The venom of the mud-dauber wasp Sceliphron caementarium

by William Rosenbrook

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 mud-dauber wasp (Sceliphron caementarium)obtained by electrical excitation of the wasp, has been investigated qualitatively and quantitatively in order that no major constituent might be overlooked. The protein fraction of the dried venom was found to be unexpectedly small and small peptides were not detected in any significant amounts. Seventeen non-protein constituents, among them three free amino acids and a lecithin-like compound, were separated by chromatographic techniques and . identified or characterized. Of particular importance was the discovery of what appears to be a series of non-nitrogenous compounds in the venom.

This is the first investigation of any wasp venom to deal with the pure venom as opposed to a venom apparatus extract. Examination of an extract of mud-dauber venom apparatuses has revealed a minimum of fifteen compounds not present in the venom itself, thus emphasizing the value of studies based on the natural venom.

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ABSTRACT

The venom of the mud-dauber wasp (Sceliphron caementarium), obtained by electrical excitation of the wasp, has been investigated qualitatively and quantitatively in order that no major constituent might be overlooked. The protein fraction of the dried venom was found to be unexpectedly small and small peptides were not detected in any significant amounts. Seventeen non-protein constituents, among them three free amino acids and a lecithin-like compound, were separated by chromatographic techniques and identified or characterized. Of particular importance was the discovery of what appears to be a series of non-nitrogenous compounds in the venom.

This is the first investigation of any wasp venom to deal with the pure venom as opposed to a venom apparatus extract. Examination of an extract of mud-dauber venom apparatuses has revealed a minimum of fifteen compounds not present in the venom itself, thus emphasizing the value of studies based on the natural venom.

INTRODUCTION

The members of the order Hymenoptera which are commonly referred to as "wasps" belong almost exclusively to the class Vespoidea, family Vespidae (social wasps), and to the class Sphecoidea (solitary or mud-dauber wasps).

Stings from wasps are so common that almost everyone is familiar with their effects. These stings are commonly considered to be harmless although quite painful. Information concerning these stings is rarely mentioned in the literature and investigations of the venom itself are even less common. During the last twenty years several papers have been prepared which survey a rather large number of deaths due to wasp and bee stings (e.g. 1,2). In the United States stings from wasps and bees cause more deaths than all other poisonous animals combined. In the 8 year period from 1950 through 1954 and 1957 through 1959 seventy-nine deaths have been directly attributed to wasps alone (2). Several of these deaths were caused by multiple stings and were probably the result of the direct action of the venom toxins. About 30 percent of the "single sting" deaths may be attributed to anaphylaxis, while most of the remaining deaths are not understood. The cause of death in the majority of the latter cases was stated simply as heart failure. Thus, death as inflicted by a wasp or bee sting is only poorly understood and the only satisfactory treatment at this time is the administration of some pressor amine such as epinephrine, phenylephrine, norepinephrine, aromine sulfate, isoproteranol, or triplennamine immediately during the shock episode.

This time limit makes it necessary to have a ready supply of one of these amines, which presupposes that the sting victim knows he is hyper-sensitive. The above survey (2) demonstrates conclusively that the majority of wasp sting victims had no prior knowledge of sensitivity to wasp sting.

Preventive medical treatment relies solely upon the method of allergic desensitization. This treatment is limited because anaphylactic shock does not appear to be the only cause of death from insect sting and because prior knowledge of sensitivity is required. Desensitization is only temporary and must be continued with a "booster shot" at least every two months. In addition, desensitization is now carried out with either whole body (3) or venom apparatus (4,5) extracts which may contain harmful antigens foreign to the venom itself. Of additional interest is the indication of common antigens among bees and wasps (3), which supports the idea of cross sensitization and suggests a definite similarity between the various venom proteins.

Bee venom has long been proposed as a therapeutic agent for such chronic ailments as arthritis and rheumatism (6), however, conclusive evidence of its effectiveness is not yet available. Statistics show that bee keepers have a very low incidence of cancer which may be due to their "bee sting immunity" (7) or to their diet of unpasteurized honey. Like bee venom wasp, venom may possess a therapeutic value.

The chemical nature of wasp venoms was first investigated in 1913 by Bertarelli and Tedeschi (8), who found that hornet venom

(Vespa crabo) resembled bee venom in its action on small animals, contained an unstable hemolysin and smelled of capryllic acid. All wasp venom investigations prior to the present research have been on crude preparations obtained by extracting the entire venom apparatus, i.e. the venom sac, acid glands and Dufour's alkaline gland. As a result, all of the constituents found to date can not necessarily be assumed to occur in the venom itself.

Various animal and insect venoms (including wasp and bee) have been found to contain a thermostable phospholipase A(9, 10), phospholipase B(9), proteases (9, 10), thrombokinases (9), lipases (9) and carbohydrases (9). The facts suggested that the toxic action of the venoms was produced by the action of the various enzymes on the tissues of the victim. Wasp "poison" (undefined) was also shown to possess a factor which saponified sodium or calcium glycerophosphates, but had no effect on plant phosphatides or natural fats (11). Riboflavin was reported in the venom glands of hymenoptera (12), but the method of analysis leaves this finding unsubstantiated. Another group of investigators (13) determined that wasp and bee venom did not contain sulfhydryl or disulfide derivatives. However, Kaiser and Michl (14) report 7.45% cystine and/or cysteine in the protein hydrolysate of bee venom. In addition 5-hydroxytryptamine and free amino acids (undefined) were found in wasp venom and acetylcholine in hornet venom (14).

Recently, the dried venom apparatus of the common wasp (Vespa vulgaris) was found to contain 1.6 to 2.0% histamine and about 0.03% 5-hydroxytryptamine (15). In the same article Jaques and Schachter made the first report of a "slow contracting substance", which was later identified (16, 17, 18, 19) as a kinin. Wasp venom kinin bears a striking resemblance in its physiological properties to the nonapeptide from human blood (20), bradykinin (kallidin), but is not identical with it. Kinin is believed to be the major pain producing factor of wasp venom (21) and its contracting effect on smooth muscle probably contributes to the toxicity of the venom. Jaques has also reported the presence of hyaluronidase (a spreading factor) (22, 23), cholinesterase and lecithinase (22) in Vespa vulgaris venom.

Michl (24) discovered the first pipecolic acid derived from animals in the protein hydrolysate of snake and wasp (Vespa germanica) venom in 1957.

Previous investigations of wasp venoms, based on qualitative analysis of venom apparatus extracts, have revealed only a fraction of the complex character of these venoms. Since the percentages of the various components found thus far is unknown, many unidentified substances may be present in wasp venoms. In addition, no attempt has yet been made to exhaustively analyse the venom of one particular species of insect. As a result, the protein fraction of these venoms has been emphasized and the possibility of other physiologically active venom

constituents has been largely ignored.

The venom of the mud-dauber wasps is of special interest because of its paralytic action on spiders (25) and its unusually mild immediate reaction on humans, i.e. negligible pain and swelling. The natural history of the paralyzing sting of various wasps gives little information concerning the physiology involved except possibly the speed of onset, duration, or completeness of the paralytic state. However, wide variations in these effects suggest that paralyzing venoms differ in type as well as in potency (26). Other evidence indicates that paralysis induced by sphecoid wasps may not result from a direct neural lesion but from a generalized "poisoning" of the blood (27). It was at first believed that the mud-dauber sting could not produce death in humans, but an authenticated case of a mud-dauber sting death has since been discovered (2), and it now appears likely that the rarity of severe sting reactions may be due to the mild temper of the wasp, rather than to the composition of its venom. Since the mud-dauber is a solitary wasp (the first of the sphecoid wasps to have the chemistry of their venom investigated) its venom might very well differ from that of the vespidae already investigated, e.g. the venom of the former paralyzes rather than kills the prey and its venom apparatus lacks Dufour's alkaline gland (28).

This research was intended to obtain a quantitative characterization of pure Sceliphron caementarium venom and to develop techniques which might facilitate the ellucidation of other complex insect venoms. The

mud-dauber wasp was particularly cooperative and their use eliminated most of the problems encountered in working with more vicious insects.

EXPERIMENTAL

Collection and Maintenance of Wasps

The mud-dauber wasp is most commonly found in areas of high humidity, although at least one species is native to every region of North America. Most of the mud-dauber wasps for this research were obtained from southeast Missouri and others of the same species were collected in the San Joaquin Valley of California. Initially, the wasps were maintained in cages under conditions similar to those of their natural habitat and fed on a diet of honey and spiders as described by Shafer (25). In the later stages of this research the venom was extracted from the wasps in the field and their venom apparatuses (with lancet attached) were immediately removed since only a fraction of the venom was released by the extraction technique. The venom apparatuses (with lancet attached) were removed from the insects by the method of Jaques and Schachter (15) and stored over phosphorous pentoxide at 5° C. The species of mud-dauber was identified by Dr. K. V. Krombein, Insect Identification and Parasite Research Branch, Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

Extraction of Venom

The methods which have been described for obtaining bee venom by electrical excitation (29, 30, 31) are not applicable to mud-dauber wasps or to certain other wasps and hornets because of insufficient

excitation voltage or because of the danger of fighting among these insects.

