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Chemistry and Pharmacology of Honey-bee Venom

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I. INTRODUCTION

The venom apparatus in social insects from the genus *Apis* is an essential part of the mechanism of defence of the colony. Stinging behaviour is usually displayed in the vicinity of the hive or nest rather than at a distance from it, since it is the stores of honey, nectar, pollen and developing bees that are variously the targets for enemies of the colony. Enemies must be recognised rapidly, and a number of factors are probably involved in the recognition process, the prime one being odour. By suspending or moving small objects in front of the entrance to a hive, it has been shown that aggressive behaviour, judged by the number and frequency of stings left in the object, can be induced by smells (e.g. of human sweat), dark colours and jerky movements (Free, 1977; Gary, 1974). A most effective agent is the highly volatile bee pheromone, isopentylacetate, that is released from cells in the sting pouch of the first bee to attack a foreign object and attracts other bees from the hive to join in a concerted attack on the intruder. The frequency of subsequent attacks is thus increased, making a very effective defence. This is important for relatively large predators, that is, birds or mammals, since an effective deterrent can only be provided by multiple stings, the amount of venom released in a single sting being very small. The sting of the bee is barbed and is left in the flesh of larger enemies together with most of the seventh abdominal segment and, of course, the cells that produce the aggression-inducing pheromone so that multiple stinging by other members of the colony will occur. Few honey-loving mammals are to be found in the vicinity of hives, with the exception of man, with whom the bee now enjoys a beneficial partnership. Beekeepers, however, are well aware of the disadvantages of the multiple-stinging defence behaviour.

The majority of the enemies of honey-bees are themselves insects, many of which are of the same order, the Hymenoptera, including ants, wasps, bumble-bees and, of course, honey-bees from different colonies, which are identified by smell. For much smaller victims, a single sting may be lethal and may not involve the mutilation of the so-called guard bee such as occurs on stinging through skin into mammalian flesh. The venom produced by the honey-bee is its principal form of defence and is of greater importance when outside food supplies are short than in times of plenty because the main problem is to defend the hive against predators from other colonies of honey-bees. Curiously enough, although honey-bee venom is now more thoroughly characterised than any other animal venom and given that insect predators, including other bees, are numerically more important than other larger predators, little appears to be known of the action of individual venom components on insects or insect tissues. An exception is the report by Owen *et al.* (1977) that the LD_{50} of histamine for honey-bees is $\sim 3.5\ \mu g$, and that

the histamine content contributes significantly to the toxicity of queen bee venom for other queens. Far more is known of the effects of particular components of venom on mammalian tissues. This may be because for humans, envenomation by bees and wasps is the most common encounter with poisonous species.

For man, bee sting is an uncomfortable experience, the degree of discomfort depending partly on the location of the sting, but most humans survive the experience without irreversible damage. Indeed, man can survive following multiple stings, up to dozens or possibly a few hundred, though this would make one feel very ill if only because of histamine release, as is the case with extensive sunburn. In spite of the unpleasantness of single (and multiple) stings, a potential therapeutic value has been attributed to the venom of the honey-bee, particularly for rheumatic and arthritic conditions. Such 'old wives' tales can be traced back to antiquity, with various bee preparations being advocated for conditions as varied as baldness, toothache, kidney stones, eye diseases and gout. A comprehensive account of the history of 'apitherapy,' as it is called, is given by Beck in his book entitled *Bee Venom Therapy*, published in 1935. Apitherapy is still practised in some parts of the world, particularly in eastern Europe, but a scientific justification for the effectiveness of such therapy is still lacking in spite of numerous efforts to identify pharmacologically active components in the venom. Some 10 years ago, antiinflammatory activity was attributed to a particular bee venom peptide (Billingham *et al.*, 1973), but in the course of subsequent studies it was shown that the presence of this component in the venom could not account for the alleged beneficial effect of bee stings in arthritic or rheumatic conditions (Banks *et al.*, 1980, 1983).

From studies of the composition of bee venom and the properties of individual components comes an understanding of the origins of the unpleasant effects of bee stings but not, sadly, an understanding of its apparent therapeutic properties. On the other hand, some of the venom components have been used extensively as tools in fundamental research on, for example, the properties of natural and synthetic membranes (melittin), the innervation of smooth muscle (apamin) and secretory and antiinflammatory mechanisms (mast cell degranulating peptide), so the efforts that have been involved in characterising the venom of the honey-bee have not been entirely wasted.

Scientific interest in the venom's composition was first apparent in the late nineteenth century (Langer, 1897) but these early studies simply showed that the venom was complex. It was not until the 1950s that Neumann and Habermann (1952, 1954) showed that it was the protein and peptide components of the venom that were associated with biological activity. In 1963, Benton and colleagues introduced an ingenious method of collection

of bee venom, which became available in large quantities as a relatively pure starting material, though no method can be guaranteed to give venom uncontaminated with pollen or other plant products or with bee products not strictly associated with the venom. Since then a number of research teams in Germany, Austria, the United Kingdom, France, Sweden, Canada, the United States, the Soviet Union and Bulgaria have made significant contributions to elucidating the composition of bee venom and examining the pharmacological effects of individual components. Earlier reviews of the field are given by Habermann (1972), Tu (1977), O'Connor and Peck (1978) and Banks *et al.* (1979c).

II. SOURCES OF VENOM AND METHODS OF COLLECTION

The venom system of the honey-bee worker does not function until after the emergence of the bee from its larval state, whereas the queen bee appears to have a fully developed venom supply on eclosion. Given that the bulk of the venom comprises proteins and peptides, the total protein content of the venom sac may reasonably be taken as a measure of maturity of venom production (Owen and Bridges, 1976). In queen-bee venom, which is needed immediately on emergence in order to kill an older queen and sibling queens in rivalry for supremacy of the hive, the protein content is at a maximum in the first (0–3) days of life and declines beyond 7 days, whereas in worker bees the protein content of the venom gland is undetectable on emergence but increases rapidly to 2 days, stays constant up to days 9 or 10 and then abruptly falls by about one-half. The decline of protein content in the queen's venom sac takes place earlier (at about 6 or 7 days) and is more dramatic. The varied patterns of venom development are consistent with the need of queens and workers to fulfill their functions. However, individual components of venom show quite complex temporal relations that are masked in the determination of the total protein content. Melittin, for instance, which is a detergent-like molecule accounting for a large percentage of crude venom, is formed as a larger precursor that is itself inactive. Transformation from the inactive to the active form does not occur in day-old worker bees but increases with maturation up to 20 days. In the queen, on the other hand, conversion of promelittin to melittin occurs on day one (Bachmeyer *et al.*, 1972). Other venom components also vary with the maturation of the bee. Histamine and its presumptive precursor histidine (Owen *et al.*, 1977) are present at peak levels in 35- and 20-day-old bees, respectively, and even in queen bees the histamine levels are low in the first week after eclosion. The change in catecholamine content of bee venom is somewhat similar in time course to histamine (Owen and Bridges, 1982). Hyaluronidase, (Owen, 1979)

a spreading factor, is present at relatively constant levels throughout life, with the queen containing less, as a proportion of total venom, than workers. This may be because the queen attacks insects rather than vertebrates, and a spreading factor is unnecessary if venom is injected straight into haemolymph. Phospholipase A_2 is also present throughout life, but, interestingly, queen bee venom contains very much less of this enzyme than does worker bee venom. While workers and queens are genetically identical, the control of venom composition in the queen honey-bee is obviously quite different from that in the worker, as is the behaviour and function of the two classes or castes (Marz *et al.*, 1981). In general, it can be concluded that the venom of the queen is fully elaborated on emergence while that of the worker develops over the first few days and that the venom glands are most active in the early part of life. Venom production is usually complete within the first 2 weeks and then the glands start to degenerate in the adults. Not only age affects venom composition, but also seasonal factors. The collection of large quantities of venom for fractionation must necessarily involve pooling of materials from bees of different ages so that discrepancies in the details of percentage composition are to be expected. Thus, it is apparent that queen bee venom is considerably different in composition from worker bee venom (Marz *et al.*, 1981).

Much of the early work on venom composition was carried out either on aqueous extracts of the whole stinging apparatus, freed by dissection, or on drops of venom extruded by applying pressure to the abdomen and drawn into fine capillaries. The development of an electric 'milking' procedure in which large numbers of bees are irritated by mild, high-frequency electric shocks on their way out of the hive (and therefore after a period of fasting), causing them to sting through a nylon net onto a glass plate (Benton *et al.*, 1963), has resulted in the collection of venom of a high degree of purity, relatively uncontaminated with plant products. The glass plate is left to dry in a vacuum desiccator, after which the thin film of dry venom is scraped off. Ultrapure venom for quantitative analysis rather than for preparative purposes can be obtained in small amounts by electrical stimulation of individual insects (O'Connor *et al.*, 1963).

