The venom of the honeybee Apis mellifera
by David Albert Nelson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Chemistry
Montana State University

Abstract:
The venom of the honeybee Apis mellifera has been obtained by electrical excitation. The nonprotein fraction of this venom was qualitatively investigated, and the identified components were -quantitatively determined. Nineteen free amino acids, fourteen small peptides, two monosaccharides, six phospholipids, and two components tenatively characterized as steroids were separated by chromatographic techniques and either identified or characterized. Of major importance in the nonprotein fraction was the characterization and identification of two histamine peptides which may contribute to the physiological effects of the venom.

This is the first investigation of a nonprotein fraction of Hymenoptera venom which has utilized the amino acid analyzer. The sensitivity of this instrument was essential in the quantitative investigation of the free amino acids and the characterization of the histamine peptides.

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ABSTRACT

The venom of the honeybee Apis mellifera has been obtained by electrical excitation. The nonprotein fraction of this venom was qualitatively investigated, and the identified components were quantitatively determined. Nineteen free amino acids, fourteen small peptides, two monosaccharides, six phospholipids, and two components tenatively characterized as steroids were separated by chromatographic techniques and either identified or characterized. Of major importance in the nonprotein fraction was the characterization and identification of two histamine peptides which may contribute to the physiological effects of the venom.

This is the first investigation of a nonprotein fraction of Hymenoptera venom which has utilized the amino acid analyzer. The sensitivity of this instrument was essential in the quantitative investigation of the free amino acids and the characterization of the histamine peptides.

INTRODUCTION

Nearly everyone is familiar with some effects of bee sting. In a majority of cases the immediate effect is an intense local pain followed by epidermal swelling and itching. However, in some cases these symptoms may be accompanied by a constriction in the throat and chest, decrease in blood pressure, coma, cyanosis and, occasionally, death. For those with a venom hypersensitivity, death can occur within ten to sixty minutes after a single sting. Most fatalities are believed to be the result of anaphylactic shock. This diagnosis, however, is usually based on nothing more than the fact that death occurred within a few minutes following a sting injury. Only a few fatalities are caused by local reaction due to stings in the throat. Effects of multiple bee stings upon a relatively immune person have been also known to cause death (1). Several reviews have surveyed the symptoms and deaths due to bee and wasp stings (2,3).

For the years 1950-1954, eighty-six deaths were reported caused by Hymenoptera as compared to seventy-one deaths by poisonous snake bite (4). Jensen and Swinny (2,5) concluded that deaths from bee and wasp stings are much more common than generally believed. Richey (6) suggests that several thousand deaths caused by Hymenoptera probably occur each year. Presumably a large number of these deaths are reported as heart failure or stroke for want of further information.

To date the most effective emergency treatment for severe sting reaction is the use of some pressor amine as: aromine sulfate, iso-

proterenol, epinephrine, phenylephrine or norepinephrine immediately during the shock episode (3). However, the hypersensitive condition must be previously known and the necessary medication available for any eventuality. Unfortunately, this venom hypersensitive condition is seldom recognized, but in cases where it is discerned, allergic hyposensitization can be used as a preventive treatment. It has been recommended that the hyposensitization program should be continued until the individual has suffered stings without accompanying reaction (3). It was also concluded that for a greater measure of protection one of the pressor amines should be carried at all times to be taken immediately in case stings are encountered.

Bee venom has long been believed to have some therapeutic applications and is an accepted pharmacological product in Canada and Europe. As a therapeutic agent it has been used for chilblains, arthritis, neuritis and trachoma (7). A certain number of arthritic patients appear to have benefited more or less by the use of bee venom therapy, but it is not yet established whether this method offers enough advantages over other methods of treatment to justify further trial. It has been shown statistically that bee keepers have a very low incidence of cancer which may be due to the large number of stings received (8) or to their diet of unpasteurized honey. Havas (9) studied the effects of bee venom on colchicine induced tumors in plants and found an anti-tumor activity.

Bee venom has been shown to have bacteriostatic and bacteri-

cidal properties with Gram-positive and Gram-negative bacteria (10). Individual strains show considerable variation in their resistance to the venom.

Langer (11) in 1897 was the first to investigate the chemical composition of the venom from the honeybee. He concluded that the active principle of bee venom was a protein-free organic base and that formic acid was present. Formic acid was later shown to be absent (12).

Histamine in honeybee venom has been reported by several investigators (13,14). However, most of the early identification utilized bioassay on crude venom extracts. Schacter and Thain (15) extracted the venom apparatus (lancet and venom sac) and identified histamine by paper chromatography and biological activity. With gravimetric elution techniques, this extract was shown to contain approximately one percent histamine. Markovic and Rexova (16) identified histamine by use of preparative electrophoresis and paper chromatography. Natural venom, obtained by electrical excitation, was employed in this investigation. Values for the histamine content were found to vary from 0.64 to 1.57% depending upon the strain of honeybee and the season in which the venom was collected. Approximately the same concentrations of histamine were found in the venom sac extracts of the common wasp (Vespa vulgaris) and the European hornet (Vespa crabo) (17,18).