The method described here (32) has been found adequate for obtaining pure venom from one to several hundred individual bees, wasps or hornets. The insect suffered no apparent damage and many were often used for repeated venom extractions.

The apparatus (Figure 1) consisted of a small (about 1/4-inch diameter) half-cylinder of fine brass mesh about 1/2-inch long soldered to the end of a 2-inch length of rigid iron wire which was bent for insertion into a rubber stopper. The insect was anesthetized with carbon dioxide or immobilized in the cold, placed in the half-cylinder, and bound in place with a 1/4-inch ribbon of aluminum foil which was twisted behind the half-cylinder. The mounted insect was supported by a clamp directly beneath a nichrome wire lead from a spark coil (about 10,000 volts). A microscope well-slide was placed under the insect in such a manner that only the sting lancet reached into the well. When the insect revived, it was excited by a brief high-voltage shock, controlled by a key switch, until venom was excreted.

After the venom was deposited on the slide it was dried over phosphorus pentoxide and stored at 5°C. With this apparatus, two or three mud-dauber wasps could be "milked" each minute with no apparent effect on the insect other than pronounced hunger and thirst.

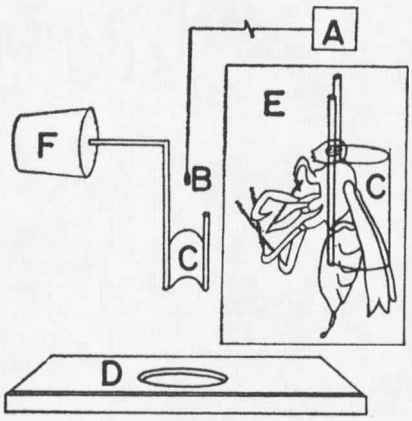

Figure 1. Procuring venom from a wasp. A, Spark coil with 6-volt d-c power supply; B, nichrome wire; C, brass mesh half-cylinder; D, microscope well-slide; E, insert showing insect mounted in half-cylinder before being wrapped with aluminum ribbon; F, rubber stopper.

It was found that the abdominal segment of the mud-dauber wasp could be removed and would remain "alive" for as long as 24 hours in this isolated state. In addition, this isolated segment made the same response to the "milking" procedure as the whole insect. Because of the increased efficiency in handling the abdominal segments as opposed to the whole insect, venom extracted from these segments was used exclusively in the later stages of this research.

Identification and Determination of Free Amino Acids (33)

The mud-dauber venom was examined for free amino acids since such compounds had been reported in other wasp venoms (14). All paper and thin-layer chromatography throughout this investigation utilized the ascending technique. The identity of one of the venom constituents as

histidine was determined by paper chromatography on Whatman No. 3 MM paper. Comparison of venom samples and known histidine spots was made using (a) 80% phenol (R_f-value of known histidine =0.65; R_f-value of venom spot =0.68). (b) 2,6-lutidine(55 ml):ethanol(25 ml):water(20 ml): diethylamine(2 ml) (R_f-value of known =0.35; R_f-value of venom spot =0.32), (c) 1-butanol:acetic acid;water(34) (R_f-value of known =0.16; R_f-value of venom spot =0.19 with detection by Pauly's reagent (35), as well as by a 2% solution of ninhydrin in acetone. Additional comparison using silica gel thin-layer chromatography developed with abs. ethanol:water (63:37 v/v) (36) gave known histidine an R_f-value of 0.44 and the venom spot an identical R_f-value. Cochromatography of venom and known histidine revealed no new spots and the ninhydrin color of the venom histidine spot was identical with that of the known histidine.

Quantitative estimation of histidine was made by chromatographing 1.4 mg of venom on a silica gel G thin-layer plate using the ethanol: water developing system. The region of R_f-value 0.42 to 0.46, previously shown to contain only histidine, was scraped from the plate and triturated in a centrifuge tupe with 1.00 ml of deionized distilled water. Residual silica gel was removed by centrifugation and 0.250 ml aliquots of supernatant solution were removed and used for the spectrophotometric determination method of Stegemann and Bernhard (37) using a histidine standard curve; a histidine content of 13 ± 2 mg was obtained corresponding to 0.8 to 1.1% of the dried venom.

Methionine was detected in the same manner as histidine. Using the 80% phenol, R_f-values for known methionine and the corresponding venom spot were 0.75 and 0.74 respectively and with the 2,6-lutindine:ethanol: water:diethylamine the known sample and venom spot gave R_f-values of 0.52 and 0.50 respectively. On silica gel thin-layer chromatography using the ethanol:water system, the known and venom spot R_f-values were both 0.64.

Quantitative estimation was made by the method employed for histidine, except that a methionine standard curve was used. A methionine content of 0.9 to 1.1% was determined.

Pipecolic acid was detected in the same manner as histidine and methionine. Using 80% phenol, R_f-values of authentic pipecolic acid (Biochemical Research Laboratories, Los Angeles, Calif.) and the corresponding venom spot were 0.86 and 0.84 respectively. With the 1-butanol: acetic acid:water system, R_f-values of the known and venom spot were 0.43 and 0.44 respectively. On silica gel thin-layer chromatography, with ethanol:water, R_f-values of both known and venom spots were 0.50. Cochromatography revealed no new spots.

Quantitative estimation was made by the method used for histidine and methionine except that elution from the silica gel was made with 1 ml of glacial acetic acid on a vortex mixer at 57°C for 15 minutes. Only 50% of the pipecolic acid was eluted by this method so that standard knowns had to be run simultaneously with the unknown, using identical

procedures. The spectrophotometric analytical method described by Schweet (38) was used and revealed a pipecolic acid content corresponding to 0.12 to 0.16% of the dried venom. An independent assay based on elution from a paper chromatograph (Whatman No. 3 MM) developed with 80% phenol gave identical results.

Protein Fraction

The paper chromatogram of a natural venom sample showed positive Azocarmine G and Amidoschwartz 10B tests only at the origin, so the protein fraction of the venom is not moved by 80% phenol.

Dried natural venom (4.41 mg) was dissolved in 1 ml of triple distilled water and applied in a series of closely spaced points along the short edge of a 23 X 50 cm sheet of Whatman No. 3 MM paper. The sheet was developed for twelve hours with 80% phenol, dried in a stream of air, and washed with ether to remove residual phenol. The dried paper was cut on a line 2 cm above the origin and parallel to it, a region previously shown to be free from the ninhydrin-positive compounds separable by 80% phenol. The residue on this strip was eluted with distilled water, lyophilyzed, and determined gravimetrically. A weight corresponding to 31% of the original dried venom was obtained. Since compounds other than proteins also remained at the origin, this figure represents a maximum protein content of the venom.

The venom from 35 insects was dissolved in 0.25 ml of distilled water and applied at a single point 2.5 cm from the bottom of a 4 X 25 cm

strip of Whatman No. 1 paper. This strip was developed for six hours with 1-butanol:acetic acid:water (100 ml of 1-butanol combined with 10 ml of glacial acetic acid and saturated with water) and dried in a stream of air. The chromatogram was then stained with Amidoschwartz 10B which revealed that the venom contains at least four different proteins. Three of the proteins showed R_f-values of 0.039, 0.080 and 0.113 while the bulk of this fraction remained at the origin.

An attempt was made to estimate the protein content of the venom from its optical density at 280 mμ. Approximately 4 mg of dried venom was dissolved in 5 ml of distilled water and filtered through a 0.22 μ Millipore membrane filter (Millipore Filter Corp., Bedford, Mass.). A spectrum of this solution was obtained on the Beckman DK-2 recording spectrophotometer. Strong absorption (O.D.=0.96) at 270 mμ by other venom constituents unfortunately obscured the protein spectrum.

A reliable estimate of the protein content was finally obtained by a gel-filtration fractionation of the venom. The various fractions were determined gravimetrically on a Mettler M5 micro balance. Approximately 3 gm of Sephadex G-25 medium (Pharmacia, Uppsala, Sweden) was allowed to swell for 8 hours in 20 ml of triple distilled water, and then it was deaerated in a suction flask.

Figure 2 shows the result of a gel-filtration of 4.0 mg of mud-dauber venom on a 7.9 ml column (1 X 10 cm) of dextran gel. The venom had been dissolved in 0.2 ml triple distilled water, and after applying the

solution to the column, elution was performed with water to facilitate the gravimetric analysis. The rate of elution was about 6 ml/hr and 0.275 ml (5 drops) fractions were collected in previously weighed 5 ml lyophilyzation bottles. Each fraction was then lyophilyzed and reweighed.

