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THE PHARMACOLOGICAL EFFECTS OF HYMENOPTERA VENOMS

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> In all things there is a poison and there is nothing without poison. It only depends upon the dose whether a poison is a poison or not.
>
> Paracelsus (1)

INTRODUCTION

The Hymenoptera form one of the largest and most highly developed orders of the class Insecta. As members of the Arthropoda phylum, which contains about 85% of the known animal world, these invertebrates exhibit segmented bodies, jointed appendages, and hard exoskeletons. The female of various Hymenoptera have evolved modified ovipositors, capable of delivering a venom to adversaries. This venom is a complex mixture of biochemical compounds ranging from simple amines to complicated proteins and enzymes. Because of their painful and sometimes fatal reactions in humans, Hymenoptera venoms are of interest to the clinician and researcher.

The terms *venom* and *poison* were used interchangeably in early writings. The word *venom* is thought to have originated from the Latin *veneneum,* meaning a magical charm relating to Venus (2). A *poison* was originally a harmless draught or drink, the term probably derived from the Greek *potos* (3). From *potos* evolved the Latin *potum,* to drink, and possibly potion, potable, and even pot, a vessel from which to drink. The fatal meaning of poison came about as a result of the ancient practice of giving a lethal potion, or drink, to one's enemies.

Works of ancient alchemists and physicians (4) described the clinical effects of venoms and envenomation, but it was not until the eighteenth century that anything approaching a scientific investigation of venom was attempted. Robert Mead, an English physician, injected venon into animals and observed and recorded their effects (5). Several venoms he tested on himself, placing small quantities on the tip of his tongue and recording the effects.

During the last two centuries, many advances have been made in the fields of analytical chemistry, biochemistry, and pharmacology. Even with our sophisticated techniques of isolation, purification, and identification of biochemical compounds, several venom components remain to be isolated and identified.

CLINICAL MANIFESTATIONS OF ENVENOMATION

Envenomation by Hymenoptera is characterized by symptoms that depend upon the inflicting species, the quantity of injected venom, the degree of hypersensitivity of the victim, and, to some extent, the site of the sting. The reaction of victims to the stings of bees, wasps, hornets, or ants is usually painful, local, and brief, but occasionally is systemic and potentially fatal.

The local reaction to single stings is described by many as a sharp prick when the sting pierces the skin, followed by mild pain lasting several minutes. A small red area, surrounded by a white zone and a red flare, gradually appears around the sting site. A wheal forms, accompanied a few hours later by itching, tenderness, swelling, and generalized erythema.

More severe reactions from multiple stings are documented (6). Systemic poisoning from the venom is manifested as swollen and tender joints with accompanying urticaria, petechial hemorrhages, dizziness, weakness, nausea, diarrhea, abdominal cramps, and constriction of the chest. These symptoms are often accompanied by involuntary muscle spasm and convulsions. A man suffering over 2000 bee stings within a period of minutes displayed generalized edema, hemoglobinuria, severe hypotension, incontinence, and shock (7). The symptoms subsided 72 hours later.

The greatest threat to life posed by Hymenoptera venom is the anaphylactic reaction elicited in hypersensitive individuals by antigens in the venom. Large molecular weight substances, usually proteins, produce the life-threatening immunological reaction in sensitive individuals. The reaction is characterized by a dry, hacking cough, labored breathing, constriction of the throat and chest, flushing of the skin, edema of the glottis, and hypotension (8). Postmortem findings in fatal cases disclosed severe visceral and pulmonary congestion, cerebral edema, and laryngeal edema with obstruction. Precipitin antibodies to Hymenoptera venom were demonstrated in the serum of these individuals by Ouchterlong technique (8).

It is suspected that insects are responsible for many more deaths than are actually recorded or recognized by physicians. Many deaths attributed to shock, allergy, or laryngeal edema were, in fact, preceded by stings from Hymenoptera (9). Hymenoptera are believed responsible for 50% of the fatalities resulting from envenomation (10).

BEE VENOM

The earliest recorded investigation of venom from the honeybee, *Apis mellifera,* was conducted by two German chemists, Brandt and Ratzburg in 1837, during the infancy of chemistry and pharmacology. They observed bee venom to be bitter to the taste, soluble in water but not alcohol, and to leave a gummy residue upon evaporation (11).

The first thorough chemical examination of bee venom, from the same species, *Apis mellifera,* was undertaken in 1897 by Langer. Dropping the poison sacs together with the sting and glands into alcohol, he found the watery extract of the dried precipitate to be acidic in nature and bitter to the taste. Subcutaneous injection of venom droplets into rabbits, hand squeezed from 12,000 bees and collected in water, produced minute localized necrosis surrounded by edema and hyperemia (12).

Analyzing the alcoholic precipitate isolated from bee venom by Langer, Flury (13) detected what he believed to be various unsaturated fatty acids, choline, phosphoric acid, and other nitrogenous compounds. Pharmacological activity, which included inflammation upon injection and hemolysis of red blood cells in vitro, was confined to a nitrogen-free fraction.

Intrigued by the discoveries of Langer and Flury, Essex and co-workers (14) allowed the venom from avulsed *Apis mellifera* stinging apparatus to diffuse into a Ringer-glycerine solution, which was subsequently injected into prepared dogs. They observed a rapid fall in systemic blood pressure, while in guinea pigs, i.v. injections of the venom solution produced bronchospasms, often terminating in death. The isolated virgin guinea pig uterus showed a maximal contraction when perfused with the venom solution, while the isolated rabbit heart ceased beating. Addition of the venom solution to heparized dog blood resulted in considerable hemolysis. These effects were attributed to histamine in combination with other protoplasmic poisons.