Freshly extruded venom is a clear, colourless liquid with a sharp, bitter taste, aromatic odour and acidic reaction. It is readily miscible with water or dilute acid and contains an estimated 12% of solid material. The amount of venom carried by an individual worker bee has been estimated to be of the order of 1 to 2 mg of liquid with ~0.1 mg of dried material per sting (O'Connor *et al.*, 1967). The venom reservoir of the queen bee has been estimated to contain roughly three times as much venom as does the reservoir of a worker bee (Owen *et al.*, 1977).

Dried venom has a powdery appearance and a light yellowish colour. Some

commercial products have a darker, even brownish colour due to photooxidation of biogenic amines, histidine and the tryptophan residues of such components as phospholipase A_2 and melittin. Photooxidation may well occur when venom is allowed to dry in sunlight and results in an apparent heterogeneity of isolated products.

Commercially available bee venom is that of the common European honeybee *Apis mellifera*, and all studies reported here are of components isolated from this source. Little work has been done on the venoms of the other three commonly recognised species (i.e. *A. dorsata, A. florea* and *A. indica*) with the exception of Kreil's observations on the major polypeptide component, melittin, which is found in all four venoms (Kreil, 1973a, 1975).

III. ISOLATION OF INDIVIDUAL COMPONENTS

The separation of individual components is not an easy task because the venom consists of a complex mixture of proteins, polypeptides and low molecular weight aromatic and aliphatic constituents in variable amounts. The main problem arises from the fact that one of the constituents, melittin, is present to the extent of nearly one-half the dry weight of crude material and this makes it difficult to separate the components of similar molecular mass that are present in much smaller quantities. When large-scale separations were attempted, it was found that it was advantageous to carry out an initial pressure dialysis step, dissolving the whole venom in 0.1 *M* acetic acid to 5 to 10% w/v and using a dialysis membrane with a cutoff of 10,000. The dialysate contains less than 10% of the main component (melittin), as this is present mainly as an aggregate (Habermann and Jentsch, 1967; Gauldie *et al.*, 1976), but nearly all the other components are of low molecular mass (Shipolini *et al.*, 1971). Further separation by gel filtration and ion exchange chromatography does not readily produce the homogeneous material necessary for pharmacological testing and therefore further steps are required, the methods depending on the nature of the component of interest and the impurities present (Banks *et al.*, 1981; Kemeny *et al.*, 1981, 1984). Additional problems arise during the purification because of the presence in the venom of formylated derivatives of some of the peptide components. This is true of melittin, peptide 401 (Mast Cell Degranulating Peptide) and apamin (Kreil and Kreil-Kiss, 1967; Doonan *et al.*, 1978). As has already been pointed out, photooxidation during or following collection of the venom may generate spurious components in which the indole or imidazole side chain groups of tryptophan and histidine residues are modified. The resulting apparent

heterogeneity makes it difficult to produce single components of phospholipase A_2, melittin, tertiapin, apamin and of the biogenic amines (R. A. Shipolini, unpublished results). High performance liquid chromatography (HPLC) in both size exclusion (SE) and reverse phase (RP) modes are proving promising for handling small amounts (a few mg) of crude venom or partially purified fractions therefrom (R. A. Shipolini, unpublished results). We have found that a solution of 0.1% trifluoracetic acid (TFA) and 30% methylcyanide is an excellent elution phase for the separation of whole venom or gel filtration fractions, using I-125 or TSK 3000 columns. A reverse phase HPLC system using 0.1% TFA and a linear gradient of *n*-propanol/water (50% v/v) with 0.1% TFA for 100 min on a Zorbox C8 column gives a good separation of all five bee venom peptides listed in Table I.

Peptide 401 (MCD-peptide) produces a double peak with nearly equal amounts of 401-1 and 401-2. When each of these fractions is separated and rerun under the same conditions, each generates double peaks again, suggesting that this peptide exists as an equilibrium mixture of two conformational isomers. This result was also found in proton nuclear magnetic resonance studies of the peptide in solution (Walde *et al.*, 1981).

HPLC may well be the method of choice for the final separation of pure peptides from bee venom, as a criterion of purity of the end products from conventional purification methods, and for the separation of chemically modified forms of individual peptides.

In the case of the higher molecular weight enzyme components, purification methods have been devised to suit the individual enzymes. Phospholipase A_2, the principal allergenic constituent of the venom, was purified to homogeneity by Shipolini *et al.* (1971) and the hyaluronidase by Kemeny *et al.* (1981, 1984). The other enzymes known to be present in the venom are an acid phosphomonoesterase, an α-glucosidase (Shkenderov *et al.*, 1979) and possibly esterases, one or more alkaline phosphatases (Benton, 1967) and a lysophospholipase (Ivanova and Shkenderov, 1982).

Altogether, some 98% of the dry weight of bee venom has been accounted for in terms of known components (enzymes, polypeptides and low molecular weight organic molecules). This does not mean that other components will not eventually be discovered, since some of the known constituents are present as considerably less than 1% of the crude material. The present data on the composition of crude venom of *Apis mellifera* are summarised in Table II, giving reference to the original observations in each case. The table does not indicate the composition of queen bee venom, which is known to differ significantly from that of worker bee venom (e.g. in relation to the phospholipase A_2 content) (Marz *et al.*, 1981).

Table I

High Performance Liquid Chromatographic Separation of Major Bee Venom Peptides

Peptide	Peptide 401		Apamin	Tertiapin	Secapin	Melittin
Retention time (min)	1 24	2 27	29	39	67	73
254/280 nm absorption ratio	3.5		5.0	0.70	0.61	0.59

IV. ENZYMES

A. Phospholipase A_2 (EC 3.1.1.4)

Phospholipase A_2 is an enzyme that catalyses the hydrolysis of natural lipids (Fig. 1), deacylating at position 2 to produce lysophosphoglycerides and long chain fatty acids (systematic name, phosphatide 2-acylhydrolase). (The lysophospholipid product may be further broken down by lysophospholipase.) Both products of reaction are powerful membrane-active agents, that is, they are able to interact with and influence the lipid bilayer structure that surrounds all cells. The turnover of the substance of biological membranes is accomplished largely by enzymes of this class, phosphoglycerides being continually broken down and replaced by newly synthesised molecules.

1. Purification

Phospholipases have been totally purified from a variety of sources including snake venoms (for review, see Rosenberg, 1979; Rosenberg *et al.*, 1983) and mammalian pancreas (de Haas *et al.*, 1968), and the presence of phospholipase A_2 activity in bee venom was first reported by Neumann *et al.* (1952). Several groups of workers attempted to purify the enzyme (Habermann and Neumann, 1957; Munjal and Elliot, 1971) with a homogeneous and crystalline product being achieved by Shipolini *et al.* (1971) after a five-stage purification procedure yielding 13 g of enzyme from 700 g of starting material. This represents an overall yield of ~3% since it is estimated that this enzyme makes up ~12% of the dry weight of crude bee venom. It is the major enzymic constituent. The purification presented some serious difficulties because it appeared that a number of subforms of the enzyme, separable on gel electrophoresis, were present in the crude venom. The origin of the heterogeneity seen on gel electrophoresis is not certainly established. It may be due either to photooxidation of the imidazoles of histidine residues or, as originally suggested (Shipolini *et al.*, 1971), to variation in the carbohydrate content, the molecule being glycosylated and

Table II

The Composition of the Venom of *Apis mellifera*, the common European Honey-bee

Class of Compound	Component	Percentage dry weight	nmol/sting	Reference
Enzymes	Phospholipase A_2	10–12	0.23	Habermann and Neumann (1957)
	Hyaluronidase	1–2	0.03	Habermann and Neumann (1957)
	Acid phosphomonoesterase	1.0	—	Benton (1967)
	α-D-glucosidase	0.6	—	Shkenderov *et al.* (1979)
	Lysophospholipase	1.0	0.03	Ivanova and Shkenderov (1982)
Polypeptides				
	Melittin	40–50	10–12	Neumann and Habermann (1954)
	Melittin-F	0.01	0.003	Gauldie *et al.* (1976)
	Apamin	3	0.75	Habermann and Reiz (1965a)
	Peptide 401 (mast cell degranulating peptide)	2	0.6	Breithaupt and Habermann (1968); Vernon *et al.* (1969)
	Secapin	0.5	0.13	Gauldie *et al.* (1976)
	Tertiapin	0.1	0.03	Gauldie *et al.* (1976)
	Protease inhibitor	—	—	Shkenderov (1973)
	Procamine A, B	1.4	2.0	Peck and O'Connor (1974)
Low molecular weight organic constituents				
	Histamine	0.66–1.6	5–10	Markovic and Rexova (1963)
	Dopamine	0.13–1	2.7–5.5	Owen (1971); Banks *et al.* (1976)
	Noradrenaline	0.1–0.7	0.9–4.5	Owen (1971); Banks *et al.* (1976)