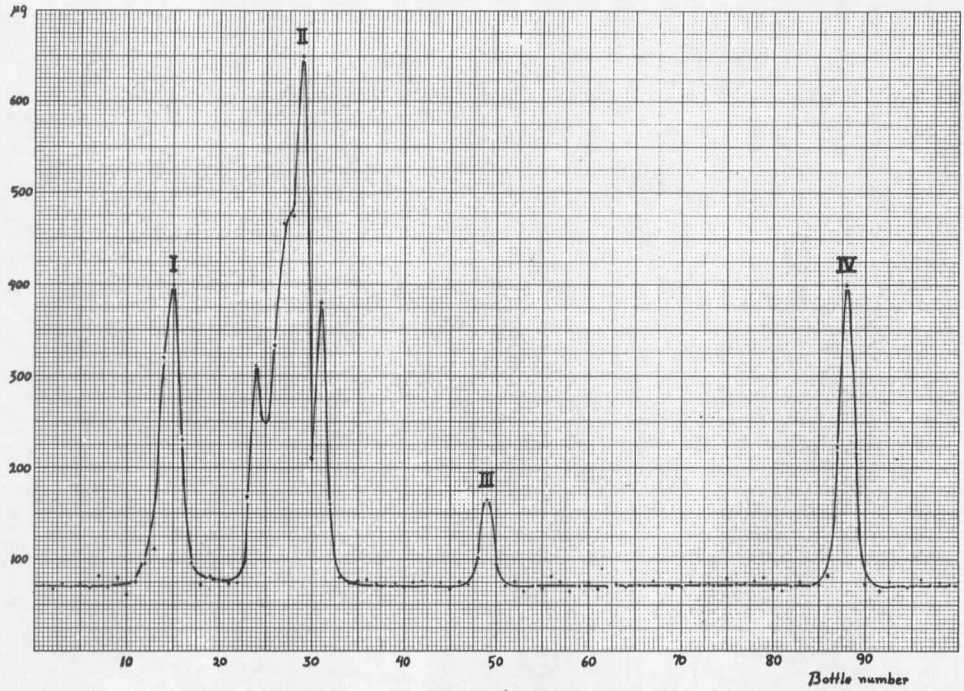

Figure 2. Gel-filtration of 4.0 mg mud-dauber wasp venom on a 7.9 ml column of dextran gel. The water regain of the gel was about 2.5 g/g. Venom was dissolved and eluted with triple distilled water. Fraction volumes 0.275 ml (5 drops). Weight of fraction (ordinate) in micrograms.

The venom was fractionated into four different bands, and a total of 4.5 mg were recovered (an error of 12%). Band I weighed 0.98 mg (25 ± 3% of the dried venom) and was Amidoschwartz 10B-positive and ninhydrin-negative. Bands II, III and IV were all ninhydrin-positive

(Amidoschwartz 10B-negative) and weighed 2.73, 0.16 and 0.64 mg respectively. Paper chromatography of the latter bands on Whatman No. 1 paper with the 1-butanol:acetic acid:water solvent mentioned above revealed a total of at least 12 different ninhydrin-positive constituents. Band II constituents showed R_f-values of 0.36 (methionine), 0.27, 0.20, 0.18, 0.12, 0.085, 0.038, 0.020, and zero; Band III: 0.058 and 0.030; and Band IV: 0.050.

Samples of pure venom protein (Band I), pure venom and a venom sac extract were separated by the disc electrophoresis method of Ornstein and Davis (39). Figure 3 shows disc electropherograms of Band I (190 µg)(No. 1), pure venom (580 µg)(No.2), and venom sac (188 µg) (No. 3). Each sample was applied in 5 µL of physiological saline, and 0.15 ml of "concentrated" large pore solution (3.3% acrylamide) was layered on top. Photopolymerization was allowed to proceed for 20 minutes. Electrophoresis was performed for one hour at 4 ma per tube using a Gelman D.C. Electrophoresis Power Supply, Model 38200 (Gelman Instrument Co., Ann Arbor, Mich.) and a glycine:2-amino-2-(hydroxymethyl)-1, 3-propanediol (Eastman 4833)(Tris) buffer (pH = 8.3; ionic strength= 0.042). The effective or "running pH" of this buffer is 9.5.

Disc electropherogram No. 2 was assumed to show the pattern and number of the active proteins, since all possible precautions for preventing denaturation were observed in handling this pure venom sample. No. 2 exhibited five protein "discs" with R_x-values (relative to the

bromphenol blue band) of 0.26, 0.36, 0.78, 0.84, and 1.00 The pattern of No. 1 compares quite favorably to that of No. 2 showning R_x-values of 0.24, 0.35, 0.48, 0.81, and 1.00. Considering that the proteins from Band I were dissolved in distilled water at room temperature, they exhibit a remarkable resistance to denaturation; only one of the five proteins from Band I possesses a R_x-value significantly different from its

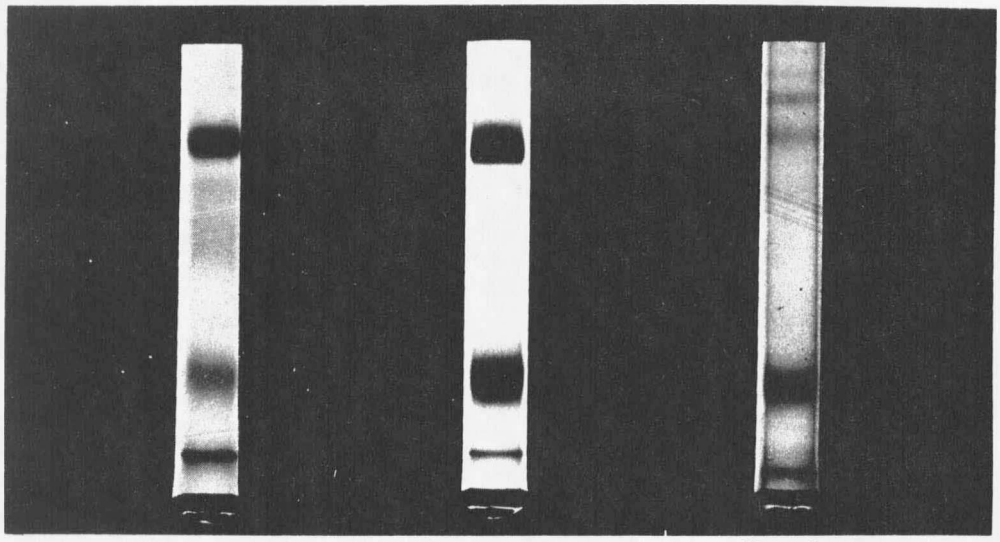

Figure 3. Disc electropherograms of venom and venom sac proteins. No. 1: Band I; No. 2: Pure venom; No. 3: Venom sacs. Photographed under water with overhead lighting.

pure venom counterpart. The relatively weak concentration of protein in the venom sac disc electropherogram (No. 3) made a comparison with No. 2 rather difficult. However, 7 discs are apparent in No. 3 with R_x-values of 0.08, 0.13, 0.23, 0.33, 0.82, 0.85, and 1.00. Thus all of the venom proteins are observed in No. 3 despite the low concentration

so that at least two "extra" discs or proteins (those possessing R_x-values of 0.08 and 0.13) are present in the non-venom portion of the venom sac.

The antigenic character of the mud-dauber venom proteins was examined by the Ouchterlony technique of immunodiffusion (40). A petri dish (6 cm in diameter) was filled with 4 ml of a 1% aqueous solution of agarose (41), the agarose was allowed to gel, and holes were cut in the gel with a Feinberg gel cutter (Consolidated Laboratories, Inc., Chicago). This cutter has a center well of 9 mm diameter and 7 mm outer wells spaced at 90° and 15 mm from the center. The center well was filled with Polistes apachus (identified by Dr. K. V. Krombein) venom sac rabbit antiserum prepared from the sac antigens [method of Loveless (5)]. All antisera were prepared by injection of a phospate buffer (5) solution of the antigen with full Freund's adjuvant into female rabbits (in triplicate). "Booster" injections were made two weeks following the initial injection. Antisera used were collected two weeks following the "booster" injection. The outer wells were charged with mud-dauber venom (150 µg in 0.15 ml), venom sacs from P. apachus (4 sacs in 0.76 ml), mud-dauber venom sacs (4 sacs in 0.10 ml), and Band I (39 µg in 1.10 ml). Each of these samples was dissolved in a phosphate buffer system (5). After 120 hours at 22°C none of the mud-dauber proteins showed evidence of a precipitin reaction with the wasp antiserum, although five precipitin lines had developed between this antiserum and the P. apachus venom sac antigens. The center well of a second immunodiffusion plate

was charged with a phosphate buffer (5) solution of mud-dauber venom (175 µg in 0.15 ml.). The outer wells were filled with P. apachus (wasp), Vespula pennsylvanica (yellow jacket), Vespula arenaria (yellow hornet), and Apis mellifera (honeybee) venom rabbit antisera. (All insects were identified by Dr. K. V. Krombein.) After 120 hours at 22°C no evidence of a precipitin reaction between the mud-dauber venom proteins and the various antisera was observed. This evidence indicates that S. caementarium venom contains no antigenic proteins in common with the venoms of the various hymenoptera referred to above.