Quantitation of the bee venom potency was attempted by Lacaillade (15) using cytolysis of *Paramecium caudatum* and hemolysis of horse erythrocytes as in vitro indices. Intradermally injected guinea pigs served as in vivo models for comparison. The degree of necrosis in guinea pigs was correlated with cytolysis of *Paramecium* and hemolysis of erythrocytes. Dilutions of 1:48,000 were effective in both systems. Bees were found to contain approximately 0.3 mg of venom, composed of an inflammatory substance, histamine, proteases, and surface-active components. Other investigators suggested that histamine, acting together with protease and other "intestinally active" principles, were responsible for the clinical symptoms of bee sting (16).

In an attempt to characterize the symptomatology of bee venom poisoning, Feldberg & Kellaway (17) attributed the hypotension observed in intact dogs, after i.v. administration of venom, to histamine released from tissues by lysolecithin. Subsequent experiments involving isolated organs of dogs and guinea pigs perfused with bee venom in a physiological solution showed that the venom depleted 40–75% of their endogenous histamine stores. Bee venom was also shown to release epineph-

rine from the isolated adrenals of rabbits, an action attributed to the lytic action of lysolecithin produced by the venom's lecithinase (18).

When whole venom was applied to the isolated guinea pig jejunum, a complex contraction composed of a fast and a slow component was observed (17). The rapid contraction was attributed to histamine, while the prolonged, slow contraction and the altered reactivity to histamine after several venom exposures was attributed to some unknown substance.

Formic acid extracts of the venom were found to contain nitrogen, sulfur, and phosphorus, while whole venom had an isoelectric point of pH 8.7 (19). The existence of pharmacologically active peptides in bee venom was becoming evident. Further purification of the formic acid extract with ethanol allowed the isolation of two components after extensive dialysis (20). The dialyzable component, 40% by weight of the crude venom, produced severe convulsions and death in mice receiving i.v. injections. The nondialyzable portion of the venom produced death in mice by respiratory paralysis, with no evidence of convulsion.

Investigating the possibility that neuromuscular blockade produced the respiratory paralysis previously observed in mice, Hofmann (21) added diluted (1:1000) bee venom to isolated rat phrenic nerve-diaphragm preparations and observed a contraction followed by partial relaxation. The block appeared to be more of the decamethonium- than of the curare-type as it was resistant to physostigmine and was accompanied by contracture. It differed from decamethonium block by lack of reversibility. Nerve conduction appeared unaffected, even by strongly contracting doses of bee venom. After prolonged contact with venom (2 hr) the muscles became unresponsive to direct electrical stimulation.

In 1953, Fischer & Neumann (22) separated the picric acid precipitate of bee venom into two fractions by paper chromatography, and found that each fraction possessed hemolytic activity. Fraction I, devoid of any S-amino acids, hemolyzed serum-free, washed erythrocytes. This direct lytic factor differed from the component of Fraction II, which required serum or lecithin for lysis of erythrocytes. Further investigation of the indirect lytic factor showed it to be phospholipase A, the lecithinase first detected by Feldberg & Kellaway in 1937 (23).

Research on venoms intensified and with the advent of new biochemical techniques, more effective separation of these highly complex substances was realized. Neumann & Habermann (24) separated whole bee venom by paper electrophoresis into five cathodal fractions: histamine, F0, FI, FII, and K. Pharmacological analysis of these fractions showed FO devoid of any biological activity, while FI, designated melittin, hemolyzed serum-free, washed erythrocytes, lowered surface tension at air-water interfaces, produced pain and inflammation on injection, and increased the permeability of skin capillaries for i.v.-administered Evans blue (25). Animals receiving i.v. melittin injections displayed a profound drop in arterial blood pressure, while isolated preparations of frog abdominus rectus muscle and guinea pig ileum responded to melittin with strong contractions. The isolated rat phrenic nerve-diaphragm showed a transient excitation followed by blockade subsequent to melittin, an effect identical with that observed earlier by Hofmann (21) with whole bee venom.

Melittin was later isolated and purified by gel filtration on Sephadex ® G-50 and chromatography on carboxymethyl cellulose (26, 27). The physiochemical properties of melittin (strong basicity, high surface activity, and absorbability to organic constituents) could be manifestations of the peptide's primary structure. Amino acid analysis revealed 26 amino acids, with a calculated molecular weight of 2840 (28). Enzyme degradation of the linear peptide by trypsin, chymotrypsin, and pepsin, as well as partial Edman degradation, revealed that amino acids in positions 1–20 were principally neutral and hydrophobic, while positions 21–26 contained basic, hydrophilic amino acids (29). Convenient associations between hydrophobic portions of the molecule and acyl chains of membrane phospholipids may be predicted, making melittin a "structural" poison (30). The broad spectrum of pharmacological activities manifested by melittin undoubtedly results from its general attack on cellular membranes.

Melittin was found to be highly toxic to *Drosophila melanogaster* larvae, manifested by violent muscle contractions occurring in the vicinity of the injection site (31). Chronic reactions to melittin, injected in the larvae's anterior three segments, included slowing of growth, lethargy, anorexia, and loss of weight. It was suggested that these effects were due to the peptide's interaction with the excitable membrane of the brain, ganglia, and ring gland, resulting in an alteration of the membrane's permeability to ions. More rigorous fractionation of melittin disclosed another peptide, minimine, found to be responsible for the lethargy and anorexia in larvae (32). Metamorphosis of treated larvae yielded miniature adult flies, reduced in size as much as 75%, of controls. They were, however, normal with respect to feeding, mobility, and production of progeny, who were of normal size.