Although two reviews (19,20) have accredited the finding of free histidine to Neumann and Habermann (21), this compound was not reported in their investigations. Histidine, methionine and pipecolic

acid were found to occur free in the venom of the mud-dauber wasp (Sceliphron caementarium) (22).

The venom apparatus of the honeybee was shown to contain 0.0021 to 0.05% 5-hydroxytryptamine (23). Neumann and Habermann (24) report the absence of 5-hydroxytryptamine in the actual venom. The venom apparatus of Vespa vulgaris was found to contain 0.03% 5-hydroxytryptamine (17), while that of Vespa crabo contains 0.7 to 1.9% (18). The hornet venom apparatus also contained acetylcholine.

Riboflavin was reported in the venom glands of Hymenoptera (25), but the method of analysis leaves this finding unsubstantiated. Tetsch and Wolff (26) reported the presence of a sterol in a chloroform fraction of honeybee venom sacs. A steroid-like component and a lecithin-like component were reported in mud-dauber wasp venom (27).

Rexova and Markovic (28) electrophoretically separated seven low molecular weight peptide components from honeybee venom. Two other components were detected which gave positive reactions with Pauly's reagent and yielded histamine upon hydrolysis.

A highly potent material which produces a characteristic delayed, slow contraction of isolated guinea-pig ileum has been isolated from the venom apparatus of the common wasp (17). This component closely resembles bradykinin, a nona-peptide found in human blood, but can be distinguished from it by its susceptibility to inactivation by trypsin and by its chromatographic behavior (15). The purification of this wasp kinin indicated that in addition to the major kinin, two other

kinins are present in low concentrations (29,30). The venom apparatus of the European hornet contains a pharmacologically active kinin which differs from the wasp kinin (18). Schacter and Thain (15) report little or no kinin activity in the venom apparatus of honeybees.

Neumann and Habermann (21,31) electrophoretically separated phospholipase A, hyaluronidase and two polypeptides designated mellitin and apamine from honeybee venom. The enzymes have been partially purified (32). Phospholipase A has a molecular weight of 19,000 (33). Mellitin, a pharmacologically active polypeptide with a molecular weight of 2840, contains twelve different amino acids and twenty-six units per molecule. The only N-terminal amino acid present was glycine. Apamine, a centrally stimulatory polypeptide, has an isoelectric point of pH 12, and consists of ten different amino acids. It has a total of eighteen units per molecule and a molecular weight of 2036. Honeybee venom contains 2 to 3% apamine.

The honeybee venom hyaluronidase has been characterized as β-N-acetylglucosaminidase (34). Characterization was performed by identification of the products of the enzyme's action on hyaluronic acid.

Phospholipase B has been found in the venom sac extract of honeybees by Doery and Pearson (35). This enzyme is extremely stable to heat and is activated by both calcium and magnesium ions. Jaques (36,37) has reported the presence of cholinesterase, hyaluronidase and lecithinase in the venom sac extract of the common wasp.

A great deal of emphasis has been placed on the protein fraction

of honeybee venom, which is consistent with the generally accepted theory that fatalities due to bee sting are the result of anaphylaxis. The presence of the pharmacologically active polypeptides, apamine and mellitin, and histamine indicate that the protein fraction cannot account for all adverse biological effects. The venom sac extracts of the common wasp and European hornet indicate this possibility since in addition to histamine and 5-hydroxytryptamine, they contain moderate concentrations of biologically active kinins.

One of the major difficulties in earlier studies has been the procurement of pure Hymenoptera venom. Previous investigations relied primarily upon venom sac extractions. Subsequently, it is not known whether many of the constituents reported are actually in the venom or originate from the sac and surrounding tissue. Two different techniques, both dependent upon electrical excitation of the insect, have been recently employed (38,39). Mud-dauber wasp venom derived from the latter technique has been shown to contain fewer components than present in venom sac extracts (27). Fewer antigens also occur in the actual venom than in the venom sac extracts of Hymenoptera (40).

This research was intended to obtain a partial quantitative characterization of the nonprotein fraction of pure Apis mellifera venom and to develop improved fractionation techniques.

EXPERIMENTAL

Venom Source

The honeybee (Apis mellifera) is found throughout North and South America and Europe. The bees used in this research were acquired from hives in the Gallatin Valley of Montana.

Pure venom was obtained by electrical excitation of individual honeybees (39). After the venom was deposited on the slide it was dried over silica gel and stored at 4°C.

Due to the limited amount of pure venom available an alternate method was used which yielded a crude venom (38,41). In order to decrease the contamination due to water insoluble material 5 ml of purified water was added to 1 g of crude venom. The resultant slurry was mechanically shaken for 15 minutes and centrifuged at 1500 rpm for 10 minutes. The supernatant was removed with a 5 ml syringe and lyophilized. This procedure was repeated three times. The lyophilized crude venom was stored over silica gel at 4°C.

