of 1:5 and 1:7 for HYAL and PHOS A in the previous study and this study, respectively. The Bureau of

Biologics reference, which is dried honeybee venom obtained from Mr. Mraz, gave a ratio of 1:2.

SDS-acrylamide gel electrophoresis showed 4 components for HMW with the same migration distances with and without prior treatment with 2-ME, indicating that the proteins constituting this fraction are not composed of subunits linked by disulfide bonds. The estimated molecular weight values of the four HMW components approximate those expected of polymers where monomer equals 16,000 D and N equals 3 to 6. Expected values would be: N = 3, 48,000 D; N = 4, 64,000 D; N = 5, 80,000 D; N = 6, 96,000 D. Values obtained were: 49,000 D; 63,000 D; 82,000 D; and 98,000 D. The apparent heterogeneity of HMW, therefore, may merely be the presence of the same substance in several polymeric forms, the majority being 98,000 D. Gel diffusion patterns of fractions from the two HMW peaks (Fig. 3) also support the concept of a single antigen in polymeric form. Precipitin lines of fractions from both peaks show patterns of identity, indicating that the two peaks are not different antigenically. This type of heterogeneity is further indicated by the earlier study of Benton,<sup>25</sup> showing that in acrylamide gel the acid phosphatase exists in at least 3 bands. Hoffman, Shipman, and Babin<sup>3</sup> also found four bands on SDS-polyacrylamide gel electrophoresis of fraction 1. Weights were similar to those presented here. However, their fraction 1B (allergen B) gave a single band at 49,000 D, whereas 1C (allergen C) gave a strong band at 105,000 D and weaker bands at 49,000 D and 71,000 D. The nature of the heterogeneity of HMW is a problem requiring further investigation.

The molecular weight obtained by elution from G-150 SF of 110,000 D is compatible with that obtained for a similar fraction by gel permeation<sup>8</sup> and for the major component by SDS-gel electrophoresis.

The average molecular weight of 105,000 D is considerably higher than that of other allergens<sup>27</sup> and may be related to the fact that this allergen is unique in

being injected by the insect, in contrast to most allergens that are either inhaled or ingested.

The importance of HMW lies in the fact that it is an allergen. Sera from 16 bee venom-sensitive individuals using the RAST, a procedure that measures specific IgE in serum, show that 94% of these individuals had reactivity to HMW, which was at least 1.9 times that of controls (Table II). Some sera (13 to 16) react equally well with HMW and LBV. These results confirm those of Hoffman and Shipman<sup>4</sup> with fraction I.

Two sera, selected because of the unusual specificity of their antibody to HYAL and PHOS A,<sup>17</sup> demonstrated their selectivity toward HMW. Serum M, whose specificity was mainly to HYAL, was also very reactive to HMW. Serum V, whose specificity was largely to PHOS A, was low in reactivity to HMW (Table III).

A second indication of allergenicity was the ability of HMW to release histamine from the peripheral leukocytes of sensitive individuals.

Release of 50% histamine was obtained at levels of  $1.7 \times 10^{-2}$ ,  $8.4 \times 10^{-3}$ , and  $2.0 \mu\text{g/ml}$  (Table IV). Leukocytes of Individual P. were unusual in that they did not release above 1% histamine with up to  $130 \mu\text{g/ml}$  of dialyzed whole bee venom.

When expressed in terms of moles, the amount of HMW required to elicit 50% HR may be compared to HYAL and PHOS A. In a previous study<sup>17</sup> using leukocytes from Individual M., 50% HR was obtained with  $150 \mu\text{g}$  PHOS A ( $3.8 \times 10^{-9}$  moles) and  $0.6 \mu\text{g}$  HYAL ( $3.0 \times 10^{-11}$  moles). The values for HMW are below these levels in terms of  $\mu\text{g}$  and moles by 10- to 10,000-fold (Table IV). By RAST, HMW has intermediate reactivity in Individual M. compared to HYAL and PHOS A. Leukocytes of Individual V. gave 50% HR with  $0.37 \mu\text{g}$  PHOS A ( $9.3 \times 10^{-12}$  moles) and  $4.6 \mu\text{g}$  HYAL ( $2.3 \times 10^{-10}$  moles). The values for HMW HR are 100- to 10,000-fold below these levels, and the order of reactivity of the fractions parallels that of the RAST. Thus, HMW can elicit 50% HR at levels as low as 10,000-fold below HYAL and PHOS A, and, like them, displays a variable relative reactivity.

Finally, the fact that HMW has acid phosphatase activity should permit easy analysis for this component in honeybee venoms and may prove useful in the standardization of venoms.

These results indicate the danger of selecting a single allergen as representing the major one in bee venom. Venom may have more than the four potential known allergens—melittin, PHOS A, HYAL, and HMW, and allergic individuals may have their

strongest reactivity to any of these. Thus, whole venom may be the best diagnostic and therapeutic reagent in cases of honeybee venom allergy.

Since the submission of this paper the acid phosphatase activity shown to be present in the HMW fraction of honeybee venom was found to be present in a similar fraction by Dr. Hoffman (J. ALLERGY CLIN. IMMUNOL. 59: 364, 1977).

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