Intra-arterial administration of melittin to cats produced a profound depression of recorded EEG activity (33). This EEG depression was followed shortly by an elevation of blood pressure and the cessation of respiration. Later, blood pressure dropped to shock levels.

Cytotoxicity of melittin on cultured cells has been observed. These include the reduction of DNA, RNA, and protein synthesis in ascites cells (34); inhibition of colony-forming ability of mouse bone-marrow cells (35); and antibacterial action against *Staphylococcus aureus* (36).

The gel filtration technique used by Fischer, Neumann, Habermann, and Reiz to separate and purify melittin also permitted the resolution of the F0 fraction into three distinct but unidentified proteins. In addition, a small basic peptide, named apamine, was eluted between histamine and melittin on the Sephadex column. This peptide was previously concealed in the FI fraction because of similar electrophoretic motility and low content relative to melittin. Unlike melittin, the pharmacological effects of apamine were rather specific and were confined primarily to the central nervous system. Mice injected with apamine exhibited long-lasting central nervous system excitation characterized by uncoordinated, uninterupted movements, culminating in generalized convulsion (37–39). Death ensued from lack of coordinated respirations.

Cats, transected at various spinal levels and injected with apamine, showed an increase in amplitude of recorded reflex potentials as compared to controls (40). This

suggested that apamine augments polysynaptic reflexes and increases the effectiveness of excitatory polysynaptic pathways over inhibitory pathways.

Biochemical analysis of apamine showed it to contain 18 amino acids, with four half-cysteine residues and no aromatic amino acids (41). Trypsin and chymotrypsin-digested fragments were examined and the amino acid sequence determined (42). The peptide has a calculated molecular weight of 2036.

The cathodal fraction FII of Neumann & Habermann (24) was found to contain the enzymes phospholipase A (PLA) and hyaluronidase. These enzymes were shown to be, in large part, responsible for the anaphylactic reaction to bee venom (43).

Purified PLA was examined for pharmacological activity (44, 45) and found to have an i.v. LD_{50} in mice of 10–20 mg/kg. Death occurred from convulsions following respiratory paralysis. Anesthetized cats receiving i.v. PLA responded with a fall in systemic arterial pressure. The isolated guinea pig ileum contracted upon exposure to PLA, but showed marked tachyphylaxis to subsequent doses. The isolated frog heart and rectus abdominus were not affected by PLA. Washed guinea pig erythrocytes, but not those from humans, were hemolyzed when incubated with PLA.

Dramatic cardiovascular changes were observed in anesthetized dogs receiving PLA intravenously (46). The enzyme produced ventricular fibrillation, increased the central venous pressure, left atrial pressure, systemic vascular resistance, and ventricular stroke work within five minutes of administration. Death followed within ten minutes. Under similar experimental conditions, Slotta and co-workers (47) found that 1 mg/kg PLA introduced intravenously in dogs produced a sharp fall in arterial pressure accompanied by a marked rise in central venous pressure. Simultaneously, a short period of apnea and profound bradycardia ensued, accompanied by complete loss of cortical EEG activity.

The activity of PLA is attributed to its reaction products, lysophosphatide and free fatty acid, produced from PLA's hydrolysis of 2-acyl bonds of natural phosphatidyl choline, ethanolamine, and serine. The surfactant character of the formed lysophospholipids is thought to result from the combination between the hydrophobic aliphatic acyl chain and the hydrophilic phosphorylated base (30). Such a molecule is capable of solubilizing tissue and disrupting membrane structures, resulting in nonspecific permeability changes. PLA constitutes a significant percentage (approximately 12%) of dried bee venom, and it is reasonable to assume that a part of bee sting symptomatology results from pharmacologically active substances liberated through nonspecific membrane damage by lysolecithin (30, 48).

It was demonstrated earlier (17) that bee venom exerted some type of histamine-releasing activity on animal tissues. PLA and later melittin were thought to be the agents in bee venom responsible for the release of histamine and the resulting physiological consequences (48). Histamine released through lysolecithin-mediated membrane destruction required no energy, as demonstrated in vitro by the failure of dinitrophenol (DNP) or the withdrawal of glucose to influence PLAs releasing action. No degranulation of mast cells was observed after PLA treatment. While degranulation of mast cells did occur after melittin treatment, histamine liberation was not affected by DNP or glucose-free medium (48).

Since the physiological release of histamine from mast cells is thought to proceed by activation of an energy-requiring degranulation process (49), the existence of a third histamine-releasing factor in bee venom was suspected. Fredholm & Haegermark (50), working with bee venom fraction obtained by gel filtration, discovered a basic polypeptide with histamine-releasing properties similar to compound 48/80. The degranulating activity of the peptide prompted the name mast cell degranulating (MCD)-peptide. The possibility that this peptide might be melittin was ruled out by its high susceptibility to the metabolic inhibitors DNP and NEM (N-ethyl maleimide) (51, 52). The lack of central nervous system stimulation in mice receiving a dose of MCD-peptide ten times the LD_{50} reported for apamine discounted apamine as the mastocytolytic component.

Concurrently, Breithaupt & Habermann (53) purified the MCD-peptide and identified it as one of the basic F0-peptides. Acid hydrolysis of the purified peptide revealed 22 amino acids with a molecular weight of 2855. Haux (54) determined the amino acid sequence and found that the amino acids in positions 3–6 corresponded exactly to the sequence of position 1–4 in apamine. However, no central neurotoxicity was detected with MCD-peptide. It was further distinguished from melittin by its inability to release serotonin from platelets, failure to hemolyze red blood cells, and by its LD_{50}, which, in mice, exceeds 40 mg/kg.