Fractionation Techniques

The venom was separated into three major fractions by extraction with solvents of varying polarity. Fraction I was obtained by extraction with purified chloroform; fraction II with anhydrous methanol; and fraction III with purified water. The extractions were performed in the above sequence at room tempera-

ture. Approximately 25 mg of venom was extracted during the procedure. The fractions were determined gravimetrically on a Metler M5 microbalance. To the dried venom contained in a 2 dram vial was added 5 ml of solvent. The vial was mechanically shaken for 5 minutes and then centrifuged at 1500 rpm for 10 minutes. The supernatant was removed by a 5 ml syringe, transferred to a weighed 2 dram vial, and dried in a stream of nitrogen. The procedure was repeated three times. Fraction III was transferred to a weighed 10 ml lyophilization bottle and lyophilized.

Previous gel-filtration of the venom has indicated a protein content of 60% (42). The protein content of fraction III and of whole venom were compared gravimetrically by this technique. Approximately 20 g of Sephadex G-10 (Pharmacia, Uppsala, Sweden) was hydrated for five hours in 100 ml of purified water and deaerated in a suction flask. All water used in this work was refluxed four hours in a dilute, acidic solution of potassium dichromate and then distilled three times in order to eliminate all ninhydrin-positive contaminants. The 52.3 ml column (1x65 cm) of dextran gel was washed with water (minimum of 1 liter) until no ninhydrin-positive gel contaminants could be detected. The venom or fraction III (25 or 19 mg) was dissolved in 0.5 ml water and applied to the column. Elution was performed with water at a rate of about 30 ml/hr. Each 5 drop fraction from the column was examined by ascending chromatography on Schleicher-Schuell

598-YD paper developed with 1-butanol:acetic acid:water (3:1:1 v/v). Detection by 2% ninhydrin in 65% ethanol and 10% Amidoschuarz-10B in 10% acetic acid (43) determined the positions of protein and nonprotein fractions.

Identification of Free Amino Acids

The honeybee venom was examined for free amino acids since such compounds have been reported in wasp venoms (19,22). The possibility of autohydrolysis was also investigated since several small peptides are present in bee venom (28). Autohydrolysis was not detected in honeybee venom. The method of this analysis will be discussed later in fraction III.

The quantitative free amino acid content of fraction II, the nonprotein part of fraction III, and the nonprotein fraction of whole venom was determined on a Technicon Amino Acid Auto Analyzer with a single column (6.3 mm I.D. x 140 cm) packed with Chromobead type A (Technicon Chromatography Corp., Chauncey, N.Y.). Aliquots of these fractions, varying in size from 2 to 6 mg, were applied to the column in duplicate. Free amino acids in the fractions were identified by comparing their retention time with those of previously chromatographed known amino acids. Quantitative results are presented in Table 2. All paper and thin-layer chromatography throughout this investigation utilized the ascending technique. Chromatograms were developed with the

following solvent systems: (a) 1-butanol:acetic acid:water (3:1:1 v/v); (b) ethanol:water (7:3 v/v); 80% phenol; (d) 2,6-lutidine (55 ml), ethanol (25 ml), water (20 ml), pyridine (4 ml); and (e) methanol (80 ml); water (20 ml); pyridine (4 ml).

Previous to sample application, all chromatograms were developed with 0.001 M ethylenediaminetetraacetate (EDTA) and dried to reduce tailing of spots. Detection of the specific amino acids was made 2% ninhydrin in 65% ethanol, 0.4% isatin in 1-butanol, platinic iodide, Pauly's reagent and Sakaguchi reagent (43).

Identification of alanine, arginine, glutamic acid, histidine and proline was confirmed by paper chromatography on Whatman No. 3 MM paper. Cystine was determined by thin-layer chromatography on Avicel microcrystalline cellulose SF (FMC corp., Newark, Delaware). The R_f-values of the known amino acids and corresponding venom spots are presented in Table 1 with the detecting spray reagents utilized. Cochromatography of the non-protein fraction of whole venom with each of the six known amino acids revealed no new spots.

The venom spot corresponding to cystine was removed from fraction II by preparative paper chromatography. Schleicher-Schuell 598-YD paper used for this procedure was washed by decending chromatography successively with: 0.001 M EDTA, distilled 5.7 M hydrochloric acid, water, and distilled 95% ethanol respectively. The paper was dried and then washed by ascending

chromatography with solvent (a). Fraction II was applied to a 10 x 50 cm sheet of paper along an 8 cm line, 3 cm from the short edge and parallel to it. The sheet was developed with solvent (a) until the solvent front reached 5 cm from the top. A small strip was cut from the long edge of each sheet and sprayed with platinic iodide reagent. A strip about 3 cm wide, parallel to the origin, and containing the desired component was removed

		solvent (a)	solvent (b)	solvent (c)	solvent (d)	Detecting reagent
alanine	venom spot	0.37	0.53			ninhydrin
	standard	0.35	0.56			
arginine	venom spot	0.19	0.28			ninhydrin
	standard	0.18	0.30			Sakaguchi reagent
cystine	venom spot	0.05	0.19	0.17		ninhydrin
	standard	0.09	0.19	0.18		platinic iodide
glutamic acid	venom spot	0.36	0.41			ninhydrin
	standard	0.36	0.43			
histidine	venom spot	0.18	0.31	0.73	0.22	ninhydrin
	standard	0.17	0.28	0.72	0.25	Pauly's reagent
proline	venom spot	0.32	0.60	0.86		ninhydrin
	standard	0.34	0.57	0.82		isatin

Table I. R_f-values and detecting reagents for free amino acids present in larger concentrations.

from the sheet. The desired component was eluted from the strip with water by descending chromatography. The preparative procedure was repeated until no accompanying ninhydrin-positive spots

were revealed.