Fraction I

The venom was separated into four major fractions by extraction with solvents of varying polarity. Fraction I was obtained by extraction with purified chloroform; Fraction II with chloroform:95% methanol (50/50 v/v); Fraction III with 95% methanol; and Fraction IV with water. The extractions were performed in that order at room temperature on approximately 4 mg of venom and with 3 ml of solvent in a 10 ml beaker stirred magnetically. After one hour the solution was filtered through Whatman No. 1 paper (3 cm in diameter) into a weighed 1 dram vial and dried in a stream of nitrogen. This extraction procedure was repeated twice. The filter paper was then cut into small pieces and placed in the beaker for extraction with the next solvent. Extraction with this series of solvents in a micro-Soxhlet extraction apparatus (Whatman 19 x 50 mm thimbles) gave similar separations except that the non-protein constituents of

Fraction IV were apparently heat labile.

The limited quantity of pure venom available (less than 20 mg were collected during this research) necessitated the use of mud-dauber venom sacs as a crude source for the isolation and purification of the various venom constituents. In order to decrease the contamination due to various fatty materials the water solubles were first extracted from 1900 venom sacs, glands and sting apparatuses. The venom apparatuses were ground to a fine powder with an agate mortar and pestle, extracted with 5 ml of water at room temperature and the resulting solution was filtered through Whatman No. 1 filter paper. This extraction procedure was repeated twice and the filtrate was then taken to dryness with a stream of nitrogen. The dried residue was subjected to the previously described series of solvent extractions at room temperature to provide crude fractions IS, IIS, IIIS, and IVS. These latter contained the pure venom fractions I, II, III, and IV respectively.

Fraction I comprises 6 ± 1% of the pure dried venom (average of two trials) and is composed of a single compound as shown by paper and thin-layer chromatography. Fraction IS of the venom apparatuses also contains only one major component which was shown to be identical with the former by thin-layer chromatography and cochromatography. Approximately 10 µg of fractions I and IS was applied to a silica gel G thin-layer plate at single points 3 cm from the bottom and developed to a height of 10 cm with chloroform:95% methanol:water (80:20:1 v/v). The plate was sprayed

with a 0.05% solution of Rhodamine 6G in 95% ethanol, which revealed single spots for both I and IS (R_f-values =0.52) while the plate was still "wet". These spots disappeared when the plate was dried and did not fluoresce under "long-wavelength" ultraviolet light. When subjected to iodine vapor the plate remained blank except for a small amount of color which appeared near the origin of the IS sample. Spraying the plate with 40% sulfuric acid (followed by heating at 130°C for 15 minutes) also produced a negative reaction. A similar plate was prepared and developed with abs. ethanol:water (63:37 v/v) and sprayed with the above reagents. Again only one spot appeared which had not moved from the origin. Thin-layer cochromatography of fractions I and IS on silica gel and developed with the chloroform:95% methanol:water system showed only a single spot (R_f-value =0.50) with the detection methods described previously. These data and the waxy appearance of this compound suggested a fatty material, such as lecithin.

A sample of β,γ-dipalmitoyl-L,α-lecithin (Mann Research Laboratories, Inc., New York) when developed with the chloroform:95% methanol:water solvent on silica gen G and sprayed with the same series of reagents showed four different spots. Three of these spots with R_f-values of 0.73, 0.57, and zero were made visible only by the iodine vapor. The fourth spot (R_f-value =0.51) was visible before the Rhodamine 6G spray had dried and did not fluoresce under ultraviolet light when dry. This spot was not positive to iodine vapor but did appear as a white spot when heated

at 130°C with 40% sulfuric acid. Vogel et al. (42) report an R_f-value of 0.50 for dipalmitoyl-L,α-lecithin in this same solvent system on silica gel G. Fraction IS and a 1.5 mg sample of lecithin were applied to separate silica gel G plates along a 17 cm line 3 cm from the bottom of the plate and developed with the chloroform:methanol:water solvent to a height of 10 cm. The silica gel between the line 4.8 and 5.5 cm from the origin and parallel to it was removed from each plate and extracted with purified chloroform in the micro-Soxhlet apparatus for two hours. Each chloroform solution was then evaporated to dryness in a stream of nitrogen. These purified samples were used to obtain the infra-red and NMR spectra.

Figure 4 represents the infra-red spectrum of fraction I (2% in carbon tetrachloride) obtained with a Beckman IR4 spectrophotometer in a micro-cell with a 2 mm path length. Figure 5 is the spectrum of the purified lecithin sample recorded under identical conditions. Compensation for the solvent was difficult to achieve due to the low solubility of these compounds in carbon tetrachloride. The frequencies of the absorption bands in these two spectra are almost identical, although the shapes or intensities of these bands do vary to some extent in the "fingerprint" region. For example, the absorption band associated with hydrogen-bonded phosphorous-oxygen bonds (P=O) occurs at 1190 cm^{-1} in fraction I and at 1205 cm^{-1} in lecithin.

NMR comparison spectra of fraction IS and lecithin were run in

deuterochloroform, however, a complete NMR spectrum of the purified fraction IS could not be obtained due to the small quantity (approximately 500 μg) of this compound available. However, a band with a chemical shift of 1.33 ppm [Ppm or δ denotes the field-independent scale of chemical shifts defined as

$$\delta(\text{ppm}) = (\nu - \nu_{SiMe_4})/60$$

where ν_{SiMe_4}, the frequency for the reference compound (tetramethylsilane), is arbitrarily taken as zero and ν is the frequency of the sample compound on the same scale] and associated with the methylene hydrogen of a long chain (dipalmitoyl methylene in the case of lecithin) appeared as the only absorption band visible in a spectrum of fraction I. These spectra were recorded in a micro-cell on a Varian A-60 NMR spectrophotometer.

Approximately 20 μg each of fraction I, the purified fraction IS and the purified lecithin sample were chromatographed on Whatman No. 1 paper with water-saturated 1-butanol. The chromatograph was treated with aqueous 0.005 M phosphomolybdic acid (35) and dark-blue spots characteristic of choline lipids appeared. The R_f-value of fraction I and IS was 0.67 while that of lecithin was 0.70. Another pair of chromatograms prepared in the same manner were sprayed with modified Hanes-Isherwood Reagent (35), heated at 85°C for 7 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.

The nitrogen content of fraction IS as determined by the submicro-

method of Belcher et al.(43) was 2.1%. The percentage nitrogen calculated for dipalmitoyl-L,α-lecithin is 1.9%

All of these data, R_f-values, infra-red and NMR spectra, positive reactions to specific reagents and a favorable nitrogen content are consistent with the assignment of a lecithin-like structure to fraction I of the pure S. caementarium venom.

Fraction II

Fraction II accounts for 40 ± 1% of the pure venom (average of two trials) and is composed of three compounds as shown by thin-layer chromatography. Chromatography on Whatman No. 1 paper with the 1-butanol: acetic acid:water solvent system revealed three ninhydrin-positive spots with R_f-values of 0.055, 0.16, and 0.23. These constituents will be referred to as IIa, IIb, and IIc in the following pages.

Silica gel thin-layer chromatography of fraction II with abs. ethanol:water (63:37 v/v) and detection with Rhodamine 6G, iodine vapor, and 40% sulfuric acid respectively revealed only one spot, which had not moved from the origin. Thin-layer chromatography with chloroform:95% methanol:water (80:20:1 v/v) using the same detection procedures showed three spots with R_f-values of 0.78, 0.61, and 0.51.

Extraction of the water solubles (less fraction IS) from 1900 venom sacs, glands and sting apparatuses with chloroform:95% methanol (50:50 v/v) yielded a total of nine ninhydrin-positive constituents. Paper chromatography with the 1-butanol:acetic acid:water system revealed six

ninhydrin-positive constituents with R_f-values of 0.50, 0.44, 0.33, 0.20, 0.18, and 0.83, which were not present in the pure venom.