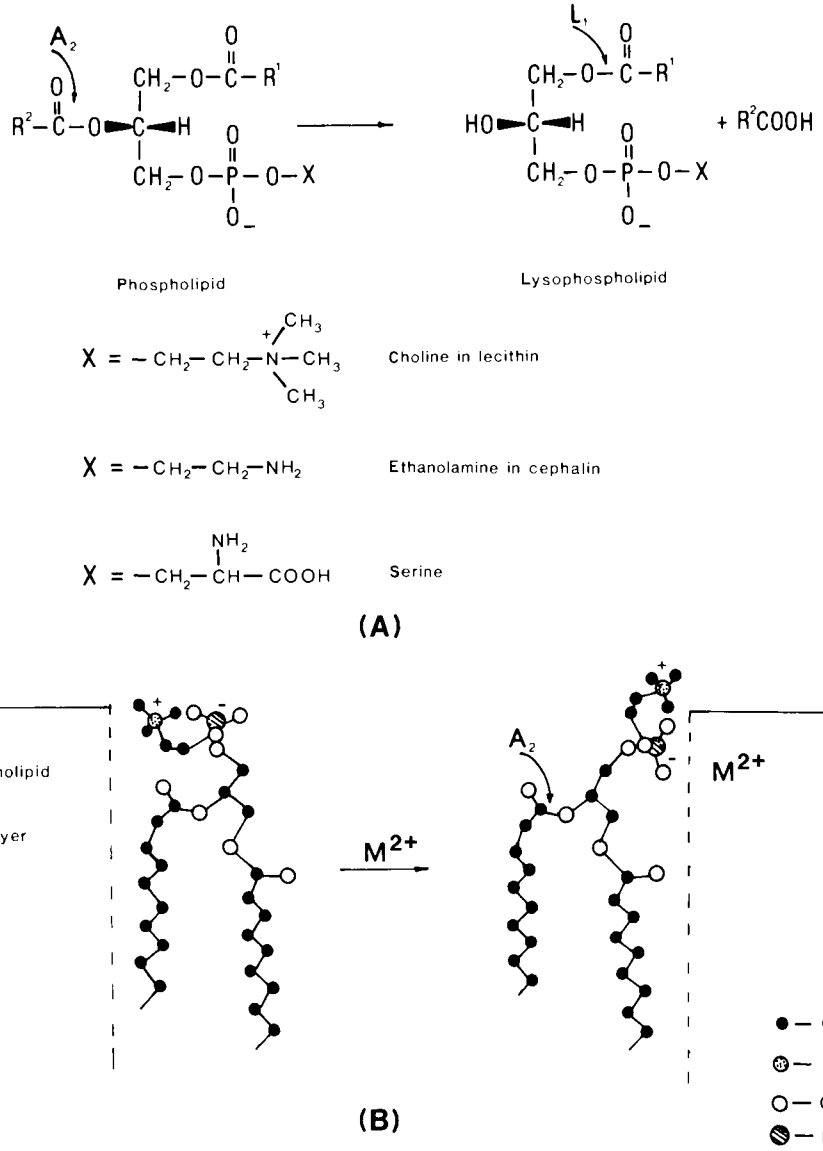


Fig. 1 Phospholipid substrate for phospholipase A_2, designated A_2 in the diagram. In (A), R^1 and R^2 are the fatty acid side chains on the sn-1 and sn-2 positions. Lysophospholipase is designated L_1. In (B), the orientation of the substrate at a lipid–water interface is indicated to illustrate the difference in location of the α-methylene groups on the sn-1 and sn-2 side chains. M^{2+} represents melittin, and the arrow represents a possible change in the orientation of the P-O-X (X in this case being choline) induced by melittin, rendering the substrate more accessible to enzymic attack by phospholipase A_2.

containing fucose, galactose, mannose and glucosamine in a 1:1:8:4 molar ratio.

2. Properties

The primary sequence has been determined (Shipolini *et al.*, 1974a), there being a single polypeptide chain of 128 amino acid residues, cross-linked by four disulphide bridges, the locations of which are illustrated in Fig. 2 (Shipolini *et al.*, 1974b). The carbohydrate moiety is attached at position 13. There are no obvious sequence homologies between the bee venom enzyme and those from pig pancreas and snake venoms though the vertebrate enyzmes are certainly homologous to each other (for review see Dennis, 1983).

The molecular mass calculated from the amino acid composition is 14,555, which increases to 15,800 when the carbohydrate moiety is included. Higher apparent values are found on gel filtration (19,500) and analytical ultracentrifugation (38,500), presumably because of the effect of the carbohydrate component on apparent size in aqueous solution and dimerisation at high concentrations. The molecule as a whole is positively charged, with an isoelectric point of 10.5 ± 0.1. The freeze-dried enzyme is stable and can be kept in the dark at room temperature for several years without substantial loss of activity.

3. Mechanism

Bee venom phospholipase A_2 has the same type of substrate specificity as do similar enzymes from different sources. The fatty acid ester bond to be hydrolysed must be adjacent to an ester-bound, negatively charged, inorganic moiety (phosphate, phosphonate, sulphate). Phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (Fig. 1) are all good substrates. The enzyme shows no marked preference for the nature of R_2 (length of chain or degree of unsaturation). Phosphatidylinositol and sphingomyelin are not substrates. All phospholipases A_2 are activated by calcium and much less effectively by other divalent cations. (Magnesium is partly active, strontium and barium poorly so.) Hydrolytic activity is found for the bee venom enzyme over the pH range 5–9 and at temperatures between 15 and 65°C. The pH optimum is at 8.0 (Shipolini *et al.*, 1971; Munjal and Elliot, 1972).

The purified enzyme has been reported (Neumann and Habermann, 1954) to be practically inactive when tested against washed erythrocytes, suggesting that the cell wall lipids cannot be attacked directly by the enzyme. If an accessible substrate such as egg yolk or serum phospholipoprotein is added to the erythrocyte suspension, the phospholipase generates lysolecithin and

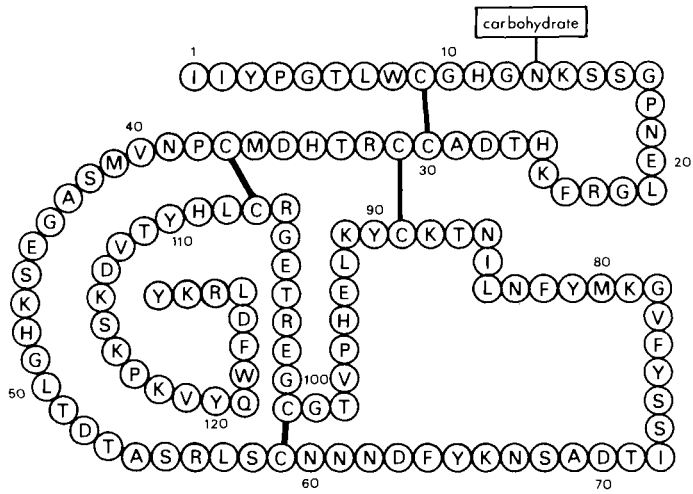


Fig. 2 Primary sequence, disulphide bridge positions and carbohydrate location in bee venom phospholipase A_2. From Tu (1984), by permission of Marcel Dekker, Inc.

fatty acids (see Fig. 1) and these interact with the cell walls, making them susceptible indirectly to attack by the phospholipase. Lysolecithin formed from added accessible substrate initially and later from cell wall phospholipids exchanges freely between cell walls, the surrounding medium (Lawrence, 1975) and between cells (Hax *et al.*, 1974). In contrast, the fatty acid products of phosopholipase A_2 action do not exchange freely between cells but remain incorporated in the bilayer in which they are generated. Within the bilayer they are free to move laterally and to interact with membrane-associated enzymes, possibly modifying enzyme activities. The functional integrity of cell membranes will be lost given hydrolysis of a proportion only of the total phospholipids, the mechanism of the enzyme constituting an autocatalytic cytolytic process which has, interestingly, been adopted in the venoms of a number of poisonous species (e.g. snakes and some insects) and in some bacteria (Alexiev and Shipolini, 1971; Mohamed *et al.*, 1968; Lewis *et al.*, 1968; Rao and Subramanian, 1970; Hayashi and Kornberg, 1954). At the same time, phospholipase A_2 type enzymes are ubiquitous in their tissue and subcellular distribution (Van der Bosch, 1974) and there is strong evidence that they play a regulatory role at the cellular level, since they catalyse a rate-limiting step in the biosynthesis of prostaglandins by controlling the release of the precursor, arachidonic acid. The function of the prostaglandins appears to be as local tissue hormones (Hong and Levine, 1976). The phospholipases appear to modulate membrane adhesiveness (Curtis *et al.*, 1975) and, as has

already been stated, are involved in the turnover of tissue phospholipids (Van Golde and Van den Bergh, 1977). Shier (1979, 1980) has suggested that a transient activation of high levels of endogenous phospholipase A_2 might produce the required alterations of lipid composition of cell membranes resulting in the functional changes observed in regulatory mechanisms associated with prostaglandin actions and in the cytotoxic effects of the direct lytic factor from cobra venom (Fryklund and Eaker, 1973) and of melittin from honey-bee venom (see Section V, A).

