Bee and Wasp Venoms

The biochemistry and pharmacology of their peptides and enzymes are reviewed.

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Bee and wasp venoms (*1*) are toxic substances that man has had to live with since prehistoric times. Nevertheless, knowledge of their composition and mode of action dates back only 10 to 20 years. There are theoretical and practical reasons for this lack of understanding. The therapeutic value of the venoms when applied to individual patients is not convincing; it has never been proved by unbiased trial. Therefore, the incentive for applied medical research is lacking. Second, bee and wasp venoms, like those of many other "actively toxic" animals (such as snakes and scorpions, which apply their venoms by biting or stinging in contrast to passively toxic animals, which are noxious by serving as prey), are highly complicated mixtures of pharmacologically or biochemically active agents, or both. The complex anatomical and functional arrangement of the apparatus producing and dispensing venoms has its counterpart in the equally complicated biochemistry of the secretions. Useful tools for analyzing these complexities were not available until a few years ago.

There are three reasons for studying animal venoms. The pharmacologist has to deal with every highly toxic compound regardless of its availability and practical usefulness. Hymenoptera venoms are—like snake venoms—concentrated mixtures of substances of general biological concern, and they can serve as models for mixtures of agents activated or released by tissue damage. Finally, they may turn out to be useful for solving biochemical and pharmacological problems. To cite one example only, the snake venom neurotoxin, α-bungarotoxin, is an important aid in identifying in excitable membranes a component thought to be the cholinergic receptor protein (*2, 3*). Therefore, the scientific significance of venoms is greater than their relevance in animal and human ecology.

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Bee Venom

Bee venom consists of only a few pharmacologically or enzymatically active components, and its complete biochemical and pharmacological analysis has been feasible. The classification of the constituents of bee venom is applicable to venoms of other hymenoptera as well as of snakes (Table 1).

Among the low molecular weight agents in bee venom histamine predominates. This substance is present in a low concentration—0.1 to 1.5 percent (*4*). Recently, dopamine and noradrenaline have been identified as constituents of the bee and yellow jacket venom reservoirs in vivo, but not of the secreted, dried venoms (*5*). The effects of the biogenic amines are obscured by the two groups of high molecular weight agents. Substances that could be called toxins in a strict sense should be differentiated from enzymes that may but need not be toxic. This classification was not obvious 15 years ago. Practically all effects of bee venom had been ascribed to its phospholipase activity. Application of electrophoretic, chromatographic, and gel-filtration procedures, together with pharmacological and biochemical analyses, led to the differentiation of two enzymes, namely phospholipase A and hyaluronidase, from a series of toxic polypeptides: the hemolyzing melittin (*6*), the neurotoxic apamin (*7*), and a mast cell degranulating (MCD) peptide (*8, 9*) (Table 1). Recently, a factor called minimine, which retards the development of *Drosophila*, has been described (*10*).

Melittin

The polypeptide melittin, with respect to weight (it is 50 percent of dry venom) and activity, is the main toxin of bee venom. Its existence became evident when in 1952 the "direct" hemolysin (at that time called F I) was electrophoretically separated from the "indirect" (*11*) hemolysin phospholipase A (*12*). The importance of this experiment reached beyond the field of hymenoptera venoms. As was shown later, some elapid (for example, cobra) venoms contain, besides phospholipase A, a "direct lytic factor" (see *13–15*) which is probably identical with cardiotoxin (*16*).

Two properties of the melittin molecule gave hints of some molecular characteristics of possible biological significance. First, melittin is strongly basic (*6*) like the other peptides of bee venom. It is, however, much more firmly adsorbed, for instance on carboxymethyl cellulose (*7*), than other venom components of comparable basicity. A second peculiarity rests on the strong surface activity of melittin. The peptide decreases the interfacial tension between air and salt solutions to a degree comparable with the hemolysins lysolecithin or digitonin (*17*); hence we have reason to believe that melittin increases the permeability of erythrocytes and other cells because it has that physicochemical action on cell surfaces.

Surface activity, however, should be reflected by the molecular structure of melittin. Amino acid analysis disclosed the lack of aspartic acid, tyrosine, phenylalanine, histidine, and amino acids containing sulfur. The minimum molecular weight calculated from acyl residues (2840) greatly differed from the apparent molecular weight evaluated by gel filtration on calibrated columns (about 12,000). Quantitative determination of the NH_2-terminal glycine proved that the lower value was correct (*7*). The four times greater value obtained by gel filtration probably indicates a micellar weight, not a true molecular weight, because (i) melittin is free from disulfide linkages, and (ii) the amino groups of the side chains are also not cross-linked, since they can be quantitatively substituted (*18*).

Formation of micelles, lowering of surface tension, and hemolysis could be manifestations of the primary structure of melittin. Determination of the amino acid sequence eventually gave the key to the understanding of the physico-

chemical and most of the pharmacological properties of the peptide. By conventional techniques, the structure (*19*) shown in Fig. 1 has been revealed.

The work on the structure of melittin has been extended by Kreil and Kiss (*20*) and Lübke *et al.* (*21*). Both groups verified our observation (*19*) that part of the natural melittin is substituted at its NH_2-terminus. They identified the substituent as a formyl group.