Cellulose column chromatography of crude fraction IIS was used to partially purify the three venom constituents. Approximately 30 gms of Whatman cellulose powder, standard grade, was allowed to stand for six hours in the eluent (the 1-butanol:acetic acid:water system used for paper chromatography). The cellulose slurry was packed into a 113 ml column (1.1 X 100 cm) and eluted with 500 ml of solvent. Fraction IIS (49 mg) was applied to the column in 0.5 ml of distilled water. The rate of elution was about 10 ml/hr and 5 ml fractions were collected to a total of 250 ml. Approximately 0.1 ml of each fraction from the column was chromatographed on Whatman No. 1 paper and developed with the 1-butanol:acetic acid:water solvent to locate the positions of the various venom constituents. IIcS, IIbS and IIaS were concentrated in tubes 16 to 30, 27 to 35 and 31 to 40 respectively. Tubes 16 to 27, 28 to 33 and 34 to 40 respectively were combined and the volumes of each of these major fractions reduced on a rotary exaporator to 0.5 ml. Each of these fractions was then applied to a 23 X 50 cm sheet of Whatman No. 3MM paper along a 20 cm line 3 cm from the short edge and parallel to it. Each sheet was developed with the 1-butanol:acetic acid:water solvent for 8 hours. A small strip was cut from the long edge of each sheet and sprayed with a solution of ninhydrin to locate the positions of IIaS, IIbS and IIcS. A strip about 1 cm wide, parallel to the origin,

and containing the desired fraction was cut from the sheet and extracted in the micro-Soxhlet apparatus for two hours. Paper chromatography showed that each of these fractions was still contaminated with a small amount of the compound adjacent to it on the paper. The preparative paper chromatographic procedure was repeated with the result that paper chromatography of 10 μg portions of IIaS, IIbS and IIcS using the 1-butanol:acetic acid:water solvent revealed no accompanying ninhydrin-positive spots. The same results were obtained with thin-layer chromatography using the chloroform:95% methanol:water (80:20:1 v/v) solvent and detection with Rhodamine 6G, iodine vapor and 40% sulfuric acid.

Constituent IIaS was obtained in a yield of 0.75 mg (21% of fraction II or 8 ± 1% of the dried venom) and when free from contaminants possesed an R_f-value of 0.042 on Whatman No. 1 paper developed with the 1-butanol:acetic acid:water solvent. Paper cochromatography of fraction II and constituent IIaS with the same solvent system and detection with ninhydrin revealed no new spots. Silica gel thin-layer chromatography with the 1-butanol:acetic acid:water solvent and ninhydrin detection revealed R_f-values of 0.025 for constituent IIaS and 0.023 for IIa. R_f-values and paper cochromatography indicate that constituent IIaS is identical with IIa.

Constituent IIaS gave a positive reaction to the alkaline m-dinitrobenzene steroid reagent (35) and for all practical purposes contained no nitrogen as determined by the submicro-method of Belcher et al. (43).

The positive ninhydrin reaction does not necessarily indicate the presence of nitrogen (44).

The infra-red spectrum of IIaS (Figure 6) is difficult to interpret due to the intensity and broadness of the band at 1600 cm^{-1}. However, this band (asymmetric stretching vibration) and the presence of a band at 1420 cm^{-1} (symmetrical stretching vibration) indicates the presence of a carboxylate ion. The absorption band at 3350 cm^{-1} indicates the presence of a hydroxyl group and is not due to a free carboxyl group since the characteristic absorption band near 1700 cm^{-1} due to the C=O stretching vibration in -COOH is absent. This spectrum is not inconsistent with the rather tenuous assignment of a steroid-like structure to constituent IIa.

Constituent IIbS with a weight of 1.4 mg (38% of fraction II or 15 ± 1% of the dried venom) had an R_f-value of 0.085 with the 1-butanol: acetic acid:water system on Whatman No. 1 paper. Paper cochromatography of fraction II and constituent IIbS with the same solvent system and detection with ninhydrin showed no new spots. Silica gel thin-layer chromatography, again with the same solvent and detection method, revealed R_f-values of 0.035 for constituent IIbS and 0.040 for IIb. This evidence confirms the identity of IIbS and IIb.

Like constituent IIaS, IIbS gave a positive reaction to the steroid reagent (35) and probably contains no nitrogen (43).

The infra-red spectrum of constituent IIbS (Figure 7) is quite similar to that of IIaS except in the "fingerprint" region and the more intense absorption band at 1730 cm^{-1} in the IIbS spectrum suggests a higher degree of unsaturation for the latter. These data are also consistent with the assignment of a steroid-like structure to constituent IIbS.

Constituent IIcS totaled 1.6 mg (41% of fraction II or 17 ± 1% of the dried venom) and had an R_f-value of 0.200 with the 1-butanol:acetic acid:water solvent on paper and 0.065 on silica gel (R_f-value of IIc on silica gel was 0.069). The identity of constituents IIcS and IIc was demonstrated in the manner previously described for IIaS and IIbS.

The percentage nitrogen of constituent IIcS was negligible (less than allowable experimental error) (43). This compound did not give a positive reaction to alkaline m-dinitrobenzene nor to any of the specific "nitrogen" reagents, such as Ehrlich's, Pauley's or Dragendorff's reagent (35), except ninhydrin.

The infra-red spectrum of IIcS (Figure 8) indicates the presence of hydroxyl groups (3300 cm^{-1}) and carboxylate ion (1600 and 1470 cm^{-1}).

Fraction III

Fraction III comprises 20 ± 1% of the dried venom (average of two trials) and is composed of four ninhydrin-positive compounds as shown by thin-layer and paper chromatography. Chromatography on Whatman No. 1 paper with the 1-butanol:acetic acid:water solvent system revealed four

ninhydrin-positive spots with R_f-values of 0.064 (IIIa), 0.096 (IIIb), 0.160 (IIIc), and 0.250 (IIId).

Thin-layer chromatography of fraction III with abs. ethanol:water (63:37 v/v) and detection with Rhodamine 6G, iodine vapor, and 40% sulfuric acid respectively showed only four spots with R_f-values of 0.020, 0.080, 0.180, and 0.530. Thin-layer chromatography on Kieselgur G with the chloroform:95% methanol:water (80:20:1 v/v) solvent system using the same detection procedures again revealed only four components with R_f-values of zero, 0.17, 0.54, and 0.86.

The venom apparatus water solubles (less fractions IS and IIS) were extracted with 95% methanol and a total of eight ninhydrin-positive constituents were obtained. The four ninhydrin-positive non-venom constituents possessed R_f-values of 0.032, 0.335, 0.440, and 0.513 on Whatman No. 1 paper with the 1-butanol:acetic acid:water solvent.

This crude fraction (IIIS) was subjected to the cellulose column chromatography described previously in order to partially purify the four venom constituents. Fraction IIIS (43 mg) was applied in 0.5 ml of distilled water. The rate of elution was about 10 ml/hr and 1 ml fractions were collected to a total of 250 ml. Collection of 1 ml fractions effected a better separation than was obtained in the column chromatography of fraction IIS. A small portion of each fraction from the column was chromatographed on Whatman No. 1 paper and developed with the 1-butanol:acetic acid:water solvent to locate the positions

of the various constituents. IIIdS, IIIcS, IIIbS, and IIIaS were concentrated in tubes 95 to 148, 130 to 180, 145 to 190, and 158 to 205 respectively. Tubes 95 to 135, 136 to 160, 161 to 175, and 176 to 205 respectively were combined and the volumes of each of these major fractions reduced on a rotory exaporator to 0.5 ml. The preparative paper chromatographic procedure described previously was applied twice to each of the major fractions from the cellulose column. Paper chromatography of 10 µg portions of the purified IIIaS, IIIbS, IIIcS, and IIIdS using the 1-butanol:acetic acid:water solvent revealed no accompanying ninhydrin-positive contaminants. The same results were obtained with thin-layer chromatography using the chloroform:95% methanol:water solvent and detection with Rhodamine 6G, iodine vapor and 40% sulfuric acid.

Constituent IIIaS was obtained in a yield of 2.50 mg (20% of fraction III or 4 ± 1% of the dried venom) and when free from contaminants possessed an R_f-value of 0.035 on Whatman No. 1 paper developed with 1-butanol:acetic acid:water solvent. Paper cochromatography of fraction III and constituent IIIaS with the same solvent system revealed no new ninhydrin-positive spots. Thin-layer chromatography with the 1-butanol: acetic acid:water solvent and ninhydrin detection showed R_f-values of 0.025 for constituent IIIaS and 0.022 for IIIa. R_f-values and paper cochromatography indicate that constituent IIIaS is identical with IIIa.

Constituent IIIaS shows negative reactions to Pauley's, Ehrlich's, and Dragendorff's reagents (35) and a negligible nitrogen content (43).

The infra-red spectrum of IIIaS (Figure 9) is of little value for the interpretation of structure beyond the suggestion of the presence of hydroxyl and carboxylate groups in constituent IIIaS.

Constituent IIIbS totaled 6.4 mg (51% of fraction III or 10 ± 1% of the dried venom) and had an R_f-value of 0.056 with the 1-butanol: acetic acid:water solvent on paper and 0.030 on silica gel (R_f-value of IIIb on silica gel was 0.033). The identity of constituents IIIbS and IIIb was demonstrated in the manner previously described. IIIbS also shows negative reactions to the specific "nitrogen" reagents (35) and a negligible nitrogen content (44). The infra-red spectrum of IIIbS (Figure 10) suggests only the presence of hydroxyl and carboxylate groups.