Purified MCD-peptide has been shown to reduce carrogeenin-induced edema in the rat paw (55). The anti-inflammatory activity of MCD-peptide is not abolished by pretreatment with antagonists of histamine, serotonin, or adrenergic agents. The anti-inflammatory activity persists after regional denervation or adrenalectomy. A direct action of MCD-peptide on vascular walls to reduce their permeability is suggested as the mechanism of action.

Several biogenic amines have been isolated from bee venom (56). Gel filtration and ion-exchange chromatography of whole bee venom yielded several fractions, one of which was found to contain norepinephrine and dopamine.

Other investigators (57) have isolated a cardioactive substance identified as cardiopep (cardiac peptide). When applied to isolated perfused hearts, the heart rate increased 50% and the force of contraction increased by 150%. In hearts with intrinsic arrhythmias, cardiopep produced an immediate restoration to normal rhythm. The identity of the compound is not known, but it is not a peptide, as originally thought.

Venom produced by the honeybee *Apis mellifera* is a highly complex mixture of pharmacologically active agents. Included among the identified constituents are the enzymes phospholipase A and hyaluronidase, the biogenic amines histamine, norepinephrine, and dopamine, and the peptides melittin, apamine, MCD-peptide, and minimine. Free amino acids, sugars, lipids, free bases, a cardioactive substance, and other unidentified compounds whose contribution to the toxicity of bee venom await determination, constitute the remainder of the venom (58).

WASP VENOM

Research on wasp venom was initiated by Jaques & Schachter (59,60) after their discovery of a potent histamine liberator in alcoholic extracts of sea anemone

tentacles. Wasp stings were known to be extremely painful and to manifest many of the symptoms of bee stings. The presence of pharmacologically active agents in wasp venom was therefore suspected.

Crude venom from the yellow jacket *Vespa vulgaris* produced a biphasic contraction of the isolated guinea pig ileum (60). Mepyramine partially antagonized the response, suggesting histamine as one of the venom components. Paper chromatography of the crude venom revealed distinct spots corresponding to histamine and serotonin standards in R_f values and color reactions. When eluates of the spots were tested on isolated preparations, mepyramine blocked the ileal contractions produced by the histamine spot. The serotonin eluate failed to contract the tryptamine-desensitized ileum. But neither block was complete, and small amounts of the crude venom still contracted the ileum rendered insensitive to histamine and serotonin. It was concluded that wasp venom contained a third substance responsible for the slow contraction seen as the second component of the biphasic ileal response. The slow-contracting substance remained after trichloroacetic acid (TCA) precipitation but was destroyed after treatment of the TCA-soluble venom fraction with boiling hydrochloric acid.

Because of the similarity of the slow-contracting substance to bradykinin, Schachter & Thain named it wasp kinin. Further studies with wasp venom, involving atropinized and mepyramine-treated rats and rabbits, confirmed the presence of a potent pharmacological agent whose effects on smooth muscle were direct and not mediated through the release of other compounds (61).

In a comparative study of wasp kinin, bradykinin, and kallidin, all three substances exhibited qualitatively similar pharmacological properties (62). Wasp kinin differed from the other kinins in that it was partially inactivated by trypsin, while kallidin and bradykinin were unaffected. Differences in rate of migration and R_f value between wasp kinin and bradykinin, kallidin and substance P on paper chromatograms were also noted.

Wasp kinin from *Vespa vulgaris* was resolved by ion-exchange chromatography into a single major and two minor components (63). All three components reacted with azocarmine and ninhydrin, exhibited similar pharmacological activity on guinea pig ileum and rat uterus preparations, and were completely inactivated by chymotrypsin. Incubation with trypsin greatly reduced activities of the three components. Like kallidin and bradykinin, wasp kinins appeared to be polypeptides.

Venom from other wasps was examined for kinins and other pharmacologically active substances. Extensive chemical analysis of venom from *Polistes annularis, P. fusicatus,* and *P. exclamanus* demonstrated three kinins readily separable on columns of carboxymethyl cellulose or carboxymethyl Sephadex (64). *Polistes* kinin 1 and 2 bore remarkable chemical and pharmacological similarities to bradykinin and kallidin. *Polistes* kinin 3, or simply *Polistes* kinin, the most abundant fraction, possessed the highest biological activity on guinea pig ileum, rat blood pressure, and rat duodenum. Treatment of *Polistes* kinin with trypsin changed its chromatographic properties and increased biological activity as opposed to the kinins from *Vespa vulgaris*, which were partially inactivated. The activity of trypsin-treated *Polistes* kinin was recovered as a single peak from carboxymethyl Sephadex. Amino

acid analysis of the active tryptic peptide revealed glycyl-bradykinin in addition to other amino acids. The structure of *Polistes* kinin has been elucidated and confirmed by synthesis to be Pyr-Thr-Asn-Lys-Lys-Lys-Arg-Gly-(Bradykinin) (65). This suggests that *Polistes* kinin is actually a kininogen-like substance whose pharmacological activity is liberated by tryptic enzymes present in the envenomated organism.

Recent investigation of venoms from various social wasps has revealed the presence of the catecholamines norepinephrine, epinephrine, and dopamine, with dopamine the most abundant (66). Small quantities of serotonin have also been identified in the venoms.