Using analytical techniques of high sensitivity for assaying phospholipase A_2 activity (Moores and Lawrence, 1972), it has been found that the bee venom enzyme does hydrolyse tightly packed phospholipids but at a very low rate that remains linear for prolonged periods (Lawrence *et al.*, 1974; Lawrence and Moores, 1975). This contrasts with Habermann's earlier observations (Neumann and Habermann, 1954). Kinetic studies have shown that the enzyme is extensively activated by one of the products of reaction (i.e. the long-chain fatty acids) or, alternatively, by fatty acyl derivatives which modify two nucleophilic sites in the enzyme. The stereochemistry involved in this activation has not been established (Drainas *et al.*, 1978; Drainas and Lawrence, 1978).

It has been found that the phospholipase A_2 and the lytic peptide melittin from bee venom act synergistically, together causing lysis of erythrocytes under conditions in which neither alone is effective (Vogt *et al.*, 1970). The nature of this cooperative action was studied in detail by Kreil and co-workers (Mollay and Kreil, 1974; Mollay *et al.*, 1976), who found that, unlike the fatty acids, melittin does not interact with the enzyme but is incorporated into the lipid bilayer that is the substrate and, in so doing, disturbs the normally close-packed array of phospholipids rendering them susceptible to enzymatic attack (Mollay and Kreil, 1973) (Fig. 1B). The effect is dependent on the amount of melittin present, maximum rate enhancement (300-fold) being found at a ratio of peptide to phospholipid of 1:16 in a system of liposomes (spherical phospholipid bilayer structures formed by fragmentation of larger cellular lipid bilayers) (Yunes *et al.*, 1977). Smaller rate enhancements (5- to 6-fold) were reported for sonicated liposomes at a ratio of melittin to phospholipid of 1:100 (Mollay *et al.*, 1976). The large difference in effect was explained by the disruptive influence of sonication producing a degree of structural disorganisation that greatly reduced the effect of melittin on the lipid structure.

Such synergism gives rise to a very effective cytolytic mechanism and may also be important physiologically for the bees. The activity of phospholipase A_2 will be influenced by the activation of the precursor of melittin, promelittin, in which form the lytic peptide is first secreted into the venom. The molar ratio of phospholipase A_2 to melittin in bee venom is approximately

1:25 and this is close to the ratio used with lipsomes. It is not possible to discuss the pharmacological action of phospholipase A_2 without reference to melittin since the cooperative actions of these two bee venom constituents are, in large measure, responsible for the overall toxicity of bee venom. Most, if not all, cells would be damaged by the two components acting in concert and tissue damage would result from local application of bee venom. It is of interest that pure been venom phospholipase A_2 is relatively nontoxic (LD_{50} ~7.5 mg kg^{-1} iv in mice), death being associated with haemolysis and microembolic blood changes in experimental animals (Habermann and Krusche, 1962). Snake venom phospholipases show marked variations in LD_{50} which have not yet been correlated with any measures of enzymic (phospholipidacyl hydrolase) activity or affinity for phospholipid substrate (Rosenberg *et al.*, 1983).

4. *Immunology*

Clinically, phospholipase A_2 from bee venom is important because this enzyme is the chief allergen in crude venom. Allergy to bee venom is an extreme example of immediate-type hypersensitivity which may be fatal (Barnard, 1973), and interest in the antigenic properties of the high molecular weight protein constituents, phospholipase A_2 and hyaluronidase, dates back to 1956, when Habermann and El Karemi demonstrated the presence of antibodies that neutralised these two enzymes in rabbits immunised by bee venom. Antibodies to the two enzymes were also detected, although at low levels, in the sera of beekeepers (Mohamed and El Karemi, 1961), but it was reported later (Barker *et al.*, 1966) that only hyaluronidase activity was significantly affected (neutralised) by the γ-globulin fraction of beekeepers' sera.

The type of allergic response to bee sting differs from that due to airborne allergens which give rise to such conditions as hay fever and asthma. Inhalation allergy is less immediately dangerous than allergy to injected substances because the cells that respond to the airborne allergens are largely localised in submucosae sited throughout the respiratory tract (Lichtenstein, 1977). In the case of injected allergens, these must pass through the circulation before coming into contact with their target mast cells, but when an individual has been sensitised to an injected allergen, the response is much more dramatic and may prove fatal as blood pressure and heart rate fall dramatically and respiratory failure may result in death (see Chapter 10). As the sensitisation to injected allergens is more dangerous than that to airborne allergens, it is as well that *de*sensitisation (by treatment with small but increasing amounts of allergen to build up the IgG levels in the serum to neutralise allergen before it can reach the IgE-tagged mast cells responsible for the systemic reaction that is so dangerous) works more effectively for injected allergens.

Desensitisation to bee venom constituents has been reported to be highly effective (Lichtenstein *et al.*, 1974; Golden *et al.*, 1982). An interesting comparison of the antibody response to bee venom phospholipase A_2 and inhaled allergens was reported by Kemeny *et al.* (1982).

The anaphylactogenic properties of whole bee venom and of some of its isolated components were studied by Shkenderov (1974), who confirmed that the principal allergens were phospholipase A_2 and hyaluronidase as judged by the ability to produce an anaphylactoid reaction by injection into sensitised guinea pigs. The lower molecular weight polypeptide components were ineffective except in a few individual animals and with the help of Freund's adjuvant.

For those who work with bee venom in the laboratory, the situation is rather different. In using freeze-dried bee venom as a starting material, it is inevitable that the light, friable powder becomes airborne and sensitises a much higher proportion of those working with it than is true of those who are stung by bees. It is an interesting confirmation of the enhanced efficiency of the inhalation route as compared with the injection route for causing sensitisation but extremely distressing for those who suffer the consequences (streaming eyes, sneezing and wheezing) whenever bee venom or its constituent phospholipase A_2 and hyaluronidase and, by inhalation, even the lower molecular weight melittin are exposed to the atmosphere near the sensitive individuals. The majority of workers in the laboratories of E. Habermann, G. Kreil and of C. Vernon have been affected in this way.

Although it is not within the scope of this chapter to deal with the immunology of bee venom in any detail, the fact of sensitisation has influenced progress in the characterisation of the venom and cannot be ignored. Further reference to the immunology will be made in discussions of recent work on hyaluronidase.

B. Hyaluronidase (EC 3.2.1.35)

The presence of a spreading factor in venoms generally is very common (Habermann, 1972). Hyaluronidase (properly called hyaluronoglucosaminidase) catalyses the hydrolysis of the viscous mucopolysaccharide hyaluronic acid, which is present in the interstitial ground substance of connective tissues. Depolymerisation and hydrolysis of the substrate removes a barrier to free diffusion of the toxic substances of the venom, hence the term 'spreading factor', which has been used for more than 40 years. Hyaluronidases were first identified as products from bacteria (Meyer *et al.*, 1960) and later their more widespread occurence in venoms was recognised. The presence of spreading factor activity in bee venom was first noted by Neumann and Habermann (1954) but the enzyme proved remarkably difficult to purify.

1. Purification

Early attempts to separate the enzymes from bee venom were reported by Habermann and Neumann (1957) but clear separations of enzymic activity were not achieved. Barker and co-workers (1966) were more successful using chromatography on a column of alumina after a preliminary separation (by size of components) on Sephadex. They separated phospholipase A_2 activity from hyaluronidase activity but, starting with only 50 mg of crude bee venom, the end products would certainly not be expected to be immunogenically pure. Shkenderov and co-workers (Ivanov *et al.*, 1972; Krysteva *et al.*, 1973) also produced partially purified samples of hyaluronidase, using conventional chromatographic techniques (i.e. gel filtration on Sephadex G-75 and rechromatography of the active material on DEAE-Sephadex). However, the specific activity of the end product reported in the 1973 paper was not noticeably higher than that of the earlier preparation (1972), which was certainly not pure. Later, King and co-workers (1976) produced yet another two-step procedure using Sephadex G-50 and CM-cellulose which again did not yield a homogeneous product, but with a purification factor of ~200. These authors claimed that the enzyme would not withstand lyophilisation, implying a fair degree of instability which, if true, could explain the difficulties in purification. Independently, Dimitrov and Natchev (1977) also reported that the enzyme was unstable when purification on a novel modified cellulose was attempted.

The difficulties experienced by all these workers, with the exception of Shkenderov and possibly King, were primarily due to starting the preparations with far too little crude bee venom. We know that hyaluronidase accounts for only ~2% of the whole venom. So, starting with 50 mg, only 1 mg of enzyme could be produced if the purification produced a 100% yield. Column chromatography is a powerful technique of purification but it must be obvious that the handling losses experienced will be proportionately larger when small amounts of starting material are used due to nonspecific absorption.