The isolated venom component and a known cystine sample were applied, respectively, to two Avicel cellulose SF thin-layer chromatographic plates. An oxidative procedure utilizing hydrogen peroxide (19) was then employed. Two dimensional chromatography with solvents (d) and (e) was then used to compare the respective products of oxidation. With solvent (d), R_f-values for cysteic acid and the oxidized venom spot were 0.31 and 0.27 respectively. With solvent (e), R_f-values for cysteic acid and the oxidized venom spot were 0.25 and 0.24 respectively. Oxidation of the isolated venom component and cochromatography with known cysteic acid indicated no new spots.

Fraction I (Chloroform)

Fraction I comprises 5 $\pm$ 1% of the pure dried venom (average of six trials) and is composed of six compounds as shown by thin-layer chromatography on silica gel G. Approximately 10 µg of fraction I was applied to a thin-layer plate at single points 3 cm from the bottom and developed to a height of 15 cm with water-saturated 1-butanol. Spraying the plate with 40% sulfuric acid (followed by heating at 130°C for 15 minutes) revealed six components with R_f-values of 0.99, 0.88, 0.70, 0.55, 0.47 and 0.0. The components were detectable after heating due to their slight fluorescence under "long-wave length" ultraviolet light. When

viewed in visible light the component with the R_f-value of 0.99 was brown, while the other components were white against the background. A similar plate was prepared and developed with chloroform: 95% methanol:water (80:20:1 v/v). Using the same detection procedure revealed spots with R_f-values of 1.00, 0.73, 0.55, 0.44, 0.10 and 0.0. When viewed in visible light after heating with 40% sulfuric acid the component with the R_f-value of 1.00 was brown, while the remainder were white. Thin-layer chromatography with benzene: ethyl acetate (5:1 v/v) revealed six spots with R_f-values of 0.90, 0.58, 0.43, 0.36, 0.17 and 0.0 after heating with the same reagent. These constituents will be referred to as Ia, Ib, Ic, Id, Ie and If in the following pages. In visible light after heating with sulfuric acid component Ia was brown, while Ib, Ic, Id, Ie and If had a white coloration. Before spraying with the reagent only component Ia had any detectable "natural" fluorescence under ultraviolet light. A plate developed with the benzene:ethyl acetate solvent was sprayed with a 0.05% solution of Rhodamine 6G in 95% ethanol, which revealed components Ia, Ib and If while still "wet". After drying only component Ia was visible against the reagent background. None of the components were detectable under ultraviolet light after this reaction. A similar plate sprayed with a saturated chloroform solution of antimony trichloride (43) and heated at 100°C for three minutes produced a negative reaction.

A positive reaction with this reagent demonstrates the presence of steroids. A plate sprayed with ninhydrin also produced a negative reaction.

Approximately 20 µg of fraction I was applied to a thin-layer plate and developed with the benzene: ethyl acetate solvent. The chromatogram was treated with 10% phosphomolybdic acid in 95% ethanol (44) and a deep-green spot characteristic of choline lipids appeared for each of the six components. Another chromatogram prepared in the same manner was sprayed with modified Hanes-Isherwood Reagent (43), heated at 85° for seven minutes and kept in air until blue spots appeared with R_f-values identical to those obtained previously. This reagent demonstrates the presence of phosphatides. These data and the waxy appearance of the components suggested a fatty material, such as lecithin.

A sample of β, γ-dipalmitoyl- L, α-lecithin (Sigma Chemical Co., St. Louis, Missouri) when developed with the benzene: ethyl acetate solvent on silica gel G and sprayed with the same series of reagents showed a single spot with R_f-values of 0.50. With the chloroform:95% methanol:water solvent the R_f-value of lecithin was also 0.50.

Approximately 20 µg of fraction I was applied to a silica gel G plate 3 cm from the left edge and bottom. A 0.5 mg sample of fraction I was applied 13 cm from the left edge allowing for a 10 cm separation of the two aliquots. The plate was developed

with the benzene:ethyl acetate solvent to a height of 15 cm. After air drying the 20 µg sample was sprayed with phosphomolybdic acid to detect the positions of the six constituents. Since the spots corresponding to components Ic, Id and Ie were barely discernible, it was assumed they were in low concentration and should be collected together. Lines were drawn 1 cm above and below the center of each major spot (Ia, Ib and If) parallel to the origin. Lines were drawn 0.5 cm above the center of component Ic and below the center of component Ie. These lines were extended to the right into the unreacted area. The silica gel between these lines in this area was removed from the plate and placed in respective 2 dram vials. To each of the four vials was added 1 ml of purified chloroform. The vials were mechanically shaken for 30 minutes and then centrifuged at 1500 rpm for 10 minutes. The supernatant was removed by a 5 ml syringe, transferred to a weighed 2 dram vial, and dried in a stream of nitrogen. The components were determined gravimetrically on a Metler M5 microbalance. The major components of fraction I account for the following amounts of whole honeybee venom: Ia; ca. 1.3%; Ib; ca. 1.7%; and If; ca. 0.7%. Components Ic, Id and Ie account for ca. 0.6%.