The parasitic solitary wasps of the suborder *Terebrantes* and *Aculeato* dispense a venom that is rather unique in its ability to paralyze, but not kill, the intended prey. Beard (67), investigating the toxicological effects of *Habrabracon juglandis* venom on wax moth larvae, discovered that one part venom in 2×10^8 parts insect hemolymph was sufficient to induce spreading paralysis. Oscillographic recordings of muscle and nerve action potentials indicated that the paralysis resulted from impairment of excitatory processes of the body-wall musculature, while internal organs remained unaffected. The effects of the venom on larvae paralleled the effects of curare on mammalian neuromuscular transmission. The venom was postulated to interfere with neuromuscular transmission in insects by chelation of copper, thus inactivating a copper-containing mediator of neuromuscular transmission. To date, no such mediator has been identified.

The paralysis observed in insects after envenomation by the paralytic wasps may be either transient or permanent, depending upon the species of wasp. The effects of *Microbracon hebetor* and *Philanthus triangulum* venoms on in vivo and in vitro insect preparations include a reversible locomotor paralysis, with the central nervous system, internal organs, and muscle fibers unaffected (68). The neuromuscular junction is cited as the venom's proposed site of action.

Electrophysiological investigation of miniature excitatory postsynaptic potentials (MEPP) recorded from the flight muscles of *Philosamia cynthia* indicate that *Microbracon hebetor* venom markedly decreases the frequency but not the amplitude of the spontaneous potentials (69). The end-plate potential measured in the neuromuscular junction is thought to be composed of a large number of smaller potentials, corresponding with the quantal release of the transmitter substance (70). The miniature end-plate potentials presumably are the direct postsynaptic result of spontaneous release of one or few transmitter-substance quanta (71). The predominant effect of *M. hebetor* venom on MEPP frequency suggested that the venom had a presynaptic site of action.

Rathmayer (72) observed a progressive paralysis in *Apis mellifera* stung by *Philanthus triangulum* and concluded that the venom was carried by the prey's hemolymph to the locomotor organs where it exerted its paralytic action. Venom from *P. triangulum* was later shown to decrease the frequency of MEPP recorded from *Shistocercia gregoria* flight muscles, similar to the effect observed by Piek & Engles (69) with *M. hebetor* venom. This led Piek (73) to propose a similar site of action for *P. triangulum* venom. It was observed that nerve stimulation during venom

produced a faster onset of muscle paralysis, suggesting that the venom might be interfering with the synthesis of transmitter or its storage (73).

Both *Microbracon hebetor* and *Philanthus triangulum* venoms have proven extremely difficult to analyze chemically. Visser & Spanjer (74) partially purified *P. triangulum* venom and recovered a toxin of 700 molecular weight from gel filtration. *M. hebetor* venom was reported to contain amino acids, enzymes, and peptides, none of which were identified (74).

Limited success has been achieved in chemical analysis of venom from *Sceliphron caementarium.* Histamine, serotonin, and acetylcholine, three low molecular weight agents previously demonstrated in other Hymenoptera venoms, were absent from *S. caementarium* venom (75). This is consistent with the mild reaction experienced by humans after receiving a sting. Small amounts of histidine, methionine, and pipecolic acid, all of unknown function, were identified by paper chromatography. Proteins isolated from *S. caementarium* venom by disc electrophoresis showed no antigenic cross-reactivity with venom proteins from *Polistes apachus* (wasp), *Vespula pennsylvanica* (yellowjacket), *Vespula arenario* (yellow hornet), or *Apis mellifera* (honeybee) (76). Solvent extraction of the purified venom followed by different chromatographic procedures disclosed 22 components, which accounted for 96 ± 8% of the dry venom weight (77). The paralyzing factor was not isolated and was believed to result from cooperative interaction of some or all of the venom constituents.

The pharmacological activities of *Vespula* and *Polistes* venoms may be attributed to histamine and serotonin acting synergistically with bradykinin-like polypeptides and venom enzymes (41). Venoms from the solitary wasps apparently exert their paralytic effect presynaptically at the neuromuscular junction, but the active components have yet to be isolated and identified (78).

HORNET VENOM

Investigation of hornet venom began in 1960, shortly after the discovery of potent pharmacological agents in the venom of wasps and other stinging Hymenoptera. Venom from the European hornet *Vespa crabo* was shown, by parallel assay, to contract the isolated guinea pig ileum and frog rectus absominus muscle (79, 80). The antagonism of these effects by atropine and *d*-tubocurarine, respectively, suggested that the venom contained an acetylcholine-like compound not previously demonstrated in venoms of other Hymenoptera. Paper chromatograms of acetylcholine standards and alcoholic extracts of hornet venom revealed spots with identical R_f's. When the venom spot was eluted and injected into rabbits, a profound hypotensive effect was observed. This effect was blocked by pretreatment of the animals with atropine.

Bhoola and co-workers (80) also detected histamine in the hornet venom by assay on the atropinized guinea pig ileum. A spot with an R_f value identical with histamine was eluted and applied to the ileum preparation. The contraction produced by the spot was completely abolished by mepyramine.

The whole-venom chromatograph also showed a spot with an R_f value very close to serotonin. The spot gave a blue-gray color when sprayed with dimethylaminobenzaldehyde, indicating a positive Ehrlich reaction for indoles. Eluates of the spot contracted the isolated guinea pig ileum and rat uterus, the latter reaction being partially antagonized with LSD. Preincubation of the eluate with chymotrypsin destroyed the LSD-resistant component. The effects observed were attributed to serotonin and a peptide with chromatographic mobility similar to serotonin in the solvent systems used (80).