Not only the finding of both unsubstituted and substituted native melittin, but also similar properties of various synthetic congeners allow the classification of melittins as a group of chemically and pharmacologically related peptides. Four substances have been synthesized (*21, 22*) by the team at the Schering Corp. (West Berlin): melittin (called melittin I), *N*α-formyl melittin I, a melittin ending with the sequence

21 27
Ser-Arg-Lys-Lys-Arg-Gln-GlnNH$_2$

called melittin II—which has been formulated tentatively (*19*) and has never been identified in animal material—and *N*α-formyl melittin II (Table 2). Synthetic melittin I was indistinguishable from native, unsubstituted melittin I by peptide mapping, surface activity, hemolytic potency, hypotensive action in rabbits, and general toxicity in mice (*22, 23*). Synthetic *N*-formyl melittin I was also identical with the native substituted peptide (*22*) and only slightly less active than unsubstituted melittin I. Melittin II and its formylated derivative yielded peptide patterns fundamentally distinct from those of melittin I and of *N*α-formyl melittin I; nevertheless, their pharmacological properties corresponded well with those of melittin I (*23*). The qualitative similarities of the various synthetic derivatives are indicative of a broader structural basis for the melittins as a group.

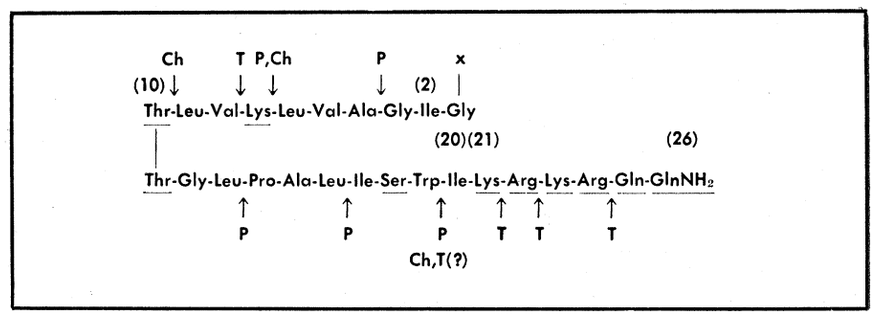


Fig. 1. Structure of natural (*19*) and synthetic melittin I. The left side of the diagram indicates the predominantly hydrophobic part (amino acids with a hydrophilic side chain are underlined), and the right side (residues 21 to 26) indicates the hydrophilic, strongly basic part. The letter **X** may be H or a formyl group; P, peptic; Ch, chymotryptic; T, tryptic attack.

The atypical arrangement of amino acids is impressive when they are classified according to neutral hydrophilic, basic hydrophilic, and hydrophobic side chains. If this classification is followed, melittin has the structure of an invert soap: a basic hydrophilic COOH-terminal part (position 21 to position 26) is connected with a predominantly hydrophobic sequence (position 1 to position 20). An often accepted fundamental concept of protein chemistry is that the interior of protein molecules is filled with predominantly hydrophobic residues, whereas mainly hydrophilic groups contribute to its shell. However, the hydrophilic residues of melittin are so unequally distributed that no such shell can be formed—except by coacervation of some monomers to form a micelle. For the same reason, surface films with consequent decrease of interfacial tension might be formed, not only at air-water interfaces, but also at the surfaces of erythrocytes.

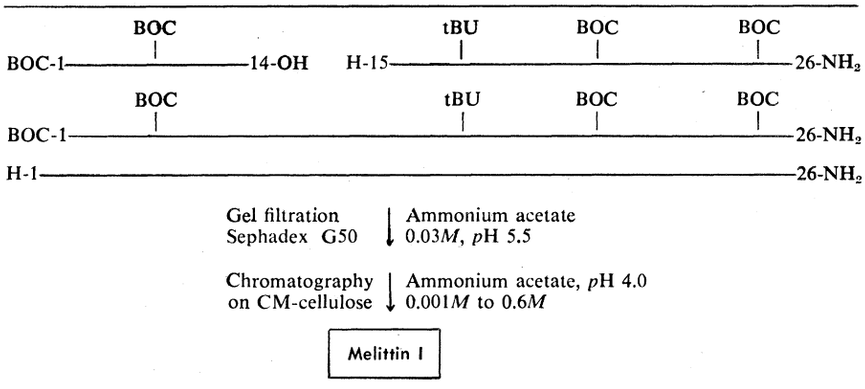
The molecular architecture of melittin in solution needs closer investigation. According to Jentsch (*24*), measurement of the optical rotatory dispersion suggests that its polypeptide chain is in a random conformation. This in turn may facilitate the arrangement of the amino acids into hydrophilic and hydrophobic areas.

There is, however, no strict parallelism between surface activity and hemolytic potency. Succinylation of the amino groups abolished the hemolytic potency (together with the basicity), without much influencing the surface activity (*18*). Some synthetic fragments of melittin possessed more than 50 percent of the surface activity of the original peptide, although they were without or of very low hemolytic potency (*22*). Besides surface activity, other molecular parameters are involved in the hemolytic activity of melittin, one of them being basicity. The single tryptophan residue does not seem to be essential, since it can be destroyed photochemically or substituted with 2-hydroxy-5-nitrobenzyl bromide without gross al-

Table 1. Pharmacologically and biochemically active constituents of various animal venoms.

Bee (*1*)	Wasp (*1*)	Hornet (*1*)	Snakes (*13, 14*)		
			Elapidae	Crotalidae	Viperidae
		Biogenic amines			
Histamine	Histamine Serotonin	Histamine Serotonin			
Dopamine, noradrenaline (*5*)	Dopamine, noradrenaline (*5*)	Acetylcholine			
		Protein and polypeptide toxins (nonenzymatic)			
Melittin Apamin MCD-Peptide Minimine (*10*)	Wasp kinin	Hornet kinin	Neurotoxins Cardiotoxin	Crotamin	Viperotoxin (*73*)
		Enzymes			
Phospholipase **A** Phospholipase **B** (?) Hyaluronidase	Phospholipase A Phospholipase B Hyaluronidase	Phospholipase **A** Phospholipase **B**	Phospholipase A Cholinesterase Hyaluronidase	Phospholipase **A** Hyaluronidase Many additional enzymes	Phospholipase A Hyaluronidase Many additional enzymes

Table 2. Synthesis of melittin I and purification procedure (*22*); CM, carboxymethyl; BOC, butyloxycarbonyl; tBU, *tert*-butyl.



teration of surface activity or hemolytic potency (*18*).