In 1981 came the first report of total purification of bee venom hyaluronidase (Kemeny *et al.*, 1981; Kemeny and Vernon, 1983). The work was published in detail in 1984 (Kemeny *et al.*, 1984). Starting with no less than 209 g of freeze-dried bee venom, they eventually prepared hyaluronidase essentially free from phospholipase A_2 activity ($<0.1\%$) after a multistep purification procedure. The end product had a specific activity of 1600 units mg^{-1} in a sample which was said to be pure.

The main problem in preparing pure hyaluronidase was in removing traces of phospholipase A_2. Since the objective was to establish whether hyaluronidase was ever the antigen responsible for sensitisation to bee venom, it was essential to have a sample that was not contaminated with the principal

allergen. The early steps in the purification were quite conventional (i.e. gel filtration and ion exchange chromatography) but later steps used less common column packing materials such as heparin-Sepharose and Sephacryl. Most importantly it was found that residual phospholipase A_2 was only removed by using immunoabsorption over antibody to phospholipase A_2 coupled to Sepharose 4B, and that several passages through such a column were needed to reduce the contaminant to less than 0.1%.

2. Properties

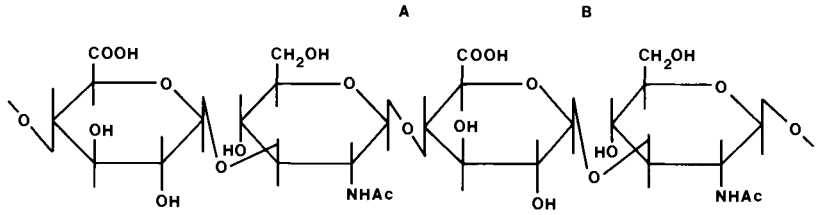
In the papers by Shkenderov's group (Ivanov *et al.*, 1972; Krysteva *et al.*, 1973), it was reported that the hyaluronidase from bee venom had arginine as its N-terminal residue, the amino acid composition was given and the end product was found to contain carbohydrate. In 1976, in a report from Lichtenstein's laboratory (King *et al.*, 1976), the end product, which was unstable, had a molecular weight of ~50,000 and the amino acid composition was given again.

When the enzyme was fully purified it was found to be remarkably stable, withstanding treatment with guanidine hydrochloride and urea, not being precipitated with trichloroacetic acid and resisting short periods of heat treatment at 100°C. The molecular weight is ~35,000 and the N-terminal residue is blocked. The amino acid composition agreed surprisingly well with that reported by King *et al.*, while Shkenderov's observation that the enzyme contained carbohydrate was confirmed. The carbohydrate content was determined and totalled some 7.5%. The enzyme has a high content of the acidic amino acid residues glutamate and aspartate (Kemeny *et al.*, 1984).

The presence of carbohydrate may in part account for the wide variety of molecular weights reported earlier, the highest of which was 60,000 (Dimitrov and Natchev, 1977) and the lowest 30,000 (Sobotka *et al.*, 1976). Glycoproteins tend to behave abnormally on gel filtration, while ultracentrifugation analysis depends on knowing the partial specific volume of the glycoprotein. This cannot be calculated accurately from the amino acid and carbohydrate composition. In the case of hyaluronidase, the partial specific volume has not been measured. The enzyme was reported to be very 'sticky', absorbing strongly to Sephadex G-75 when highly purified. (Kemeny *et al.*, 1984).

3. Mechanism

Hyaluronidases, defined as enzymes catalysing the hydrolysis of the polymer hyaluronic acid, which contains alternating units of *N*-acetylglucosamine and glucuronic acid [β 1:4 linked] (Fig. 3) are divided into



Hyaluronic acid

Fig. 3 Substrate for hyaluronidase. Alternating units of D-glucuronic acid and 2-acetamido-2-deoxy-β-D-glucose, β(1-3)-linked and β(1-4)-linked as shown. Hyaluronoglucosaminidase (bee enzyme) acts at alternate 1:4 linkages (A) while hyaluronoglucuronidase (leech enzyme) acts at alternate 1:3 linkages (B).

two classes depending on substrate specificity, which is either to cleave in alternate positions A, generating the tetrasaccharide glucuronic acid/*N*-acetylglucosamine/glucuronic acid/*N*-acetylglucosamine as the main product (hence the name hyaluronoglucosaminidase) or, in alternate positions B, generating predominantly *N*-acetylglucosamine-glucuronic acid-*N*-acetylglucosamine-glucuronic acid as the tetrasaccharide product (hence the name hyaluronoglucuronidase). The first class (EC 3.2.1.35) of hyaluronidases is also called the testicular type, since mature bull testicles are the major source, the enzyme being located specifically in the spermatozoa. It is characteristic of this class of enzymes that they catalyse the hydrolysis of chondroitin, (a polymer like hyaluronic acid but with *N*-acetylgalactosamine in place of *N*-acetylglucosamine and the sulphated derivatives of chondroitin, the sulphates located on positions 4 or 6 of the *N*-acetylgalactosamine units). Chondroitin and its derivatives are present in skin, so the function of the hyaluronidase from bee venom, which is of this class, is not restricted to spreading the toxin through deep tissues but will start undermining the structure of the dermis. The specificity of the bee venom enzyme was established long before the enzyme was purified (Barker *et al.*, 1963). Other testicular-type hyaluronidases are found in snake venoms, submandibular glands (canine) and lysozomes from liver. The only example of the other class of hyaluronidases (the hyaluronoglucuronidases, EC 3.2.1.36) is that isolated from leeches. This is specific to hyaluronic acid itself. A summary of the earlier work on hyaluronidases is given in Volume 5 of *The Enzymes* (Meyer, 1971).

4. *Immunology*

The main interest in bee venom hyaluronidase lies in its association with hypersensitivity and the occurence of anaphylactic reaction to bee sting. Identification of bee venom allergens has been attempted by a number of

laboratories (e.g. Shepherd *et al.*, 1974; Shkenderov, 1974; Sobotka *et al.*, 1976; Hoffman *et al.*, 1977), but in the absence of immunogenically pure hyaluronidase, free from contamination with the main allergen, phospholipase A_2, the techniques of solid phase radioimmunoassay could not be used to determine the IgE antibody levels in the sera of sensitive individuals. Since these are the most sensitive techniques, the availability of immunogenically pure hyaluronidase was highly desirable. Pure hyaluronidase has been used to demonstrate that while phospholipase A_2 is the major allergen in bee venom and IgE antibodies to this are found in ~90% of sensitive individuals, half of these patients also have IgE antibodies to hyaluronidase. In 17%, the IgE antibodies to hyaluronidase had higher titres than those to phospholipase A_2, and in a small number of individuals (9% of the total number of patients), hyaluronidase appeared to be the only antigen to which IgE antibodies were directed (Kemeny *et al.*, 1983a,b).

C. Acid Phosphomonoesterase (EC 3.1.3.2)

This enzyme, commonly called acid phosphatase, represents ~1% of the dried venom (w/w). The presence of both acid and alkaline phosphatases was reported by Benton (1967) as a result of analysis by disc electrophoresis but the isolation was not reported until later (Shkenderov *et al.*, 1979). Purification by chromatography on SP Sephadex-C25, DE-52 Cellulose and Sephadex G-200 led to a product with acid phosphatase activity that had a strong tendency to aggregate, a pH optimum at 4.8 with *p*-nitrophenyl phosphate as substrate, an isoelectric point at 5.1, contained some carbohydrate (<3%) but, from the amino acid analysis, contained no valine, methionine, leucine or isoleucine. The protein was strongly antigenic. In 1977, Hoffman *et al.* reported the presence of an allergenic component in bee venom that was distinct from phospholipase A_2 and hyaluronidase and had acid phosphomonoesterase activity, but this component contained all four of the common amino acid residues missing from Shkenderov's sample. The acid phosphatase from bee venom has recently been isolated (E. Barboni, M. Kemeny and C. A. Vernon, unpublished results) by ammonium sulphate precipitation, gel filtration on Sephadex G-100 and chromatography on CM Sephadex-C50. A single peak was eluted from the ion exchange column, indicating that the higher molecular weight aggregate of the enzyme certainly does exist (as judged from gel filtration results) and does not differ significantly in charge.

The final material could again be separated into two active fractions by gel filtration. The specific activities of the two fractions were roughly the same. The molecular weights of the two forms as judged by SDS gel electrophoresis are about 45,000 and 90,000. Treatment of the active fraction from CM Sephadex-C50 with dithioerythritol abolished the formation of the

higher molecular weight form, which may therefore be a dimer held by a disulphide bridge. The glycoprotein nature of the phosphatase was confirmed by use of the standard periodate reagent.