Constituent IIIcS totaled 2.3 mg (19% of fraction III or 4 ± 1% of the dried venom) and had an R_f-value of 0.150 with the 1-butanol: acetic acid: water solvent on paper and 0.045 on silica gel (R_f-value of IIIc on silica gel was 0.044). The identity of constituents IIIcS and IIIc was demonstrated in the manner previously described. IIIcS also shows negative reactions to the specific "nitrogen" reagents (35) but possesses a rather high nitrogen content (21%) (43).

The infra-red spectrum of IIIcS (Figure 11) indicates the presence of hydroxyl and/or amino or imino groups (3350 cm^{-1}) and carboxylate

ion (1580 and 1420 cm^{-1}). This spectrum and the relatively high nitrogen content is quite suggestive of an amino acid. However, the absorption band present in most amino acids (2140 to 2080 cm^{-1}) (45) is absent and the R_f-values from paper and thin-layer chromatography of constituent IIIcS do not correspond to those of any known amino acid.

Constituent IIIdS was obtained in a yield of 1.3 mg (10% of fraction III or 2 ± 1% of the dried venom) and had R_f-values of 0.250 on paper with the 1-butanol:acetic acid:water solvent and 0.080 on silica gel (R_f-value of IIId on silica gel was 0.078). The identity of constituents IIIdS and IIId was shown in the manner described previously. Negative reactions to the specific "nitrogen" reagents (35) and a low nitrogen content (1.3%) (43) were obtained.

The infra-red spectrum of IIIdS (Figure 12) is suggestive of hydroxyl and carboxylate groups.

NMR data for the constituents of fractions IIS and IIIS were not obtainable due to the small quantities available and/or to their relative insolubility in deuterochloroform, carbon tetrachloride and deuterium oxide.

Fraction IV

Fraction IV comprises 34 + 1% of the dried venom (average of two trials) and is composed of twelve different compounds. Three of these constituents are the free amino acids histidine, methionine and pipecolic acid which have already been discussed and account for a total of

2.1 ±0.3% of the pure venom. The five proteins of the venom (25 ± 3% of the dried venom) are also present in this fraction but in a denatured form due to the relatively harsh conditions of the extraction procedure.

The remaining 7 ± 4% of the pure venom consists of six different compounds as shown by paper and thin-layer chromatography. Three of these compounds are separated by paper chromatography on Whatman No. 3MM paper developed with 80% phenol and possess R_f-values of 0.19, 0.29 and 0.36 respectively. All of these constituents give weak colors with ninhydrin, fluoresce strongly under "long wavelength" ultraviolet light, and give strong positive reactions to Ehrlich's Reagent (indoles) and Dragendorff's Reagent (quaternary ammonium ions) (35) for alkaloids.

These compounds are not changed by prolonged reflux in 6 M hydrochloric acid. The R_f-values of these compounds on silica gel thin-layer chromatography with the abs. ethanol:water system are very low (0.083, 0.080, and 0.093 respectively) and the compounds will not move from the origin on Whatman No. 1 paper developed with the 1-butanol: acetic acid:water solvent. In addition, no movement from the origin could be detected on thin-layer chromatography using 1-butanol saturated with water.

Three other components of fraction IV react only with ninhydrin, possess R_f-values of 0.076, 0.131, and 0.254 on Whatman No. 1 paper with the 1-butanol:acetic acid:water solvent, but are not moved from the origin by 80% phenol. Thin-layer chromatography with abs. ethanol:

water gave these compounds R_f-values of 0.50, 0.595 and 0.700.

Thin-layer chromatography of fraction IV with abs.ethanol:water and detection with Rhodamine 6G, iodine vapor and 40% sulfuric acid revealed only six spots which had moved from the origin. With the water-saturated 1-butanol on silica gel only two spots appeared, which had not moved from the origin (R_f-values of 0.02 and 0.03 respectively) as shown by the same series of detection reagents.

Extraction of the venom apparatus water solubles (less fractions IS, IIS and IIIS) gave the crude fraction IVS. Fraction IVS contains at least three non-venom ninhydrin-positive constituents with R_f-values of 0.019, 0.041 and 0.380 on Whatman No. 1 paper developed with the 1-butanol:acetic acid:water solvent.

Because of the complexity of this fraction and the extremely small quantities of the non-protein components available no further characterization has been attempted.

Absence of Histamine, Serotonin and Acetylcholine

Since histamine, serotonin (5-hydroxytryptamine) and acetylcholine have been reported (14, 15) for hornet and common wasp venoms, attempts were made to detect them in the S. caementarium venom samples.

Acetylcholine was chromatographed on Whatman No. 1 paper using 1-butanol saturated with water. The acetylcholine, detected with phosphomolybdic acid (35) showed an R_f-value of 0.10. In 80% phenol, acetylcholine's R_f-value was 0.76 to 0.84. Chromatography of increasing

amounts of the venom repeatedly failed to reveal any spot corresponding to acetylcholine and calculations show that any amount greater than 0.01% of the venom must have been detected. Hence, acetylcholine appears to be absent in any detectable amount.

Serotonin may be detected in quantities of 1 μg or more using Ehrlich's reagent (35) or by the ninhydrin reaction. Serotonin, both alone and in admixture with the amino acids found, was chromatographed on Whatman No. 3MM paper using 80% phenol (R_f-value = 0.045) and 2,6-lutidine (55 ml)-ethanol (25 ml)-water (20 ml)-diethylamine (2 ml) (R_f-value = 0.87). Chromatographs of venom samples under identical conditions using increasing amounts of venom revealed no spot characteristic of serotonin, so that no more than 0.01% of the venom could be serotonin.

Histamine analysis was made in a manner similar to that used for serotonin, except that detection was by Pauley's Reagent (35). No spot characteristic of histamine was obtained using increasing amounts of venom, so that no histamine could be present to the extent of 0.01% or more of the dried venom.

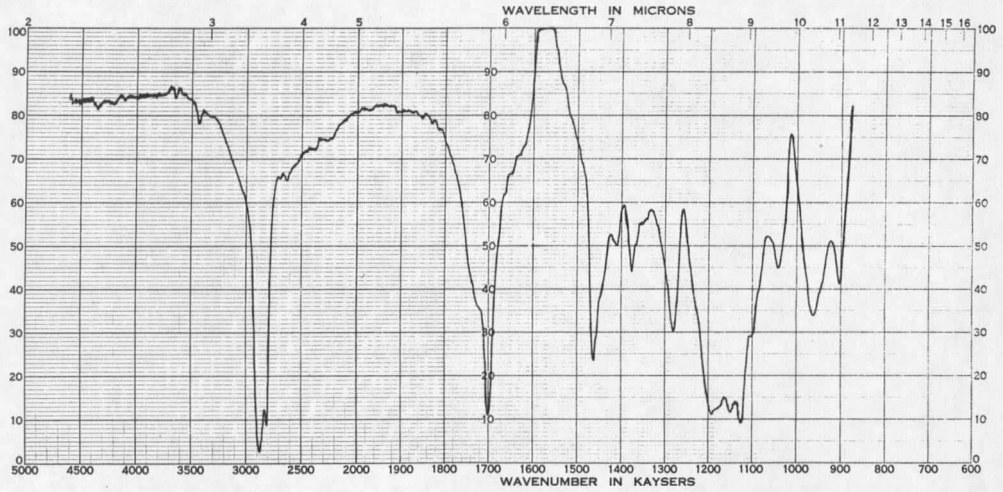

Figure 4. Fraction IS (2% w/w in carbon tetrachloride)

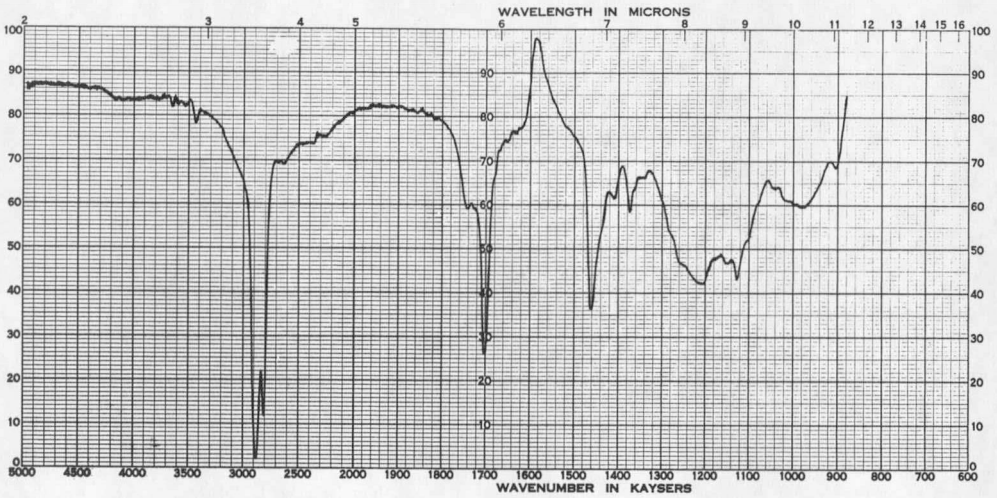

Figure 5. Dipalmitoyl-L, α-lecithin (2% w/w in carbon tetrachloride)