Melittin has a moderate antibacterial and antifungal activity (in concentrations between 0.05 and 0.2 percent) against many kinds of organisms. A series of melittin fragments prepared by solid phase synthesis (residues 1 to 8, 1 to 17, 7 to 17, and 14 to 17) proved to be devoid of the activity which is apparently connected with the whole molecule (*25*).

It would be erroneous to assume that the intimate mechanism of melittin hemolysis must be the same as that of other detergents, such as lysolecithin or digitonin. Indeed, many differences in kinetics, in behavior against inhibitors, and in osmotic influences point to the contrary (*26*). The best evidence, however, for a specific attack by melittin stems from the subsequent appearance of the erythrocyte ghosts. Lysolecithin is a typical "solubilizer"; with high concentrations, erythrocyte ghosts are totally dissolved. Digitonin, reacting with the erythrocyte cholesterol, produces clodlike changes when applied in high concentrations. Melittin, in contrast, renders the ghosts shrunken and more strongly contrasting in the dark field, phase contrast, or electron microscope. Osmium-fixed shadows appear netlike, the interior appears empty (*27*). Lipid spherules have been introduced as models for biological membranes, especially those of erythrocytes (*28*). At concentrations above 10^{-6} molar, melittin releases marker anions (CrO_4^{2+}) or glucose from these liposomes, regardless of whether they were prepared with a net negative (dicetyl phosphate) or net positive (stearylamine) charge, or whether they contained cholesterol. Studies with lipid monolayers indicated that melittin had a remarkable affinity for the air-water interface and more so for the lipid-water interface. From the foregoing experiments it is generally concluded that the surface activity and convenient apolar associations between the hydrophobic portions of the peptide and the acyl chains of phospholipids are more important than are ionic interactions.

Hemolysis is a very simple model for studying the action of melittin. Melittin has been effective on nearly every other pharmacological system tested, provided that it is not bound by compounds of nutrient solutions (such as blood) before reaching the sensitive structures. It damages not only erythrocytes but also leukocytes and their lysosomes with subsequent release of enzymes. Electron microscopy of isolated leukocyte granules shows a reduction of their diameter, an increase in the density of the matrix, and membrane fractures (*29*). Thrombocytes release serotonin; rat mast cells release histamine; striated musculature releases potassium ions and organic and inorganic phosphates. Thus in addition to its direct effects, melittin may act indirectly by releasing the tissue components mentioned, and in this respect, a "concerted action" is effected by venom constituents and tissue factors.

Depending on dose and organ, melittin constricts or dilates blood vessels. It depolarizes and shortens skeletal and heart musculature. It produces pain, increases vascular permeability on local application, and elicits slow contraction of smooth musculature. Nevertheless, melittin is not a typical slow reacting substance like bradykinin or so-called SRS-A (*30*). The shortening is more gradual. Higher doses are followed by diminished responses, not only against melittin, but also against other chemical or electrical stimuli. Excitation followed by inexcitability is a typical feature of the pharmacological action of melittin. This phenomenon occurs in the neuromuscular block of the rat phrenic nerve–diaphragm preparation in vitro and in the isolated perfused superior cervical ganglion of the cat; it also occurs in the respiratory and circulatory disturbances that appear when the peptide is applied to the floor of the fourth ventricle [see (*1*)]. The ubiquitous pharmacological effects of melittin render its pharmacology uninteresting, like that of other detergents. Particularly fascinating is, however, the possibility of understanding the effects mentioned on the basis of the chemical properties of the melittin molecule.

The same is true for the actions of melittin on biochemical systems. Like other detergents, melittin damages enzyme systems bound to cell membranes, such as cation activated adenosine triphosphatases (*31*). It diminishes the electron transport in mitochondria and uncouples oxidation from phosphorylation (*32*). Melittin inhibits the thromboplastic potency of tissue preparations [for example, from lung (*33*)] as well as acetylcholine esterases (*34*, *35*). The turbidity of particle suspensions from liver or egg yolk is increased by melitttin—again quite contrary to the action of lysolecithin, which solubilizes them (*17*).

Many of the melittin effects mentioned could be involved in intoxication of experimental animals. In fact, the precise event leading to death is unknown. Neither intravital hemolysis (*36*) nor neuromuscular or ganglionic block (*37*) is prominent in vivo.

Melittin is also very toxic to *Drosophila* larvae in amounts of more than 0.1 microgram per animal. Local damage at the injection site is evident from the strong muscle contraction and the appearance of black bodies. The latter is presumed to be due to activation of phenol oxidase after cell death. In *Drosophila* larvae Mg^{2+}, K^+, and Na^+—but not Ca^{2+}—seem to be antagonistic to melittin (*34*).

The biosynthesis of melittin was studied in vivo by feeding radioactive amino acids to honey bees. The amino acids were first incorporated into another peptide which is present in glandular extracts and which is thought to be a precursor of melittin (promelittin). This "precursor" component differs from melittin in having a prolonged, negatively charged amino terminus. It could not be detected in

Table 3. Neurotoxic actions of some basic polypeptides in mice.