März *et al.* (1983) have shown that phospholipase A_2, hyaluronidase and acid phosphatase from bee venom all behave as glycoproteins on chromatography on a column of Sepharose 4B–concanavalin A except that one subform with phospholipase A activity was not retarded on passage through this column and therefore was presumed to be free of carbohydrate. Mannose and *N*-acetylglucosamine but not *N*-acetylgalactosamine or galactose were present, suggesting that the glycoproteins are of the asparagine-linked *N*-glycan type (Montreuil, 1980), as found for the phospholipase A_2 component (Shipolini *et al.*, 1974a).

D. α-D-Glucosidase (EC 3.2.1.20)

The presence of an α-D-glucosidase in the fraction from bee venom that was excluded on gel filtration along with the acid phosphomonoesterase was established by Shkenderov *et al.* (1979). The amount of this enzyme in crude venom was estimated to be 0.6%. The isoelectric point was 4.9, the pH optimum 5.5 using *p*-nitro-α-*D*-glucopyranoside as substrate, the carbohydrate content 2.6% and, as with acid phosphatase, this enzyme was also strongly antigenic. The enzyme is probably associated with the complex of enzymes involved in transforming nectar into honey and may well be present in commercial venom samples only because of the method of collection (i.e. by electric stimulation).

The enzyme is thermolabile and its presence in venom samples can be used as a marker for proper storage conditions.

E. Lysophospholipase (EC 3.1.1.5)

A low phospholipase B activity in bee venom was first detected by Doery and Pearson (1964). The substrate, commonly lysolecithin, is hydrolysed to form glycerophosphocholine, releasing the remaining fatty acid anion from the C_3 position (Refer to Fig. 1). The observation was supported in a brief report from Shkenderov's group (Ivanova and Shkenderov, 1982). A three-step purification procedure gave a product that hydrolysed lysolecithin with a specific activity of $5\mu M\ min^{-1}\ mg^{-1}$ but did not hydrolyse lecithin. The molar mass found by gel filtration was 22,000 ± 2000, the pH optimum 9.0 and the isoelectric point, 8.8. As with the phosphomonoesterase (Shkenderov *et al.*, 1979) the amino acid composition of the lysophospholipase was found by these workers to be odd in that it did not contain tyrosine, methionine or cysteine. A 2.6% carbohydrate content and thermolability were also

reported, as was also the case for the α-glucosidase. No detailed account of this work has yet been published.

The presence in bee venom of an enzyme with phospholipase B activity could enhance the effectiveness of the major enzyme, phospholipase A_2, by removing the lysolecithin product of the reaction catalysed by the latter (see Fig. 1), since phospholipase A_2 is inhibited by lysolecithin at concentrations above 0.2 $\mu g\ ml^{-1}$ (Drainas *et al.*, 1981). In addition, the extra fatty acid anion generated by phospholipase B would be expected to enhance the activation of phospholipase A_2 that is known to occur in the presence of fatty acids (Drainas and Lawrence, 1978).

V. PEPTIDE COMPONENTS

The peptide components of bee venom, that is, those molecules in the molar mass range of 2000 to 6000 are listed in Table II. Together they comprise some 50% of the weight of freeze-dried venom and account for a number of interesting properties of whole venom, although for man and possibly other large mammals, the main problem of bee envenomation lies in the larger allergenic enzymes and not so much in the interesting physiological properties of the peptides. Nonetheless, three of the peptides, melittin, apamin and mast cell degranulating peptide, otherwise known as peptide 401, have been used as tools in a large number of scientific investigations of specific biological phenomena that are not directly related to the effects of bee venom in mammals. Whether these actions are relevant to the effects of bee venom in insects remains unknown since, as has already been pointed out, the effects of bee venom on the common enemies of bees remain largely uninvestigated.

It is not possible to give a comprehensive review of the extensive literature on all the major components in the space available but, for the interested reader, the references will enable more extensive searches of the literature to be accomplished readily.

A. Melittin

Melittin is the major component of bee venom. It was first identified as a direct haemolytic factor by Neumann and Habermann (1954) and its membrane-active properties are most readily observed in the lysis of red blood cells (Habermann, 1972). The peptide was sequenced in 1967 (Habermann and Jentsch) and was shown to contain 26 residues, mostly with hydrophobic or at least uncharged side chains except for the C-terminal sequence of six hydrophilic residues. The molecule therefore has a primary structure reminiscent of the basic form of a detergent-like molecule, rendering

explicable its membrane-active properties. It lowers the surface tension of water (Habermann, 1958) and shows a tendency to aggregate in aqueous solution to form a tetramer (Habermann and Reiz, 1965b). The molar mass of melittin is 2840 but the molecular weight, determined by analytical ultracentrifugation, is 12,500 (Gauldie *et al.*, 1976), which is consistent with a tetrameric form in concentrated aqueous solution at high ionic strength, (the conditions used for ultracentrifugation). The peptide acts synergistically with phospholipase A_2 on phospholipid structures, whether of natural membranes (Mollay *et al.*, 1976) or liposomes (Mollay and Kreil, 1974). Although considerably smaller than phospholipase A_2 (molar mass 17,500), separation of the peptide (about 40–50% w/w of bee venom) from the enzyme is complicated by the tendency of the peptide to aggregate. In order to study the properties of the peptide and the mechanism of its disruptive action on cell membranes, it is obviously necessary to free the molecule from significant contamination with phospholipase A_2 since this has important effects on the structures of cell membranes.

Overall, the significance of the presence of a natural detergent in bee venom and the extent to which it is found is presumably related to its ability to disrupt cell membranes and to act as a spreading factor, facilitating the entry into the bloodstream of bee venom components that are more toxic than is melittin itself [LD_{50} by intravenous injection into mice is in excess of 4 mg kg^{-1} (Habermann, 1972)].

1. *Purification*

Purification procedures have been described by Habermann and Jentsch (1967), Gauldie and co-workers (1976), Mollay *et al.* (1976) Banks *et al.* (1981) and DeGrado *et al.* (1982). The problems of removing traces of phospholipase A_2 were first solved by Mollay *et al.* (1976). They made use of the fact that whereas phospholipase A_2 contains disulphide bridges, melittin has none, so under conditions in which -S-S- bridges are reduced, the structure of melittin is unaltered. Banks *et al.* (1981) made use of the observed strong interaction between melittin and heparin to develop a column chromatographic separation of melittin from two more minor peptides, apamin and peptide 401, by using heparin–Sepharose CL-6B as the packing material. Very high salt concentrations are needed to elute melittin once it has been bound to the column. DeGrado and co-workers (1981) successfully purified melittin by high pressure liquid chromatography in the reverse phase mode, on Zorbax C_{80} columns, eluting with 0.1% phosphoric acid, 0.1 *M* $NaClO_4$ and 56% v/v acetonitrile.

Whatever choice is made of the final steps in the purification, the early steps nearly always involve gel filtration to separate peptides on the basis

of size and ion exchange chromatography (SP-Sephadex, pH 4.2) to separate on the basis of charge. The care with which traces of phospholipase A_2 are then removed depends on the purposes to which the peptide component is to be put. Apart from phospholipase A contamination, it is also necessary for most purposes to remove the α-*N*-formyl derivative of melittin, in which form ~10% of the peptide exists in crude venom (Kreil and Kreil-Kiss, 1967). A more minor, partly degraded form of melittin, melittin-F, (Gauldie *et al.*, 1978) is also present in crude venom, but is less than 1% w/w. It is not known whether this fragment can be separated from melittin on heparin–Sepharose but it would be surprising if it were not, since the affinity of the peptide for the column material depends on hydrophobic interactions.

2. Primary Structure

The amino acid sequence of melittin from *Apis mellifera* venom (Habermann and Jentsch, 1967) is given in Fig. 4. This structure and the structure of the α-*N*-formyl derivative were both confirmed by synthesis a few years later (Schröder *et al.*, 1971; Lübke *et al.*, 1971). The melittins from three other subspecies of *Apis* were also sequenced by Kreil (1973b, 1975), who found that the peptide from *A. cerana* was identical to that from *A. mellifera*, while those from *A. dorsata* and *A. florea* showed, respectively, three and five conservative amino acid substitutions. All three sequences are given in Fig. 4. The substitutions in *A. dorsata* and *A. florea* melittins would not be predicted to cause significant changes in the secondary structures nor the activities of these molecules relative to the melittins of *A. mellifera* and *A. cerana*.

Since the peptide has a linear sequence without disulphide bridges, sequence determination was a straightforward matter by classical methods. The fragment called melittin-F lacks the first seven residues of the native molecule (Gauldie *et al.*, 1976).