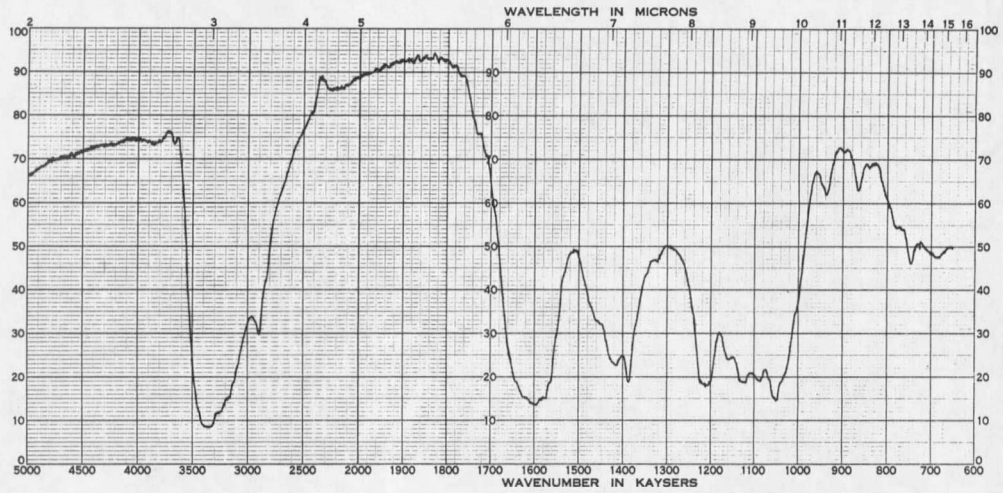

Figure 6. Constituent IIaS (micro KBr)

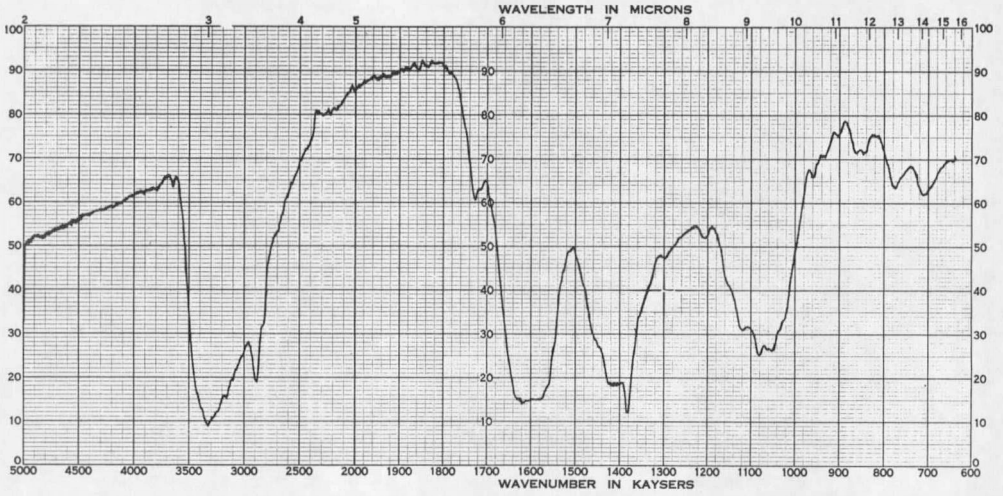

Figure 7. Constituent IIbS (micro KBr)

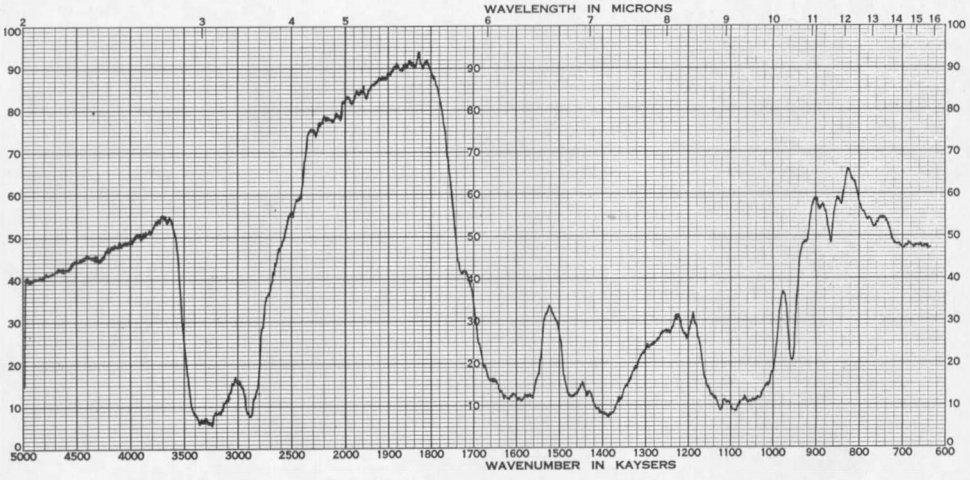

Figure 8. Constituent IIcS (micro KBr)

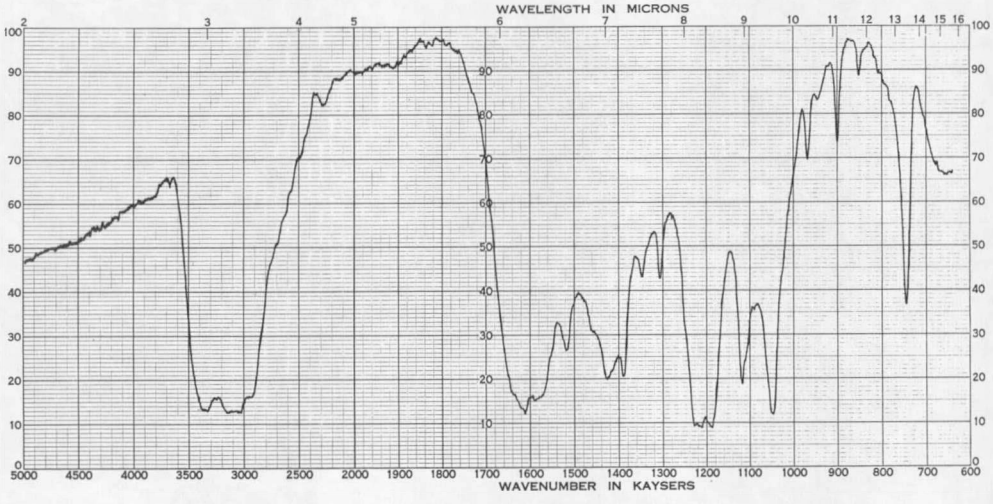

Figure 9. Constituent IIIaS (micro KBr)

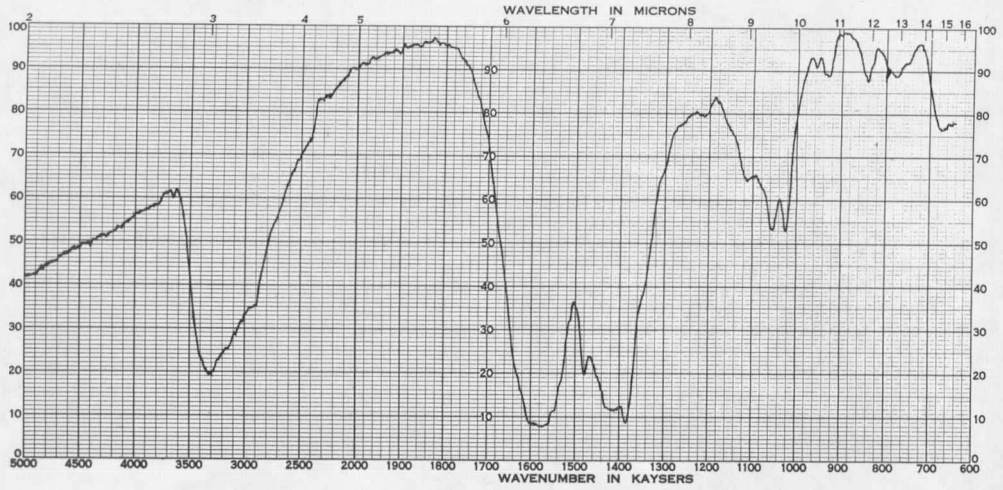

Figure 10. Constituent IIIbS (micro KBr)