Substance	Source	Action
Apamin	Bee	Uncoordinated movements increasing to spasms and convulsions of central origin
Scorpamin	*Androctonus australis* (scorpion)	Convulsions, spasms, excessive salivation, paralysis of hind legs, polypnoea, respiratory paralysis (*74*)
Crotamine	*Crotalus terrificus* (rattlesnake)	Persistent spasms of the hind legs, probably of peripheral origin (*75*)
Neurotoxins	*Naia* species (cobra)	Curarelike peripheral actions (*76*)

the ejected venom. Formylation apparently takes place after the active peptide has been released from the precursor (*38*). This may be the first report about a peptide that appears to be a "protoxin."

Apamin

In contrast to melittin, apamin is a drug with a highly specific mode of action that cannot yet be explained on the basis of its chemical structure. For many years, the neurotoxic action of bee venom has been known. Whole bee venom can sensitize mice to various stimuli and also decrease the sleeping time induced by barbiturates (*39*). A fraction of bee venom that passes through cellophane membranes produces convulsions in mice (*40*). It is, however, not clear whether the excitatory manifestations are due to an interaction of the already known ingredients of the venom, whether they are caused by terminal asphyxia, or by a novel neurotoxin. The effects are obscured by the toxic effects of melittin and phospholipase A.

When screening the peptide fractions of whole venom obtained by gel filtration, we encountered a strong neurotoxin that could be purified by additional chromatography on carboxymethylcellulose. This neurotoxin, a peptide, was called apamin. When injected into mice at doses of 1 milligram per kilogram of body weight, or more, the animals succumbed, after a lag period of about 15 minutes, to uncoordinated, uninterrupted movements culminating in generalized convulsions. They died of lack of coordinated respiration. Surviving mice (the lethal dose, LD_{50}, was about 4 mg/kg) were extremely hyperexcitable for up to 60 hours. This was especially noticeable when the intoxicated animals were kept in groups (*7*). There was no significant lack of ability to balance on a rotating bar, and no abnormal behavior on the inclined plane, except when the animals lost the control of their motility. Transection experiments proved useful for excluding the muscles or the peripheral nerves as primary target organs. When the spinal cord was dissected, the central parts of the nervous system proved to be more susceptible to apamin than the disconnected length of the spinal cord.

More recently, closer insight into the synaptic actions of apamin has been gained in cats that have been subjected to spinal transection (*41*). Only 15 minutes after intravenous injection of the peptide (0.5 to 1.0 mg/kg), the amplitudes of the monosynaptic extensor reflex potentials and of the polysynaptic flexor reflex potentials were increased. No influence of the peptide on the spatial and temporal summation, on the direct inhibition, and on the recurrent inhibition could be observed when the conditioning technique was used. Apamin mainly augments polysynaptic reflexes and renders excitatory polysynaptic pathways more effective than inhibitory polysynaptic mechanisms.

Our current knowledge of the primary structure of apamin does not yet explain the specificity of its action. The peptide consists of 18 amino acids, not less than four of them being half-crystines. Sequential analysis performed independently by a British group (*42*) and our group (*43*) led to the same structure (Fig. 2). The British investigators (*42*) localized an amide group at the carboxyl terminus; our recent evidence points to the same conclusion. One disulfide bridge connects a half-cystine in position 3 with a half-cystine in position 15; since there is no free sulfhydryl group, the second link is to be assumed between half-cystine in position 1 and half-cystine in position 11 (*44*) (Fig. 2). Opened by reduction, the bridges can be restituted by cautious reoxidation. This is important for synthesis. On chromatography on carboxymethyl-cellulose columns, apamin emerges in two neighboring peaks (*45*). The amino terminus of some apamin molecules is possibly substituted, as with melittin.

Apamin is the smallest neurotoxic polypeptide known, and it is the only one whose interaction with the spinal cord is well established. It differs from other neurotoxins, such as scorpamins in scorpion venoms, crotamine in the venom of the Brazilian rattlesnake and venom neurotoxins from the cobra, bungarus, and erabu. These toxins all consist of about 60 amino acids, and they act exclusively at the neuromuscular junction (Table 3). Both apamin and the above-mentioned neurotoxins have a high basicity and a high sulfur content. Both types of neurotoxins are long-acting as compared to other drugs affecting the central or the peripheral nervous system.

Mast Cell Degranulating Peptide

We already mentioned the mast cell destroying (MCD) potency of melittin, and lysolecithin, the reaction product of phospholipase A, is also a mastocytolytic agent. The novel peptide to be discussed in this section is, therefore, the third bee venom component that releases histamine either directly or indirectly. Rothschild (*48*) has stated that, when applied to washed cells, the phospholipase component is not the main histamine liberator in bee venom, whereas melittin is very potent in this test. This agreed with our own, unpublished findings. Fredholm and his co-workers (*47*) confirmed these observations but emphasized the lack of parallelism between histamine release and the amount of melittin and phospholipase A in bee venom. They found that fractions rich in phospholipase A released histamine from rat skin and lung tissue and from guinea pig lung tissue, but not, except when present in very high concentrations, from rat peritoneal mast cells. This is easily understood because phospholipase A is able to form lytic compounds from tissue but not from intact cells. The en-

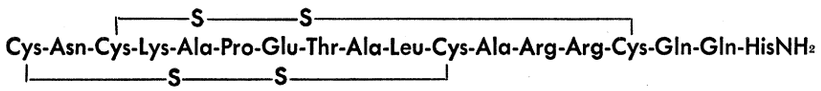


Fig. 2. Structure of apamin (*42–44*).

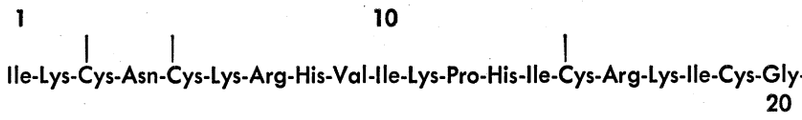


Fig. 3. Structure of MCD-peptide (*50*).