3. Crystal Structure

Because melittin is both freely soluble in water and has a powerful effect on natural and synthetic membranes, apparently becoming closely integrated with the lipid components, and has also been shown to form micelles with synthetic detergents (Knöppel *et al.*, 1979), it is probably the smallest representative of the class of so-called amphipilic proteins. These important substances such as apolipoprotein, (associated with high density lipoprotein) and certain polypeptide hormones are all water soluble but can nonetheless show marked affinity for and profound effects on phospholipid vesicles or cell membranes. Knowledge of the crystal structure of such substances might

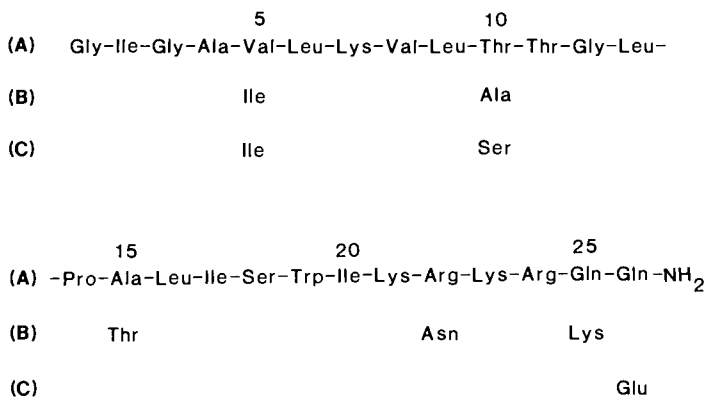


Fig. 4 Amino acid sequence of melittin (A) from *Apis mellifera* and *A. cerana*. Variant residues in melittins from *A. florea* and *A. dorsata* shown in (B) and (C), respectively. From Tu (1984), by permission of Marcel Dekker, Inc.

give some information on the process of the assembly of proteins into essentially phospholipid structures, as must occur in the biosynthesis of functional cell membranes.

The first report of the crystallisation of melittin in forms suitable for high resolution X-ray crystallographic analysis came from Eisenberg's laboratories a few years ago (Anderson *et al.*, 1980). Two crystal forms were grown from solutions of melittin containing ammonium sulphate and sodium formate and the X-ray diffraction patterns indicated that the peptide was present as a tetramer with at least one two-fold axis of symmetry. The crystalline melittin was shown to retain lytic activity against blood cells embedded in agar plates.

From the same laboratory came the full crystal structure analysis (Terwilliger and Eisenberg, 1982a,b; Terwilliger *et al.*, 1982) at 2-Å resolution. The polypeptide chains are packed in a tetrameric unit with a nearly perfect pseudo 222(D_2) symmetry. Each melittin monomer has two α-helical segments running roughly from the first to the tenth residue and from the thirteenth to the twenty-sixth residues (Fig. 5). The proline in position 14 had, on the basis of calculations, been thought to cause a bend in the middle of an essentially rod-like structure, according to Drake and Hider (1979). The helical disposition gives rise to a structure with two distinct faces or surfaces. That of the inner part of the bent rod is very hydrophobic with all the bulky, apolar side chains of valine, leucine, isoleucine and tryptophan while on the outer surface occur the more hydrophilic side chains of serine and threonine, the polar side chains of the N-terminus, the lysine residues and the smaller apolar side chains of residues up to position 20. The C-terminal six residues are all polar. The melittin α-helix therefore has a polar and a nonpolar surface, and within the tetramer subunit interaction is largely

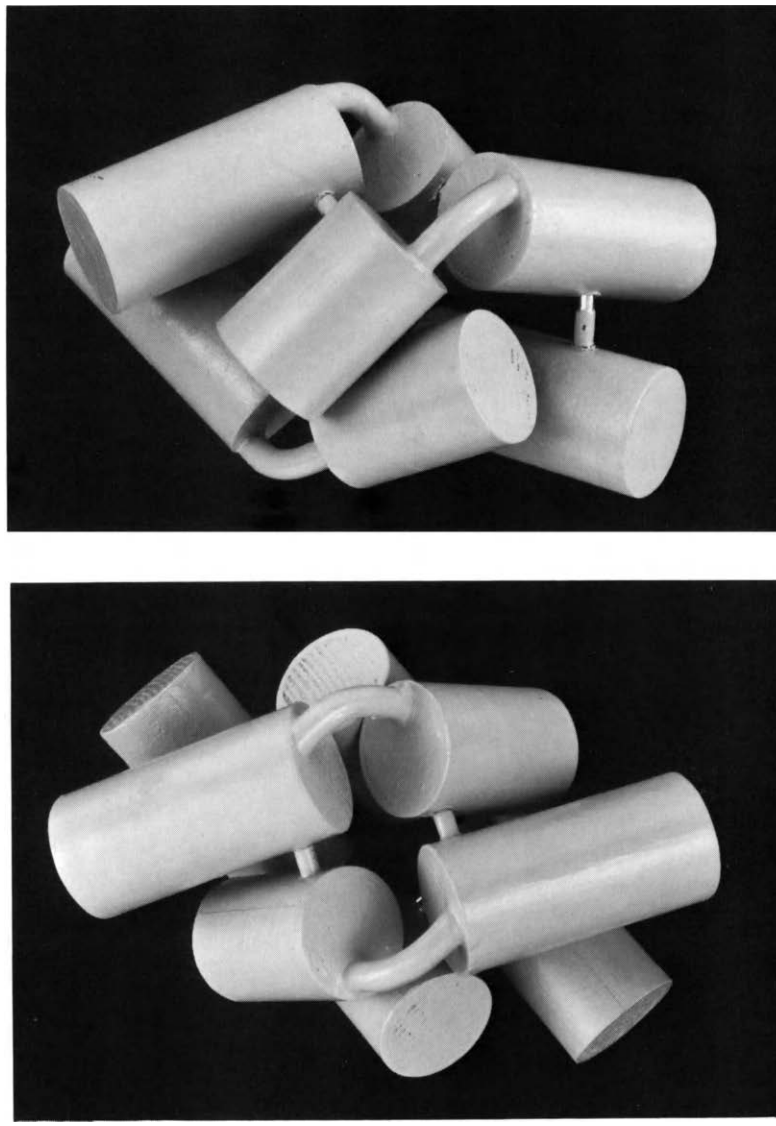


Fig. 5 Model of tetrameric melittin photographed from two angles. Each monomer is represented by two unequal cylinders connected by a bent rod. The cylinders represent the α-helical segments and the polar side chains protrude from the ends of the smaller cylinders.

between the hydrophobic surfaces (Fig. 5). Electrostatic repulsion between the C-terminal sections of the four subunits opposes the hydrophobic interaction forces and the balance is such that the structure of melittin in solution is markedly dependent on concentration, pH, temperature and ionic strength and cannot be deduced from the three-dimensional crystal structure.

4. *Structure in Solution*

The tetrameric nature of melittin at high concentration in solution has already been referred to (Habermann and Reiz, 1965b; Gauldie *et al.*, 1976) but it is also clear that at low concentrations and low ionic strength the molecule is monomeric, with the tryptophan at position 19 totally exposed to the solvent water, as indicated by intrinsic fluorescence measurements. As the concentration of melittin is increased in aqueous solution, aggregates are formed up to a tetramer, leading to a change of environment of the tryptophan residues from aqueous to hydrophobic (Talbot *et al.*, 1979; Faucon *et al.*, 1979) and marked changes in secondary structure as followed by optical rotary dispersion measurements. The conformation of the monomeric form has been studied by techniques of circular dichroism (CD) (Dawson *et al.*, 1978) and high resolution proton nuclear magnetic resonance (NMR) (Lauterwein *et al.*, 1979). Both studies have shown that the polypeptide chain is of an extended, flexible form resembling a random coil but from the proton NMR spectra, it was concluded that two regions of the molecule were more ordered than the remainder, residues 5–9 and 14–20. At concentrations above 4×10^{-3} *M* oligomers are formed in which the N-terminal region of each subunit takes up a more rigid structure held by intrachain hydrogen bonding (Brown *et al.*, 1981), as judged by ^{1}H NMR, while circular dichroism spectra indicate an increase in the α-helical content within the subunits (Dawson *et al.*, 1978; Lauterwein *et al.*, 1979). In the monomer, nearly 90% of each peptide is in the random coil conformation and the remaining 10% is α-helical whereas in the tetramer, ~65% is α-helical and only 20% is a random coil (Talbot *et al.*, 1979). The monomer–tetramer equilibrium is profoundly influenced by ionic strength, high salt concentrations favouring the aggregate and also by the concentration of melittin. Not only does a high ionic concentration favour tetramer formation but phosphate and other ions in solution have been found to stabilise the tetramer (Drake and Hider, 1979; Tatham *et al.*, 1983), with phosphate being the most effective. Another study by Bello *et al.* (1982) has shown that the conformation of monomeric melittin is influenced by pH, with high pH inducing α-helix formation and favouring aggregation. The C-terminal part of the molecule, which is strongly polar, is in a spatial arrangement that is very sensitive to solvent interactions and exhibits large chemical shifts as the

temperature is changed. Because of the apparent flexibility of this part of the molecule in solution, ion–ion or ion–dipole interactions with the immediate environment are probable and the architecture of the tetramer in solution is therefore not predictable from the structure of the tetramer deduced from crystallographic studies.