Figure 1 represents the infra-red spectrum of β, γ-dipalmitoyl- L, α-lecithin obtained with a Beckman IR 4 spectrophotometer using a micro potassium bromide (KBr) pellet. Suitable

material for pellets could not be produced when lecithin and KBr were ground in an agate mortar due to the wax-like consistency of the former. When ground under purified diethyl ether, however, lecithin was dispersed to a high degree in the KBr. Unfortunately, water condensed on the mixture during this procedure and consequently was present in the pellet. Thus, the band occuring at 3450 cm^{-1} is due to water. This band is also present in the spectra of the fraction I occuring at 3400-3450 cm^{-1}. If any of the fraction I components have structures similar to lysolecithin, the absorption band associated with oxygen-hydrogen bonds will be obscured by those from the water. Figures 2, 3 and 4 are the spectra of components Ia, Ib and If respectively, which were recorded under identical conditions. The frequencies of the absorption bands in these and figure 1 are very similar, although the shapes or intensities of these bands differ to some extent.

The infra-red spectra and positive reactions to specific reagents appear to be consistent with the assignment of a lecithin-like structure to components Ia, Ib and If of pure Apis mellifera venom. Without further data components Ic, Id and Ie can only be assigned a phospholipid structure.

Fraction II (Methanol)

Fraction II accounts for 18 $\pm$ 2% of the pure venom (average of six trials). The amino acid analyzer indicates the presence of eighteen free amino acids, six of which have been confirmed

by paper chromatography. Qualitative and quantitative data for these free amino acids are presented in Table 2.

Two samples of fraction II (3 and 4 mg) respectively were hydrolyzed in 5.7 M hydrochloric acid for 30 hours. The hydrolyzates were taken to dryness on a rotary evaporator, moistened with water and again evaporated to dryness. This procedure was repeated twice. Each hydrolyzate sample was dissolved in 0.5 ml water and applied to the analyzer column. Amino acids in this fraction were identified by comparing their retention times with previously chromatographed known amino acids. The minimum quantity of small peptides in this fraction was determined by subtracting the free amino acid content from the hydrolyzate amino acid content. The number of peptides was determined by comparing the unhydrolyzed and hydrolyzed fraction II analyzer graphs. If a peak present on the unhydrolyzed fraction II graph was decreased or disappeared upon hydrolysis, it was assumed to represent a peptide. Ten such peaks were found. There were eight additional peaks which did not decrease upon hydrolysis and could not be identified as common amino acids. Although methionine did not occur free, it was present in the hydrolyzate of fraction II.

A 4 mg sample of the nonprotein part of whole venom, as obtained by gel filtration, was hydrolyzed in 13% barium hydroxide for 18 hours. The hydrolyzate was cooled and dry ice was added.

The barium carbonate precipitate was removed by centrifugation at 1500 rpm for 10 minutes. The sample was then applied to the analyzer column. Tryptophan was identified by comparing its retention time with a known tryptophan sample. Thus, tryptophan is present in the nonprotein hydrolyzate, although its presence in the fraction II hydrolyzate was not specifically determined.

Paper chromatography with Whatman No. 3 MM using the 1-butanol:acetic acid:water system revealed twelve ninhydrin-positive constituents with R_f-values of 0.71, 0.60, 0.57, 0.53, 0.49, 0.46, 0.42, 0.38, 0.36, 0.30, 0.17 and 0.11 which were present in fraction II. A similar paper chromatogram developed with the 1-butanol:acetic acid:water system revealed six constituents positive to Pauly's reagent with R_f-values of 0.60, 0.57, 0.49, 0.42, 0.17 and 0.11. The constituents with R_f-values of 0.17, 0.42 and 0.57 will be referred to as IIa, IIb and IIc in the following pages.

Constituent IIa was isolated from fraction II by preparative paper chromatography. The technique used in this isolation was similar to that previously employed to isolate cystine from the nonprotein part of whole venom. However, the small strip cut from the long edge of each sheet was sprayed with ninhydrin to locate this constituent. The preparative procedure was repeated until paper chromatography with the 1-butanol:acetic acid:water solvent revealed no accompanying ninhydrin-positive spots. The

identity of this fraction II constituent as histamine was determined by chromatography on Schleicher-Schuell 598 YD-paper. Using the 1-butanol:acetic acid:water solvent, R_f-values for known histamine and the venom constituent were 0.21 and 0.20 respectively and with the 80% phenol the known sample and venom spot gave R_f-values of 0.63 and 0.62. Cochromatography of fraction II and known histamine revealed no new spots, and the ninhydrin color of the venom histamine spot was identical with that of the known histamine.