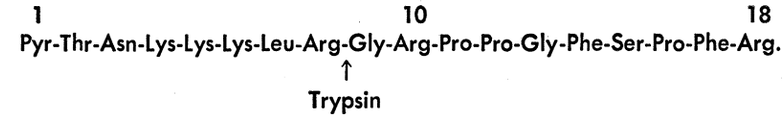


Fig. 4. Structure of a wasp kinin (*55*).

zyme fraction, and also a fraction probably rich in melittin, promoted the release of histamine from granules obtained by sonic distintegration of rat peritoneal mast cells. Fredholm's fraction II, whose active principle is probably identical to our MCD-peptide, released histamine from peritoneal mast cells, skin, and lung tissue of the rat, but not from guinea pig lung tissue.

We localized a specific histamine-releasing action in a hitherto neglected fraction, previously called FO. From this fraction, a peptide (MCD-peptide) has been purified, which is—depending on the test system—10 to 100 times more active than melittin against rat mast cells. Since, however, bee venom consists of about 50 percent of melittin and only of 1 to 2 percent of MCD-peptide, both factors contribute to the mastocytolysis caused by whole venom (*46*). When tissue phospholipids are accessible, phospholipase A may interact as a third factor. The differences between the various histamine releasers are evident from Table 4.

The MCD-peptide differs from melittin not only in its specific activity but also by its mode of action. Melittin is a strong hemolysin and releases serotonin from thrombocytes. The MCD-peptide is inactive in both tests. Melittin increases the capillary permeability in the rabbit as well as in the rat. Like the synthetic reference compound 48/80, MCD-peptide is much more effective in rats than in rabbits. Further results of ours (*46*) and Fredholm's group (*47*) also rank MCD-peptide in the group of "48/80-like" substances, quite in contrast to melittin. Other pharmacological effects, apart from those on mast cells and capillary permeability, are not prominent. Injected intravenously in mice, MCD-peptide is practically nontoxic (LD_{50}, above 40 mg/kg). Rats develop, however, extreme cyanosis after 0.5 mg/kg. In 10-μg amounts, the peptide lowers the blood pressure of rats, possibly by releasing histamine. Higher doses produce short-lasting excitation with spasms: the picture is very different from that produced by apamin (*46*).

Mast cell destruction is the earliest visible process resulting from the subcutaneous injection of bee venom in mice. Heparin diminishes the systemic toxicity and hemolytic potency of bee venom. Thus, it has been assumed that mast cell destruction with release of endogenous heparin might protect against the effects of exogenous bee venom or of endogenous (lysosomal) basic polypeptides (*49*).

MCD-peptide consists of 22 amino acids (melittin contains 26). It differs from melittin in containing at least two disulfide bridges, thus resembling apamin. Like apamin, it seems to exist in two arrangements of different basicity, which can be separated by ion-exchange chromatography. The structural formula, as proposed by Haux (*50*), is given in Fig. 3. The position of the two disulfide bridges is still unknown. Positions 3 to 6 of MCD-peptide correspond to positions 1 to 4 in apamin. It should be mentioned that in the three bee venom peptides whose structure is known the carboxyl terminus is in the amide form ($-CONH_2$).

Minimine

Recently (*10*), another "minor" peptide has been prepared from bee venom. It is insoluble in methanol, but soluble in 33 percent acetone. After its adsorption on carboxymethyl-25 Sephadex, it can be eluted with 0.1*M* tris-(hydroxymethyl)aminomethane hydrochloride. About 3 percent of the original bee venom was recovered in this fraction. The basic polypeptide (molecular weight, about 6000) contains all of the usual amino acids. *Drosophila* larvae which survive the LD_{50} (about 0.005 μg) stop feeding and growing and are lethargic, but not paralyzed. They develop into flies as small as one-fourth the normal size, but they are normal with respect to feeding, mobility, and progeny (*10*). In my opinion, minimine should be checked for phospholipase activity, since it appears, during gel filtration, with the beginning of the melittin peak. Phospholipase A elutes in the same region (*7*).

Low Molecular Substances in Wasp Venoms

The formal analogy between bee venom and wasp venom is evident from Table 1. Among the biogenic amines, serotonin is, in addition to histamine, present in wasp [*Vespa vulgaris* (*51*)] and hornet [*Vespa crabro*] venom. Hornet venom contains considerable amounts (about 5 percent, dry weight) of a third amine: it is the most concentrated biological source of acetylcholine (*52*). Without doubt, each of the three amines produces pain. The combination of acetylcholine with histamine is not unique for hornet venom; the stinging nettle uses the same mixture—which has been found to be much more painful than expected by simple addition of the individual components (*53*).

Kininlike substances take an intermediary position between biogenic amines and compounds of high molecular weight. They occur in various wasp venoms (*54*), but only the kinins in polistes venom have been sufficiently purified. The main difficulty in working with wasp venoms is obtaining enough venom. Only a few milligrams of purified wasp kinin have been isolated from the venom apparatus of many thousands of wasps. At least three fractions with kininlike activity have been separated in vespid (*V. vulgaris; V.*

polistes) venoms; there is also evidence for differences between wasp and hornet kinins.