Further chromatographic investigation with different solvent systems resulted in the isolation of the new peptide. Named hornet kinin by Bhoola because of its pharmacological activity and sensitivity to chymotrypsin, it differed from wasp kinin by its insensitivity to trypsin inactivation (61). Hornet kinin demonstrated a remarkable similarity to bradykinin when tested on the rat uterus, rat duodenum, and rat arterial pressure, but was only one tenth as active on the guinea pig ileum. Hornet venom has been shown to contain phospholipase A (30) and is suspected to contain other peptides, amino acids, and enzymes as yet unidentified.

A pharmacological evaluation of *Vespa orientalis* venom was started by Edery and co-workers in 1972 (81) and has been continued by Ishay. Venom injected into mice produced akinesia and paralysis which lasted until the death of the animals. While the paralyzing effect of Hymenoptera venom on other insects has been well documented (68, 69, 73, 82), there have been no reported instances of insect venom causing neuromuscular disturbances in mammals. Intra-arterial injections of the venom into cats blocked directly and indirectly induced single twitches of gastronemius-soleus and tibialis anterior muscles, without affecting conduction in the sciatic or semitendinosus nerve. A blockade of neuromuscular transmission was suggested as the mechanism of action.

Whole venom produced bronchiolar constriction when injected into guinea pigs and increased capillary permeability in rabbits and rats receiving intradermal injections. The presence in the venom of histamine or histamine-releasing agents as well as other potent biogenic amines was suspected, since these substances had been demonstrated in other hornets and wasps. Histamine, serotonin, and acetylcholine were identified by their actions on the guinea pig ileum and rat uterus, with antagonism by mepyramine, 2-bromolysergic acid tartrate, and atropine, respectively. The venom continued to elicit a slow, sustained contraction of smooth muscle preparations in the presence of the three antagonists. Incubation of the venom with chymotrypsin abolished the ileal contraction while trypsin did not affect the kinin's activity. It appears that kinin from *Vespa orientalis* is related to that of *Vespa crabo,* but differs from wasp kinins which are sensitive to trypsin. The presence in *V. orientalis* venom of a histamine-releasing agent was explored using rat peritoneal mast cells incubated with hornet venom or compound 48/80. No histamine remained in the mast cells after incubation, while substantial quantities of histamine could be liberated from controls.

Edery and his group found norepinephrine, epinephrine, and dopamine in small quantities after biochemical analysis of the venom and venom sacs of *V. orientalis*

(81). These agents had been previously demonstrated in the venom sacs, but not venom, of bees and wasps (83). It was suggested that they prolong the local action of the venom by constriction of cutaneous capillaries and venules.

Ishay and co-workers have continued the investigation of the venom and venom sac extract (VSE) of the Oriental hornet, *Vespa orientalis*. Intravenous administration of VSE to cats produce a drop in blood sugar. This effect was attributed to a nondialyzable protein or protein-bound substance in the VSE that is heat sensitive (84).

The hornet VSE was shown to have in vivo and in vitro anticoagulant properties (85). VSE inhibited in vitro formation of thrombin and thromboplastin when incubated with human plasma. Intravenous administration of VSE to dogs reduced the clottability of whole blood. The anticoagulant factor has not been identified, but it is inactivated at high temperature and alkaline pH.

The hornet venom produced dramatic cardiovascular effects when administered intravenously to prepared dogs (86). Effects seen 10 sec after administration of the venom included increased respiration and cardiac output, while mean aortic pressure and peripheral resistance decreased. Pretreatment of dogs with methysergide prior to the administration of the venom abolished all of the effects except vasodilation. This appears to confirm the presence of serotonin in hornet venom as previously reported by Edery (81). The vasodilation seen after blockade of the serotonin effects were attributed to a kinin-like substance, previously isolated by other investigators (61).

The local reaction to hornet sting, characterized by pain, inflammation, and edema result from the potent amines and polypeptides present in the venom. In addition, the presence of the enzymes hyaluronidase and phospholipase A, as well as a histamine liberator, probably contribute to the reaction by promoting the spread of the venom.

ANT VENOM

Ant venoms fall into two categories, proteinaceous and nonproteinaceous, and are found among species of primitive subfamilies where the sting mechanism is highly developed. The majority of ant stings are only slightly painful but those inflicted by *Myrmecia* or *Solenopsis* ants result in both localized necrosis and systemic reactions of severe intensity. Within the classification of ant venom, *Myrmecia* produces a proteinaceous venom, while *Solenopsis* secretes a venom devoid of detectable protein. As with venom from other stinging Hymenoptera, the greatest threat to human life lies in the allergic reaction exhibited by sensitive individuals. The severity of the reaction depends on the degree of sensitivity of the individual, with death occurring as a consequence of anaphylactic shock.

Initial studies on the venom of the fire ant *Solenopsis saevissina* revealed the absence of ninhydrin-positive reactants, suggesting a nonproteinaceous venom (87). The pure venom was insoluble in water, dispersing as fine, milky-white globules on the water surface. The venom was soluble in most organic solvents. While the fire

ant is not susceptible to its own venom, several species of mites and weevils, as well as *Drosophila melanogaster* and *Musca domestica*, are.

Pharmacological investigation of crude *Solenopsis* venom was undertaken with the aim of isolating and identifying the necrotoxic principle(s) (88). The venom showed marked hemolytic activity on washed rabbit erythrocytes, and the active component was thought to be nonprotein in nature because no loss of activity was observed after exposure to 100°C for 1 hr. Isolation of the hemolytic component was accomplished by solvent extraction which yielded white crystals melting at 144–145°C. These crystals gave a positive test for a tertiary amine.

The pustules produced by experimental ant sting being bacteriologically sterile (89), the crystalline hemolytic component was screened for antimycotic activity utilizing 21 different human pathogens (90). Susceptible pathogens included *Candida albicans, Blastomycese dermatitis*, and *Cryptococcus neoforus*, as determined by the zone of inhibition surrounding a crystal on the agar plate.