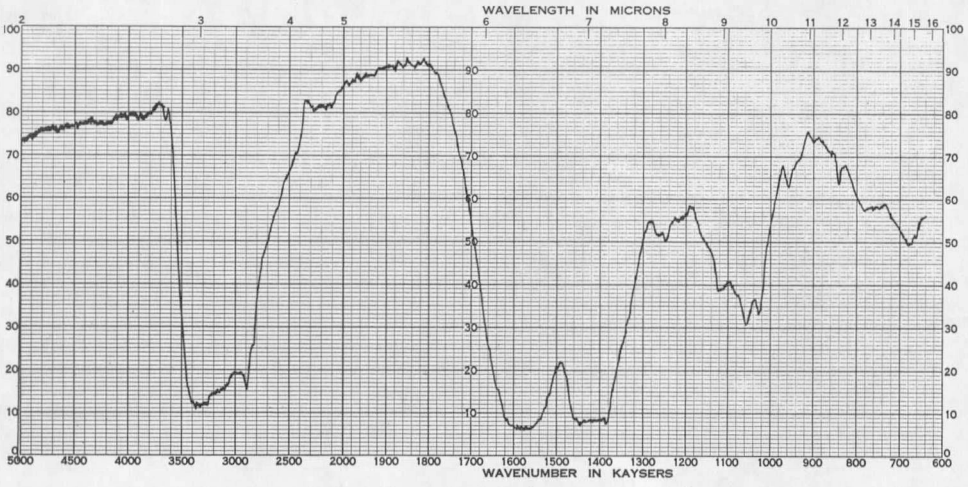

Figure 11. Constituent IIIcS (micro KBr)

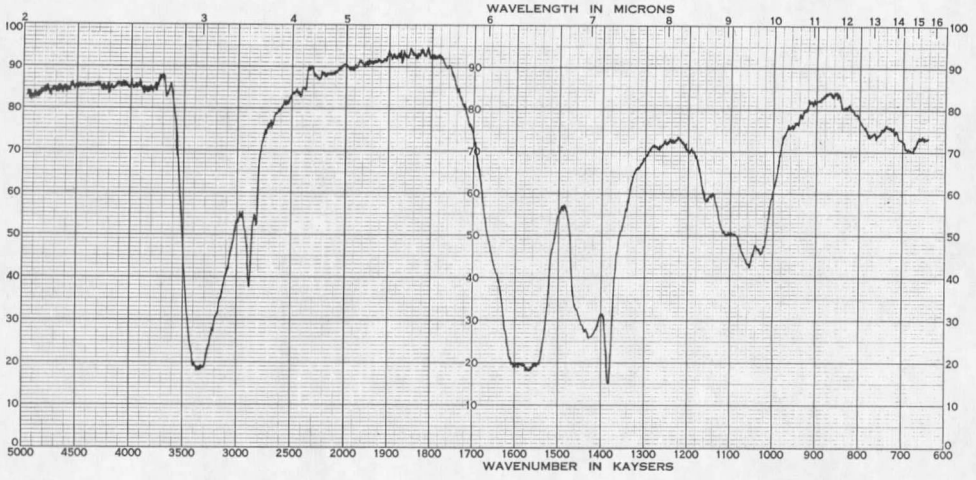

Figure 12. Constituent IIIdS (micro KBr)

SUMMARY AND CONCLUSIONS

The venom of the mud-dauber wasp (Sceliphron caementarium) has been characterized quantitatively in this investigation. Twenty-two constituents which account for 96 ± 8% of the pure dried venom were revealed by various chromatographic techniques and their percentage in the venom determined in order that no major component might be overlooked. The venom constituents are summarized in tabular form:

Fraction	Percentage	Characteristics
I	6 ± 1%	Lecithin-like substance.
IIa	8 ± 1%	Contains no N; appears to be the salt of a hydroxy acid with a steroidal character.
IIb	15 ± 1%	Similar to IIa.
IIc	17 ± 1%	Similar to IIa and IIb but apparently has no steroidal character.
IIIa	4 ± 1%	Contains no N; salt of a hydroxy acid.
IIIb	10 ± 1%	Similar to IIIa.
IIIc	4 ± 1%	21% N; possibly an unknown amino acid.
IIId	2 ± 1%	Similar to IIIa.
IVp	25 ± 3%	5 proteins.
IVaa	2%	Histidine, methionine and pipecolic acid.
IVx	7 ± 4%	6 unidentified compounds.

Eight of these constituents, which were found in solvent fractions I, II and III, account for 62 ± 7% of the venom and are definitely not polypeptide in nature. The exhaustive purification of each of these

fractions by silica gel G thin-layer chromatography must be relied upon to have detected all of the various compounds which compose each fraction. The submicro-method for the analysis of nitrogen by Belcher et. al. (44), although thoroughly tested, may not be effective in reducing all types of nitrogen. As a result, the percentage nitrogen determined for the various venom constituents throughout this investigation could be in error.

The protein fraction which comprises 25 ± 3% of the pure venom and is the major portion of solvent fraction IV was isolated by gel filtration. Inherent in this fractionation technique is the reasonable assurance that the protein material thus obtained will be free from small molecule contamination and indeed, the presence of non-protein material in this fraction was not indicated by paper chromatography. The separation of venom proteins by disc electrophoresis (39) had not been attempted previously but its application to this particular case appeared to be quite effective.

Schachter has recently suggested (46) that some 30 to 40% of Vespa vulgaris venom sac extract is composed of small peptides. Nothing of this nature was found in the mud-dauber venom and no more than 7% of this venom could conceivably be attributed to small peptides. The "alkaloid-like" constituents of fraction IV could, however, contain peptide bonds resistant to 6 M hydrochloric acid and the other three fraction IV non-protein constituents which could not be separated from

protein contaminants in quantities sufficient for investigation may possess peptide characteristics. Schachter's report of the high concentration of peptides in wasp venom sac extract emphasizes the importance of using only pure venom for investigations of this nature, for these peptides cannot be attributed to the venom itself with any degree of certainty. The water solubles of V. vulgaris amount to 29% of the venom sacs and glands (15) and those of the mud-dauber wasp to about 17% of the venom apparatuses. There is no basis for assuming that these water solubles are true components of the venom as evidenced by the discovery of 15 "non-venom" compounds in the water extract of S. caementarium venom apparatuses. Two of these "non-venom" compounds were protein in nature.

The three free amino acids histidine, methionine, and pipecolic acid account for about 2% of the venom and were quantitatively determined by a method which appears to be quite reliable in the submicro-range of amino acid analysis. The use of thin-layer chromatography for submicro-scale separations leading to a quantitative estimation had been suggested as a general procedure but had not been applied to amino acid analysis. This method has decided advantages over the more complex and time-consuming Moore and Stein method, even as modified by Stegeman and Bernhard (37). The thin-layer chromatographic method is capable of determining as little as 2 μg of an amino acid while the sensitivity of the Stegeman and Bernhard method is in the 100 μg range. In addition,

the use of micro-cuvettes in the spectrophotometric estimation of the amino acids could conceivably increase the sensitivity of the thin-layer method by a factor of ten.

This method of quantitative analysis has not been applied to amino acids other than those investigated in this thesis but a general applicability seems quite possible. Although the thin-layer chromatographic method is quite useful in analyzing simple mixtures of amino acids its application to more complex mixture, e.g. protein hydrolysates, would not always be successful for the simple reason that good resolution of 15 to 20 amino acids can not be assured with the support media and solvent systems now in use. Thin-layer chromatography of amino acids has, however, received relatively little attention and future developments may be favorable to the quantitative procedure described here.

The failure to find histamine, serotonin (5-hydroxytryptamine), acetylcholine and small peptides (kinins) in S. caementarium venom is certainly consistent with the low pain production of this wasp sting compared to that of other wasps, bees or hornets.

At least one of the compounds detected in mud-dauber venom must account for the paralytic action of the venom on spiders. Methionine and pipecolic acid have no known activity related to the apparent function of the venom. Histidine, also found in honeybee (Apis mellifera) venom (47), may be converted to the active histamine by a histidine decarboxylase present in the spider or in an inactive form in the venom itself.

Histamine is a smooth muscle contractant but has no known paralytic activity. Lecithin could be degraded to lysolecithin which is physiologically active but again has no apparent paralytic activity. The paralytic activity of this venom must lie with one or more of the partially characterized constituents.

The surprisingly large number of compounds discovered in this venom and their apparent failure to belong to any one class of organic compounds emphasizes that any future explanation of a venom's activity will most probably be based on a cooperative effect of all or most of the various venom components. The failure of past researches to investigate much beyond the protein and peptide components of a venom has placed an undue emphasis upon these venom constituents.

SUGGESTIONS FOR FUTURE RESEARCH

The complete characterization of the various venom constituents is undoubtedly worthy of further attention. If the high concentration of a particular constituent is significant of its contribution to the venom's activity, fractions IIb (15%) and IIc (17%) warrent immediate investigation both chemically and physiologically.

However, if further investigations are to be conducted on the mud-dauber wasp, whose venom content is quite low, further improvements on the venom extraction technique must be made. Quantities of the various venom constituents sufficient for structural analysis will otherwise be most difficult to obtain.

Of additional interest is the identification of the volatile components of the natural venom, since non-aqueous volatile constituents may also contribute to the venom's activity.

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