Only one kinin, which accounted for more than 90 percent of the activity, has been obtained in the purified state (polistes kinin 3). The substance behaves like bradykinin in the usual pharmacological tests on smooth musculature and the circulation and is, indeed, a higher homolog of it (Fig. 4). It is activated by trypsin which yields glycyl-bradykinin (*55*). The peptide might be active as such or only after previous hydrolysis to glycyl-bradykinin by enzymes of the envenomated organism. A similar question has been put forth for phyllokinin which is active per se (*56*). The relative potency of the wasp kinin, on a molar basis (if bradykinin is 1), is as follows: rat uterus, 2; rat duodenum, 2; guinea pig vascular permeability, 5; rat blood pressure after intravenous injection, 20, and after intra-arterial injection, 0.5; pain production in man, 10. Suitable experiments with hornet kinin are still lacking. The relative importance of kinins in envenomation awaits clarification. As yet, no peptides similar to melittin, apamin, or MCD-peptide have been found in wasp and hornet venoms.

Enzymes

Animal venoms, especially snake venoms, are sources of many enzymes. Their abundance obscures the correlation of enzymatic and pharmacological events. Hymenoptera venoms can serve as relatively simple model mixtures containing, besides the amines and peptides mentioned, only two or three enzymes: hyaluronidase, phospholipase A, and phospholipase B (Table 1). Generally they can be regarded as agents altering tissue structures by enzymatic hydrolysis, in contrast to melittin action, which is based on physicochemical properties. Needless to say, various interactions can be expected between enzymatic and nonenzymatic venom constituents.

Hyaluronidase. This spreading factor occurs in bee venom in high concentration (about 2 to 3 percent of dry venom) and activity. The crude venom is, by weight, more active than commercial hyaluronidase preparations from bull testes. It differs from similar enzymes by its *p*H optimum in the acid range (*p*H 4 to 5) (*57*), but produces the same split products as does testicular hyaluronidase (*58*). Without possessing remarkable local or systemic toxicity alone, the enzyme opens the way for the other venomous constituents.

Table 4. Different modes of action of histamine releasers (compiled from *46*, *47*, *77*).

Item	MCD-peptide and compound 48/80	Melittin	Phospholipase A
Intact mast cells	+	+	0
Mast cell granula	0	+	+
Mode of action	Triggering of degranulation	Destruction of cellular and subcellular structures	Indirect by formation of the mastocytolytic lysolecithin

Phospholipase A. Phospholipase A of animal venoms hydrolyze the 2-acyl bonds of the natural phosphatidyl cholines, phosphatidyl ethanolamines and phosphatidyl serines as well as those of the corresponding plasmalogens. The bee venom enzyme is also of the A_2 type which has been shown with 1-oleoyl-2-isolauroylphosphatidyl ethanolamine (*59*). The reaction conditions of phospholipases and the chemistry, biochemistry, and physicochemical peculiarities of lyso compounds, have been reviewed (*1*, *13*, *15*, *60*). Pertinent for the understanding of the biochemical pharmacology of phospholipase A are three statements:

1) The enzyme attacks structural phospholipids. Since phospholipids are integral parts of biological membranes, mitochondria, and other cellular constituents, their loss will cause failure of cellular functions requiring such structures.

2) One group of the reaction products consists of the lysophospholipids. Their biologically crucial surfactant character stems from the combination between the hydrophobic aliphatic acyl chain and the hydrophilic phosphorylated base. It is understood that such a molecule not only lowers the surface tension of water and forms micelles, but also solubilizes cholesterol, lecithin, and particulate tissue fractions (*17*). Like melittin, lysolecithin is a "structural poison." In most cases, it is not clear whether (i) the enzymatic destruction of structural phospholipids or (ii) the production of lyso compounds or (iii) both are decisive in the biological activity of phospholipase A.

3) As possibly important intermediary products, the fatty acids released from the β position should not be neglected. Some pharmacological effects of lyso compounds or phospholipase A can be imitated by higher concentrations of fatty acids. Degradation products, such as peroxidized unsaturated acids, behave like slow reacting substances (*61*); nothing is, however, known about the occurrence of peroxidation in vivo.

With respect to the ubiquitous occurrence of phospholipids, one could assume that phospholipase A is a strong, universal toxin. This is, however, not the case. For example, phospholipids built into surfaces of intact red cells are resistant to the enzyme (*6*). There are continuous transitions between such inaccessible structures and highly susceptible suspensions of lipoproteins. The enzymatic attack depends not only on the physicochemical state of the substrate, but also on the kind of phospholipase A used. Enzymes from viperid venoms seem to be less active on structural lipids than those of elapid (cobra) venoms (*15*); the bee venom enzyme seems to be similar to that of cobra venom.

A striking example of the mode of action of phospholipase A and the cooperation between various venom components is the hemolysis caused by bee venom. As mentioned above, melittin is a "direct" hemolysin when mixed with washed human erythrocytes. In contrast, purified phospholipase A does not induce any hemoglobin release at all. Only if accessible substrate is added, such as egg yolk or serum lipoproteins, does the enzyme initiate "indirect" lysis by formation of lysolecithin. Substrate can, however, be made available in another way: Combined addition of melittin and phospholipase A leads to initial "direct" hemolysis, the ghosts becoming accessible to phospholipase A. The resulting lyso compounds render more cells susceptible to phospholipase A, and so on (*26*). The same cooperative action may occur with other biological structures.