Constituent IIb was isolated from fraction II by preparative paper chromatography on Whatman No. 3 MM developed with the 1-butanol:acetic acid:water system. After the initial separation the purity of IIb was determined by paper electrochromatography on a Karler-Kirk Continuous Flow Preparative Spectrolator (Microchemical Specialties Co., Berkeley, Calif.). A Schleicher-Schuell 598-YD paper curtain previously washed with 0.001 M EDTA was equilibrated for 30 minutes in a pH 5.3 pyridine:acetic acid:water buffer (45). Approximately 20 µg of the isolated constituent was applied to the curtain by a motor-driven syringe. The electrochromatogram was then allowed to develop for two hours at 110 volts (1.5 mamp). Detection by ninhydrin revealed four contaminants in addition to the desired component. Constituent IIb was purified by preparative paper chromatography on Schleicher-Schuell 598-YD with 95% ethanol:water (7:3 v/v).

Paper electrochromatography with the previous conditions revealed a single spot of red coloration when detected with ninhydrin. When IIb was applied to Whatman No. 3 MM and developed with the ethanol:water, the R_f-value was 0.59; with 80% phenol the R_f-value was 0.38.

A 52.3 ml column (1 x 65 cm) of Sephadex G-10 dextran gel was prepared as previously described and washed with water until no ninhydrin-positive contaminants could be detected. A series of known compounds of varying molecular weights were applied to the column in order to determine the number of drops required for the elution of each. These elutions were performed in triplicate with water at a rate of 30 ml/hr. A 2 mg sample of glycylphenylalanylphenylalanine (Mann Research Laboratories, Inc. New York, N.Y.) having a molecular weight of 405 was dissolved in 0.5 ml of water and applied to the column. Each 5 drop fraction was applied to a sheet of Whatman No. 3 MM, dried and sprayed with ninhydrin in order to detect the first appearance of the compound as it passed the column. The color reaction due to the known occured at 460 drops. A 2 mg sample of flavin mononucleotide (Nutritional Biochemicals Corp., Cleveland, Ohio) having a molecular weight of 514 was similarly applied to the column. Each 5 drop fraction was viewed under "long-wavelength" ultraviolet light. The fluorescence due to the known occured at 450 drops. A 2 mg sample of adenosine triphosphate (Nutritional Biochemicals Corp., Cleveland,

Ohio) having a molecular weight of 623 was applied to the column. Each 5 drop fraction was applied to a sheet of Whatman No. 3 MM paper and reacted with the silver nitrate-sodium bichromate reagent (46). The color reaction due to the known occured at 445 drops. A 2 mg sample of constituent IIb was dissolved in 0.5 ml of water and applied to the column. Each 5 drop fraction from the column was applied to a sheet of Whatman No. 3 MM paper and sprayed with Pauly's reagent. The first detectable spot corresponded to the fraction which appeared at 450 drops. Consequently, the molecular weight of constituent IIb is approximately 500.

A 1 mg sample of IIb was hydrolyzed in 5.7 M hydrochloric acid for 30 hours. The hydrolyzate was taken to dryness on a rotary evaporator, moistened with water and again evaporated to dryness. The procedure was repeated twice. Approximately 30 µg of the hydrolyzate was applied to a sheet of Schleicher-Schuell 598-YD paper and developed with the 1-butanol:acetic acid: water solvent. Detection by ninhydrin revealed four spots with R_f-values of 0.13, 0.26, 0.33 and 0.38. These R_f-values correspond to known histamine (R_f = 0.12); glycine (R_f = 0.26); glutamic acid (R_f = 0.32) and alanine (R_f = 0.38). The remainder of the hydrolyzate was applied to the analyzer column. Glycine, glutamic acid and alanine were found to be present in the µmole ratio of 2:1:1. Histamine was not detected since it is not eluted through

a column packed with Chromobead type A.

The C-terminal amino acid of IIb was determined by hydrazinolysis (46). A 1 mg sample of IIb was refluxed with 4 ml of anhydrous hydrazine at 100°C for 12 hours. Excess hydrazine was removed by vacuum dessication over concentrated sulfuric acid. The hydrazides were then removed by reaction with 5 ml of benzaldehyde. The residue remaining was dissolved in 0.1 ml of water and applied to a sheet of Schleicher-Schuell 598-YD which was developed with the 1-butanol:acetic acid:water solvent. Detection by ninhydrin revealed a single spot with an R_f-value of 0.12 which corresponds to known histamine (R_f = 0.12).