The first chemical analysis of the hemolytic component (91) suggested the compound 2-methyl-3-hexadecyl-pyrrolidine. More recent experimental analysis, utilizing gas chromatography, mass spectroscopy, and NMR, has led investigators to propose *trans*-2-methyl-6-*n*-undecylpiperidine named *Solenopsin A*, as the active hemolytic component (92). The structure was verified after synthesis of Solenopsin A.

In contrast to *Solenopsis* venom, the clear, colorless liquid obtained from the venom reservoirs of *Myrmecia gulosa* was readily soluble in water, was insoluble in organic solvents, and gave a positive ninhydrin reaction (93). Its protein nature was further supported by absorbance at 277–282 mμ of the UV spectrum.

Separation of the crude venom into eight fractions was accomplished with paper electrophoresis. Fraction I corresponded to histamine by comparative paper chromatography. Hyaluronidase activity was detected in Fractions IV and V, as was a kinin-like substance which contracted the guinea pig ileum and rat uterus. Fraction VII contained a direct hemolytic factor found to be heat labile. No cholinesterase, 5-nucleotidase, or protease was detected in the venom.

Similar investigative efforts have been directed toward venoms from other members of the Myrmeciinae subfamily. Precise identification of the extract species sometimes proved difficult. Venom collected from *Myrmecia forficata*, later identified as *M. pyriformis*, produced a contraction of the isolated rat uterus characterized by a prolonged delay in recovery when compared with serotonin and acetylcholine (94). Incubation of the venom with trypsin abolished the activity on the uterus, but had no effect on the response of the guinea pig ileum. Mepyramine completely antagonized the latter action. The presence of histamine was confirmed by paper chromatography of whole venom. One spot produced a positive reaction to ninhydrin, had an R_f identical with the histamine standard, and was resistant to boiling in strong mineral acid. This latter treatment had no effect upon the ileum-contracting ability of the spot eluate, although mepyramine completely abolished the activity. The prolonged response seen with the other chromatogram spot on the rat uterus was suggestive of a kinin-like substance.

Myrmecia sting is characterized by a pronounced local reaction, suggesting that the venom might contain a histamine-releasing compound (95). Normal saline extracts of *Myrmecia pyriformis* venom sacs, when incubated with rat peritoneal mast cells, showed considerable histamine-liberating activity. Ten micrograms of venom, about the amount present in one good ant bite, released 56% of the mast cells' histamine, as compared to 74% released by an equal concentration of compound 48/80. Increasing the venom concentration tenfold resulted in 95% depletion of mast cell histamine. This histamine-releasing activity might be involved with the prolonged spasmogenic activity of ant venom on smooth muscle and the long-lasting erythema and localized edema seen after envenomation.

A more detailed investigation of *M. pyriformis* venom, aimed at characterizing the various components, was undertaken by Lewis & de la Lande (96). Crude venom contracted the isolated rat uterus, toad rectus abdominus muscle, and guinea pig ileum, and constricted the vessels of the isolated rabbit ear. A marked hypotensive effect was observed in anesthetized cats after administration of the venom. Histamine identified previously in other experiments was believed partially responsible for the isolated organ activity. The persistence, however, of a slow contractile response on histamine-insensitive uterus and mepyramine-treated ileum suggested another active component. The crude venom released histamine from isolated peritoneal mast cells, lysed washed red blood cells, and showed hyaluronidase activity. All four activities were destroyed by boiling the venom, while a marked loss of all activities was noted after incubation with trypsin or chymotrypsin. The involvement of polypeptides as mediators of the above responses was suspected.

Paper chromatography allowed the separation of histamine from the other active substances, but failed to separate the smooth-muscle stimulant from either the red-cell lysing fraction or the histamine-releasing component.

A possible correlation of the single substance in ant venom that stimulates smooth muscle, lyses red blood cells, and releases histamine to the bee peptide, melittin, has been suggested (97).

The ability of purified *M. pyriformis* to split lecithin was demonstrated by in vitro incubation of the venom with plant and liver lecithin (98). Thin-layer chromatography of precipitated lysolecithin from the ant venom incubation produced spots identical in R_f with lysolecithin from phospholipase A hydrolysis. The components of ant venom appear very similar in activity to the substances found in the venom of the honeybee, *Apis mellifera* (99).

Venom is but one of the exocrine secretions produced by ants. Ant venom has been shown to contain histamine, a histamine-releasing substance, a nonhistamine smooth-muscle stimulant, a direct red-cell lysing factor, hyaluronidase, phospholipase, and other complex chemical substances (97).

SUMMARY

Venoms from the hymenopteran superfamilies Apoidea, Vespoidea, Sphecoidea, and Formicoidea constitute a group of toxic substances that have been of concern to man since prehistoric times. Even now, after seventy-five years of research on

hymenopteran venoms, it is difficult to make any meaningful generalizations concerning their composition except to say that they contain pharmacologically active components that fall into one of three general categories: (a) biogenic amines, (b) nonenzymatic proteins and polypeptide toxins, and (c) enzymes. Venoms from different families and species of Hymenoptera have been biochemically separated, and some components isolated and evaluated pharmacologically by a multiplicity of techniques, resulting in a rather fragmentary picture of venom composition.

A highly specialized apparatus serves to secrete, store, and eject the venom. Venom, produced in the venom glands of the female, is stored in a venom reservoir until needed, at which time it is ejected through a hollow, pointed aculeus, or stinger, into the intended victim. The entire maneuver is controlled by a series of muscles located in the abdomen of the Hymenoptera. The morphology of the sting apparatus varies slightly between different families.