The resistance of erythrocytes to phospholipase A contrasts to their sensitivity to the bacterial alpha toxin of *Clostridium perfringens*, which is a phospholipase C. This difference may, however, help in explaining some fundamental properties of biological membranes. The present concepts of their structure rest on the—admittedly to be

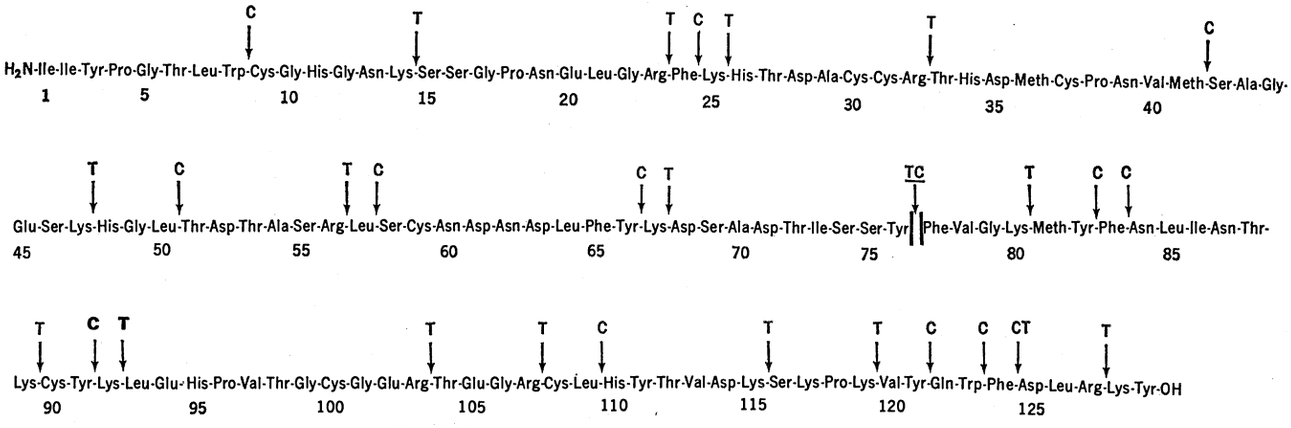


Fig. 5. Sequence of bee venom phospholipase A (*67*).

modified—model of a lipid bilayer. The hydrophobic fatty acid residues are directed inward; the hydrophilic phosphoryl-base groups form the outer surfaces. Such membranes are perhaps sensitive to phospholipase C because the phospholipid molecules offer to the outside just those (glycerophosphoryl) bonds that will be hydrolyzed by this enzyme. The part sensitive to phospholipase A (ester bond in position 2) is hidden in the interior of the membrane, provided that the membrane is intact. The enzymological difference manifests itself in rabbits in that moderate doses of phospholipase A depress the level of plasma phospholipids, whereas hemolysis is not prominent. Phospholipase C, however, produces massive hemolysis without affecting plasma phospholipids to a significant degree. Only with higher doses of phospholipase A (6 to 7 mg/kg, in mice), will the animal be killed by hemolysis and microembolic blood changes. Lysolecithin, originating from plasma lipids, may be involved as considered earlier (*62*).

Phospholipase A is capable of producing a series of pharmacological effects: contraction of smooth muscles, lowering of blood pressure with consecutive tachyphylaxis, increase of capillary permeability (*63*), and destruction of mast cells. Much more prominent, however, are the modifications of structure-bound enzymes or enzyme chains. Without advanced knowledge concerning the composition of animal venoms, their enzymatic activities and especially their interactions with enzyme systems of the envenomated animal were thought to be the cause of death. Bee venom again was the material whose toxic constituents were distinguished from phospholipase. This relatively nontoxic enzyme destroyed succinate dehydrogenase and tissue thromboplastin activity in vitro (*6*). At the same time, it had been shown (*64*) with cobra venom phos-

Table 5. Synopsis of some biochemical and pharmacological properties of bee venom components and lysolecithin [from (*1*)]; ++, strong; +, significant; ?, not yet tested; 0, not demonstrable.

	Histamine	Melittin	Apamin	MCD-peptide	Hyaluronidase	Phospholipase A	Lysolecithin
Content (percent)*	0.1–1	50	2	2	1–3	12	0
Molecular weight	111	2840	2038	2593	⩾ 20000	14500	524
Sulfur content	0	0	++	++	+	+	0
Surface activity	0	++	?	?	0	0	++
General toxicity†	192–445‡	4	4	>40	0	7.5	150
Local toxicity; pain production	++	++	?	?	0	?	?
Increase of capillary permeability	++	++	+	++	Indirectly	+	+
Cellular damage	0	++	?	+§	0	+	++
Neurotoxicity	0	+‖	++	(+)	0	0	0
"Direct" hemolysis	0	++	0	0	0	0	++
Circulatory effects	++	++	0	+	0	+	+
Neuromuscular effects	0	++	0	?	0	0	?
Smooth muscular effects	++	++	0	0	0	+	+
Ganglionic blockade	0	++	0	?	0	0	++
Histamine release	0	++	0	++	0	+	++
"Indirect" hemolysis	0	0	0	0	0	++	0
Thromboplastin inactivation	0	+	?	?	0	++	+
Interruption of electron transport	0	+	?	?	0	++	+
Interruption of oxidative phosphorylation	0	+	?	?	0	++	+
Spreading	0	0	0	0	++	0	0
Antigenicity	0	?	?	?	++	++	?

* Approximately, dry venom. † Milligrams per kilogram, mouse, intravenously. ‡ See (*78*). § Only known for mast cells. ‖ Only known for local application.

pholipase A that only desmoenzymes—that is, particle-bound enzymes—are susceptible, not the lyoenzymes. This was true for the various steps of electron transport in mitochondria, for membrane-bound adenosine triphosphatases and for tissue thromboplastin (*1*). In some of these systems the defect could be repaired by the addition of phospholipids, even of those with a composition different from the original ones. This means that—as a cautiously denaturated protein tends to be refolded into the original arrangement—certain physical forces exist which favor the rearrangement of desmoenzymes into an ordered state (*65*).