The N-terminal amino acid of IIb was determined by the phenylisothiocyanate method of Edman (47). The reaction was carried out in a tube 20 cm in length, sealed at one end and with a 12/30 standard taper female joint at the other. Lyophilization and anhydrous reactions were performed in this tube, therefore avoiding unnecessary transfers. The reaction tube was immersed in a water bath at 40°C. A 0.5 mg sample of IIb was dissolved in 2 ml pyridine:water (1:1). The solvent contained 3 mg bromthymol blue per 100 ml. By addition of 0.5 M sodium hydroxide the color was adjusted to that corresponding to pH 8.6. For comparison another tube was placed alongside containing solvent and indicator, the pH of which was determined to be the desired 8.6 by means of a glass electrode. To the solution of IIb was added 70 µl of phenyl-

isothiocyanate (K & K Laboratories, Plainview, N.Y.). The reaction mixture was mechanically shaken and the pH maintained at 8.6 for 12 hours. After completion of the reaction pyridine and excess phenylisothiocyanate were removed by three extractions with 2 ml aliquots of benzene. The aqueous solution was then taken to dryness by lyophilization. To the reaction tube was added 2 ml of 3% hydrogen chloride in anhydrous nitromethane (w/w). The tube was sealed, immersed in the 40°C water bath and mechanically shaken for 12 hours. At the end of the reaction the mixture was centrifuged at 1500 rpm for 10 minutes. The solution was removed with a 5 ml syringe and evaporated to dryness in a stream of nitrogen. To the residue was added 0.1 ml of dichloroethane. This solution was applied to a sheet of Whatman No. 1 which had been previously impregnated with a formamide:acetone (1:9 v/v) solution. After development with n-butyl acetate:propionic acid:formamide (48), the chromatogram was sprayed with an iodine-azide reagent (46) to reveal the phenylthiohydantion (PTH) amino acid. The PTH derivative of constituent IIb had an R_f-value of 0.87, while known PTH-alanine had an R_f-value of 0.88.

The identity of the N-terminal amino acid of IIb as alanine was confirmed by the dinitrophenylation method (46). A 0.5 mg sample of IIb was dissolved in 0.1 ml of 1% trimethylamine to which was added 10 µl of 2,4-dinitrofluorobenzene (Sigma Chemical Co., St. Louis, Mo.) in 0.2 ml ethanol. After 2 hours at 40°C a

few drops of water and 1% trimethylamine were added and the solution was extracted three times with ether. The aqueous solution was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 2 ml of 5.7 M hydrochloric acid and hydrolyzed in the dark for 12 hours. The liberated dinitrophenyl (DNP) amino acid was extracted from the aqueous solution with 5 ml of ethyl acetate. The solvent volume was decreased to less than 0.5 ml under a stream of nitrogen, applied to a buffered sheet of Whatman No. 1 paper and developed with a system using t-amyl alcohol: pH 6 phthalate buffer (46). The R_f-value of the DNP derivative of constituent IIb was 0.25, while that of DNP-alanine was 0.27.

A 0.5 mg sample of IIb was applied to the analyzer column to determine the chart position and the gravimetric conversion factor of the peak due to it. With this information and the molecular weight of IIb the amount of this compound in fraction II and the nonprotein fraction was obtained from the respective charts of these fractions. It was found that constituent IIb accounts for 3.9% of fraction II or 0.6 $\pm$ .05% of the dried venom.

Constituent IIc was isolated from fraction II by preparative paper chromatography on Whatman No. 3 MM developed with the 1-butanol:acetic acid:water solvent. The paper electrochromatography procedure indicated that it was necessary to repeat the isolation since two additional components were present. Constituent IIc was purified by repeating the preparative pro-

cedure with the 1-butanol:acetic acid:water solvent. Paper electrochromatography then revealed a single red spot with ninhydrin. When IIc was applied to a sheet of Whatman No. 3 MM and developed with the ethanol:water solvent the R_f-value was 0.63. With 80% phenol the R_f-value was 0.59.

With the same 1 x 65 cm column of Sephadex G-10 gel and procedure used previously constituent IIc was detectable by Pauly's reagent in the eluted fraction which corresponded to 450 drops. The three known compounds (glycylphenylalanylphenylalanine, flavin mononucleotide and adenosine triphosphate) were eluted from the column in the fractions corresponding to 460 drops, 450 drops and 445 drops respectively. Therefore the molecular weight of constituent IIc is approximately 500.

A 1.3 mg sample of IIc was hydrolyzed in 5.7 M hydrochloric acid for 40 hours. After removal of the acid a 30 µg aliquot was applied to a sheet of Whatman No. 1 paper and developed with the 1-butanol:acetic acid:water solvent. Detection by ninhydrin revealed six spots with R_f-values of 0.05, 0.15, 0.20, 0.28, 0.36 and 0.40. These values correspond to known histamine (R_f = 0.08); glycine (R_f = 0.17); glutamic acid (R_f = 0.21); alanine (R_f = 0.30) and proline (R_f = 0.36). The compound with the R_f-value of 0.40 was shown to be unhydrolyzed IIc since it had the characteristic red color with ninhydrin. A 30 µg sample of the IIc hydrolyzate was applied to a point 3 cm from the bottom

and side, on a 25 x 25 cm sheet of Whatman No. 3 MM paper. The two-dimensional chromatogram was developed in the first direction with the 1-butanol:acetic acid:water solvent. After air drying the chromatogram was turned at a right angle to the first direction and developed with the ethanol:water solvent. Detection with ninhydrin revealed the six spots which had been previously identified. The remainder of the hydrolyzate was applied to the analyzer column. Alanine, glycine, glutamic acid and proline were present in the μmole ratio of 2:1:1:1.