The venom of the honeybee, *Apis mellifera*, has received the greatest research attention over the years, and as a result, more is known about this venom than the others. Histamine was the first pharmacological agent identified in bee venom, along with other substances that produced slow, sustained contractions of the guinea pig ileum and released epinephrine from perfused cat adrenals. The latter action was attributed to lecithinase, previously discovered in cobra venom, and later found in bee venom. Other factors present in bee venom included a hemolytic component and a convulsant factor.

Among the more sophisticated techniques of separation employed by venom researchers, paper electorphoresis proved the most fruitful. Whole bee venom was separated into five fractions: FO, FI, FII, H, and K. Analysis of these fractions revealed histamine, phospholipase A, hyaluronidase, and a polypeptide, melittin, as components of bee venom. Comprising 50% of total dry bee venom by weight, melittin was believed to be responsible for most of the pharmacological actions of whole bee venom. These included direct hemolysis of washed erythrocytes, liberation of histamine and serotonin from mast cells and platelets, contraction of smooth muscle, and ganglionic blockade. The amino acid sequence of melittin has been determined and the peptide recently synthesized. It has been suggested that pharmacological properties of melittin are directly related to its primary structure, that of an invert soap, giving melittin strong surface-active characteristics.

Continued investigation of bee venom has led to the discovery of several other peptides of pharmacological interest. The dialyzable convulsant factor, first recognized by Hahn and Ledelschke in 1937, was isolated and named apamine. This peptide had rather specific actions; it produced long-lasting central excitation in mice and increased the permeability of rat cutis for Evans blue dye. The central excitation is thought to result from apamine's augmentation of central excitatory pathways. The amino acid sequence of apamine has been described and the peptide synthesized.

A specific mast cell degranulating peptide (MCD-peptide) was isolated by Breithaupt and Habermann from their electrophoretic fraction FO, previously described as biologically inactive. At the same time, Fredholm discovered a similar active principle in his bee venom fractions. The two substances were found to be identical

and to exert their histamine-releasing action in a manner similar to compound 48/80. The amino acid sequence of MCD-peptide has been elucidated. Bee venom, therefore, acts to release histamine by three mechanisms: (*a*) phospholipase A, acting indirectly by forming lysolecithin from extracellular lecithin; (*b*) melittin, disrupting mast cells directly by surface activity; and (*c*) MCD-peptide, producing degranulation similar to 48/80.

A fourth peptide, minimine, has been detected in bee venom. Minimine produced lethargy and anorexia when injected into *Drosophila melanogaster* larvae. Adult flies emerging from the treated larvae were one fourth the normal size, although they appeared normal with respect to feeding, motility, and progeny.

Recently, a cardioactive substance, cardiopep, has been found in bee venom. It has been shown to increase the rate and the force of contraction of the heart.

Venom from the wasp *Vespa vulgaris* was found to contain histamine, serotonin, phospholipases, and a substance that produced slow, sustained contractions of the guinea pig ileum. The slow contracting substance bore a remarkable pharmacological resemblance to bradykinin and kallidin, but differed from these peptides in that it was susceptible to trypsin inactivation. The substance was named wasp kinin and was shown to be a peptide.

A peptide with properties similar to the wasp kinin from *V. vulgaris* was isolated from the venom of *Polistes* wasps by fractionation on carboxymethyl cellulose. *Polistes* kinin differed from the Vespa kinin in that its activity was enhanced by trypsin. Amino acid analysis of the trypsin fragment showed it to be glycyl-bradykinin, suggesting that Polistes kinin is actually a kininogen-like substance whose pharmacological activity is unmasked by the host's enzyme system. The complete structure of *Polistes* kinin recently has been described and confirmed by synthesis.

Wasps of the family *Sphecoidea* elaborate a venom that is paralytic in action and devoid of the usual pain-producing substances. The paralytic component reportedly acts presynaptically at the insect neuromuscular junction and has proven resistant to isolation attempts.

Hornet venom of the species *Vespa crabo* and *Vespa orientalis* contain histamine, serotonin, phospholipase A, hyaluronidase, and substantial quantities (10% by weight of dried venom) of acetylcholine. Both venoms contain a kinin sensitive to chymotrypsin but insensitive to trypsin. Norepinephrine, epinephrine, and dopamine, previously reported as constituents of bee and wasp venom sacs, were present in small quantities in hornet venom. The amines are thought to prolong the local action of the venom by constricting cutaneous capillaries and venules.

Ant venoms may be classified as proteinaceous and nonproteinaceous. *Myrmecia gulosa* produces a proteinaceous venom that was separated electrophoretically into eight components. Histamine, hyaluronidase, and a direct lytic factor were identified among the constituents. Another species of *Myrmecia, M. pyriformis,* elaborates a venom containing a potent histamine liberator, a hemolytic component, a smooth-muscle stimulant, and hyaluronidase. Except for hyaluronidase, none of the components have been identified.

Venom devoid of detectable protein is produced by the fire ant, *Solenopsis saevissina*. It contains a powerful hemolytic agent that has been crystallized and identified as *trans*-2-methyl-6-*n*-undecylpiperidine.

Hymenoptera venoms are highly concentrated mixtures of pharmacologically active substances that may be of value in the study of agents activated or released by tissue damage. Venoms may be for the pharmacologist

> . . .an instrument which associates and analyzes the most delicate phenomena of the living machine and in studying attentively the mechanism of death . . . [reveals] by an indirect route the physiological mechanism of life.
>
> Claude Bernard (100)

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