It is doubtful that the impressive effects in vitro of phospholipase A play any role in vivo. Envenomated rabbits do not show any significant prolongation of clotting time (*36*); there are, however, still unconfirmed claims concerning the decrease of oxidative phosphorylation in mitochondria of mice intoxicated with cobra venom (*66*). Even membrane enzymes of isolated organs seem to be relatively resistant to phospholipase A, as suggested by a lack of effect on cardiac or skeletal muscle in vitro.

The close cooperation between phospholipase and melittin is evident from Table 5. As direct and indirect cytolysins, melittin and phospholipase A damage erythrocytes or mast cells; both uncouple oxidative phosphorylation, break the respiratory chain, inhibit tissue thromboplastin, and destroy lysosomes. These effects may participate in tissue damage resulting from local application of venom. The venom injected by stinging insects is extremely concentrated (about 30 percent, weight to volume), and hence the components exert actions usually not reproducible by diluted solutions under laboratory conditions.

Bee venom phospholipase has been purified by dialysis, subsequent gel filtration on Sephadex G25, and repeated chromatography on SE-Sephadex G25. The enzyme can be brought to crystallization. Isoleucine has been identified as the amino terminal amino acid. No free sulfhydryl groups exist. The 12 half-cystines of the molecule probably are interconnected by disulfide bridges. One phospholipase molecule contains four glucosamine (or acetylglucosamine) and at least eight mannose residues. From gel filtration or quantitative end group analysis, a molecular weight of 19,000 has been calculated (*59*), whereas amino acid analysis and sequence determination (129 residues) revealed a molecular weight of 14,500. The discrepancy is apparently due to the carbohydrate content of the molecule, which increases the molecular size relevant in gel filtration. Recently, the sequence of bee venom phospholipase A (see Fig. 5) has been established (*67*).

Conclusions

A few years ago, ingredients of venoms were dismissed as mere curiosities without relevance for general biology. Today, the view has changed. Teleologically, venomous animals practice high-level biochemical pharmacology; they have invented a series of very active, specific, pharmacologically and chemically novel drugs that may be useful in elucidating basic mechanisms of central nervous (apamin) or membrane (melittin, phospholipase A, MCD-peptide) functions.

Moreover, most of the components discussed have been observed, admittedly more diluted or modified, in higher animals. Histamine, serotonin, and acetylcholine need no special discussion. The "active region" of wasp kinin is identical (*55*) with bradykinin, the peptide which is the terminal link of the kinin system in the mammalian body. Lysolecithin is a normal constituent of blood and many tissues; it can be produced by phospholipase A present in the body and metabolized by organ phospholipase B or by acylating enzymes (*1*, *15*, *60*). There are many hypotheses and theories concerning the relation between endogenous lysolecithin and membrane permeability, lysosome disruption (*68*), mast cell destruction (*69*), and the pathogenesis of acute pancreatitis (*70*). Lysolecithin is to be regarded as a possible mediator in various kinds of tissue injury.

The same is true for basic polypeptides. Such components have been isolated from cell nuclei and lysosomes (*71*). They are certainly different from melittin which is much more of a detergent. Increase of vascular permeability and mast cell destruction are, however, common effects of lysosomal polypeptides, MCD-peptide from bee venom, and melittin (*72*). It is not premature to postulate that basic polypeptides and phospholipases not only play a role in envenomation, but also in physiological or pathological conditions in mammals.

References and Notes

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Residuals Charges for Pollution Control: A Policy Evaluation

Residuals charges are more effective and efficient than current policies of environmental regulation.

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As environmental degradation has emerged as a public policy issue, interest in alternative strategies for controlling environmental pollution has increased. Our growing awareness of the failure of present pollution control strategies to cope effectively with the problem has contributed significantly to this willingness to examine alternatives (*1–3*). Some of the proposed alternatives would not alter the basic thrust of present policies, but would only do more, do it better (we hope), and do it faster. However, there has also been growing interest in one alternative which would involve a significant departure from the present policies. This is the strategy of creating economic incentives for pollution control by levying taxes or charges on wastes (residuals) discharged to the environment.

Five years ago, this residuals charge strategy was opposed by businessmen, political leaders, and environmental groups. Only academic economists (and some of their number who had infiltrated government) could be found to espouse this position. Yet today, the President and his Council of Environmental Quality, environmental groups, and numerous members of Congress have endorsed this strategy, at least in principle (*4*, pp. 136–139, 287, and 303–305; *5*).

Congress now has before it a wide range of pollution control bills based on an economic incentive strategy. The Administration has proposed both a tax on lead additives in gasoline and on sulfur oxide emissions from fossil fuel combustion (*6*). Since November 1969, Senator William Proxmire has gathered increasing support for his bill to impose charges on industrial effluents discharged to public watercourses (*7*). Charges on effluents causing water pollution have been or are being considered in several states, including Maine, Wisconsin, New Jersey, and Illinois. And Vermont has enacted a modified form of charging for effluents that is designed to accelerate compliance with the provisions of state-issued licenses for the discharge of wastes (*8*). All of these proposals have the following common characteristic: either directly or indirectly they would raise the cost of discharging harmful wastes to the environment. Thus their aim is to induce firms, municipalities, and individuals subject to the charge to curb their discharges of wastes and ultimately to reduce the damages caused by these discharges.

At an abstract level the logic of the argument in favor of residuals charges is impeccable. Even at the practical level of policy implementation, the case for such a strategy appears very strong indeed. Yet despite the increasing interest in the concept and the growing support for specific proposals, the public debate has been clouded by confusion and misconceptions. This has allowed some assertions questioning the efficacy, feasibility, and effectiveness of residuals charges to gain an unwar-

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