The C-terminal amino acid of IIc was determined with a 0.5 mg sample by the previously described hydrazinolysis procedure. The residue remaining after the benzaldehyde wash was dissolved in 0.1 ml of water, applied to a sheet of Whatman No. 1 paper and developed with the 1-butanol:acetic acid:water solvent. Detection by ninhydrin revealed a single spot with an R_f-value of 0.04. The R_f-value of histamine under similar conditions was 0.06.

The N-terminal amino acid of IIc was determined with a 0.5 mg sample by the dinitrophenylation method. The liberated DNP amino acid was applied to a buffered sheet of Whatman No. 1 paper and developed with the t-amyl alcohol:pH 6 phthalate buffer system. The R_f-value of the DNP derivative of constituent IIc was 0.32, which was the same as that of known DNP-alanine.

The sequence of the amino acids present in constituent IIc

was determined by the phenylisothiocyanate method. A 2 mg sample of IIc was dissolved in 2 ml of the pyridine:water solvent. The procedure which followed was the same as that previously used for the determination of the N-terminal amino acid of IIb. After centrifugation of the cleaved phenylthiocarbamyl peptide, both the supernatant and precipitate were recovered. The supernatant which contained the PTH amino acid was evaporated to dryness in a stream of nitrogen. After the addition of 0.1 ml of dichloroethane the PTH amino acid was applied to a sheet of formamide-impregnated Whatman No. 1 paper and developed with the n-butyl acetate:propionic acid:formamide solvent. Detection with the iodine-azide reagent revealed a spot with an R_f-value of 0.85. The R_f-value of known PTH-alanine was 0.86. The insoluble precipitate remaining in the reaction tube was washed with three 5 ml aliquots of dry ether in order to remove excess hydrogen chloride. The precipitate was then dissolved in 2 ml of the pyridine:water solvent and the entire procedure was repeated. The next PTH amino acid detected with the iodine-azide reagent had an R_f-value of 0.72, while known PTH-glycine had an R_f-value of 0.74. After repetition of the procedure the third PTH amino acid had an R_f-value of 0.91, which was the same as known PTH-proline. The R_f-values of both the fourth PTH amino acid and known alanine were 0.81. The R_f-values of the fifth PTH-amino acid sequenced from IIc and known PTH-

glutamic acid were both 0.30. When the procedure was repeated for the sixth time, only the background spot present at the solvent front was detected. It was therefore assumed the sequence was terminated.

The fourth amino acid from the N-terminal end of IIc was confirmed as alanine by repeating the phenylisothiocyanate method for the first three units. The insoluble residue remaining after the three stepwise degradations was dissolved in 0.1 ml of 1% trimethylamine to which was added 10 μg of 2,4-dinitrofluorobenzene in 0.2 ml ethanol. The procedure for dinitrophenylation was then performed. The liberated DNP-amino acid was applied to a buffered sheet of Whatman No. 1 paper and developed with the t-amyl alcohol:pH 6 phthalate buffer system. The R_f-values for the DNP-amino acid and known DNP-alanine were both 0.24. Therefore, constituent IIc is a histamine peptide with the structure alanylglycylprolylalanylglutamylhistamine.

By applying a 0.4 mg sample of IIc to the analyzer column and comparing its intensity and chart position to the chart of fraction II and the nonprotein fraction of the venom, it was found that IIc accounts for 5.0% of fraction II or 0.8 ± 0.04% of the dried venom.

Paper chromatography of fraction II with Whatman No. 3 MM using the 1-butanol:acetic acid:water solvent revealed two components with R_f-values of 0.18 and 0.22 which were detectable with aniline-diphenylamine-phosphoric acid (46). This reagent in-

dicates the presence of carbohydrates. The constituents with R_f-values of 0.18 and 0.22 will be referred to as IId and IIe in the following pages.

The identity of constituent IId as glucose was determined by paper chromatography on Schleicher-Schuell 598-YD. Comparison of venom samples and known glucose were made using (a) the 1-butanol:acetic acid:water solvent (R_f-value of known glucose = 0.32; R_f-value of venom spot = 0.34), (b) the ethanol:water solvent (R_f-value of known = 0.70; R_f-value of venom spot = 0.68) and 80% phenol (R_f-value of known = 0.41; R_f-value of venom spot = 0.40). Detection of the venom spot with p-anisidine hydrochloride (46) and alkaline triphenyl tetrazolium chloride (43) produced coloration identical with that of known glucose. Cochromatography of fraction II and known glucose revealed no new spots.

The identity of constituent IIe as fructose was determined in a similar manner. Using the 1-butanol:acetic acid:water system, R_f-values of known fructose and the corresponding venom spot were 0.36 and 0.38 respectively and with the ethanol:water the known sample and venom spot gave R_f-values of 0.74 and 0.75 respectively. With 80% phenol, R_f-values of the known and venom spots were 0.55 and 0.53 respectively. The colors produced by the venom spot with the above reagents were identical with those of fructose. Cochromatography of fraction II and known fructose produced no