5. Melittin in Contact with Lipids

The foregoing comments are relevant to the structure and hence the activity of melittin as it exists in the venom sac of the bee. Once released into the victim the melittin is first diluted and then comes in contact with cells, the membranes of which are constructed largely of phospholipid bilayers. Sessa and co-workers (1969) first demonstrated that melittin has a high affinity for a lipid–water interface as represented by lipid monolayers on aqueous solutions. A large number of studies since then have been devoted to melittin–lipid interactions, the entire arsenal of physical techniques used in membrane research being applied to what is a very interesting system, amenable to all manner of experimental approaches. The molecular basis of the interaction, the effect of a lipid environment on the conformation of the peptide and the spatial relations between the peptide, the lipid and the aqueous surroundings have all been investigated.

Monomeric melittin binds very rapidly and with a high affinity to micellar structures of detergents or phospholipids. In the case of phospholipids, the length of the acyl side chain (see Fig. 1) does not significantly alter the affinity constants above 10 carbon atoms but melittin binds to diacyl lipids much more strongly than to monacyl lipids (Mollay and Kreil, 1973; Lauterwein *et al.*, 1979). The physical state of the lipids is profoundly important for binding if the lipid is zwitterionic (e.g. of the phosphatidylcholine type) rather than negatively charged (e.g. phosphatidylglycerol) (Faucon *et al.*, 1979). Binding is facilitated by raising the temperature, giving a semifluid state to the lipid such as exists normally in cell membranes under physiological conditions. On cooling below the transition temperature, melittin already incorporated into a lipid layer is released, suggesting that the main forces anchoring the peptide into the micelle or bilayer are hydrophobic. Similar properties have been attributed to a number of intrinsic membrane proteins (e.g. cytochrome b_5 and cytochrome b_5 reductase).

6. The Effect of Phospholipid on the Structure of Melittin

When bound to lipid or in association with detergent micelles, melittin assumes a predominantly α-helical conformation, as it does when self-associated in a tetramer or in the crystalline state. This conclusion is based

on CD measurements, intrinsic fluorescence and ^{1}H NMR (Drake and Hider, 1979; Faucon *et al.*, 1979; Lauterwein *et al.*, 1979; Brown *et al.*, 1981, 1982) and was suggested earlier as a possibility by Dawson *et al.* (1978) and also by Strom and co-workers in the same year. Interaction with lipid thus induces a structural change in the melittin so that the peptide resembles more closely its structure out of an aqueous environment, which is not unreasonable. The helical structure for residues 1–20 with a bend preceding the proline in position 14 will give the same amphiphilic properties associated with the tetramer but now in a monomer, the inner surface of the bent rod being hydrophobic and presumably predominatly associated with the lipid while the other surface is, or may be, oriented towards the aqueous phase. Such a structure would have a high affinity for a lipid–water interface, with the dipole moment of the helix itself contributing to long-range attractive forces with the phosphate groups of phospholipids (Hol *et al.*, 1978).

Although the structure of melittin associated with lipid is more similar to the structure of the subunit in the tetrameric crystal than that in water, the C-terminal, positively charged residues (C_{21}–C_{26}) have a reduced flexibility because of the proximity of the negative charges of the phospholipids.

7. *The Effect of Melittin on the Structure of Lipids*

Just as lipids markedly influence the structure of melittin, so does melittin alter the structure and hence the function of lipid mono- and bilayers, particularly because of electrostatic interaction between the C-terminal end of the peptide and the polar head groups of the lipids or phospholipids. De Bony *et al.* (1979) studied the binding of melittin to lysophosphatidylcholine micelles using NMR techniques and showed that both monomeric and tetrameric melittin influenced the lipid structure over the restricted range of peptide:lipid ratios examined (0.01–0.1) and found the maximum extent of perturbation in the methylene groups α and β with respect to the ester bond of the lipid. A great deal of work on the effects of melittin on lipid structure has been reported by Mollay and various colleagues, starting in 1976 with observations on the gel–liquid phase transition of dipalmitoyl phosphatidylcholine and the amount of lipid-bound water which, under the influence of melittin at a mole ratio as low as 1 melittin to 100 lipid increases from 10 to 23 mol mol^{-1} of lipid (Mollay, 1976). Most of the physicochemical studies using NMR (De Bony *et al.*, 1979; Lauterwein *et al.*, 1979), infrared and Raman spectroscopy (Verma and Wollach, 1976; Levin *et al.*, 1982), fluorescence spectroscopy (Dufourcq and Faucon, 1977; Faucon *et al.*, 1979; Talbot *et al.*, 1979) and circular dichroism (Strom *et al.*, 1978; Drake and Hider, 1979; Knöepel *et al.*, 1979) have been made at relatively high molar ratios of melittin to lipid but in fact it is in the profound influence of small numbers of melittin molecules on large numbers of lipid molecules that the

real power of melittin as a disruptive agent is to be seen. As a lytic agent, melittin is extremely efficient. At ratios of peptide to lipid of 10^{-4}, melittin causes the release of entrapped substances from phospholipid vesicles. It has recently been shown (Posch *et al.*, 1983) that below a peptide:lipid ratio of 10^{-3}, melittin increases the cooperative phase transition temperature in model membranes whereas at higher peptide:lipid ratios (>1 mol %) the transition temperature is lowered. These results have been obtained by temperature scanning densitometry and indicate the importance of the mole ratio of melittin to the interpretation of results in the many studies of peptide–lipid interaction. The perturbation of highly organised lipid by very low concentrations of melittin has been discussed by Mollay and colleagues (Posch *et al.*, 1983) in terms of a 'cluster' model, a mosaic of lipid clusters within which a single melittin molecule in the centre of a large cluster can perturb adjacent small and large clusters while having a major disruptive effect on the lipid organisation of the cluster into which the melittin is inserted.

At higher mole ratios of melittin:lipid the perturbations of lipid structure are complex, as evidenced by the observations of Levin *et al.* (1982) on temperature effects using Raman spectroscopy. The melittin is apparently surrounded by an immobilised annulus of lipid so that two phase changes can be observed, the one of the bulk of lipid changing from gel to liquid crystal and the other 'melting' or fluidising of the immobilised lipid ring.

The orientation of melittin associated with lipid bilayers has been the subject of much discussion and much of the evidence prior to 1982 favoured the view that the peptide did not span the membrane (Schoch and Sargent, 1980; Brown *et al.*, 1982). Dawson *et al.* (1978) proposed that the bent-rod structure of melittin in its α-helical form acted as a wedge, intruding itself into the membrane. The NMR data of Lauterwein *et al.* (1979) indicate that whereas most hydrophobic residues in melittin change their signals on binding to micelles, most polar groups do not, and this is interpreted as meaning that the interaction of melittin with lipid involves primarily a hydrophobic interaction, leaving the hydrophilic C terminus free and the hydrophilic surface of the amphiphilic helical segments largely free of a hydrophobic environment. It is difficult to reconcile the wedge theory of Dawson *et al.* (1978) with what is now known of the tertiary structure since the rod is bent as in Fig. 6. A wedge attack would require the rod to be bent the opposite way. The presence of protonated amino groups at the aqueous interface is confirmed by pH titration data and the assignment of six positive charges per bound melittin is consistent with the measurements made by Schoch and Sargent of the surface potentials of lecithin bilayers in an aqueous environment. When melittin was added to one side only of a bilayer it apparently stayed on that side. Schoch and Sargent (1980) firmly excluded the possibility that melittin could span the lipid bilayer. Nonetheless, the orientation of the melittin is not unambiguously established and, in any case,

the association is not necessarily static, particularly if one is concerned with the interaction with real membranes as opposed to synthetic bilayers or phospholipid vesicles, since the membrane potential that exists across a natural membrane will surely influence the affinity and interaction between the charged peptide and its fate once bound to the phospholipid. This apart, the results of Vogel *et al.* (1983) on the use of polarised infrared spectroscopy to examine the interaction of melittin with synthetic lipid membranes at low peptide:lipid ratios ($<1:50$) have provided evidence that the polypeptide either spans the bilayer or is present as an inverted wedge. The method gives information about the orientation of the α-helix segments of the peptide, which could in principle be oriented largely at right angles to the lipid plane or largely within it. The results were consistent either with Fig. 6a or (b) (Terwilliger *et al.*, 1982).

The influence of membrane charges on melittin orientation has been addressed by Kempf *et al.* (1982). Using black lipid membranes (bilayers as opposed to vesicles) with melittin added at low concentration on one side, the resulting conductance change (Schoch and Sargent, 1980) was shown to be voltage dependent, increasing with a negative potential trans to the melittin face and decreasing with a positive trans potential. The proteolytic enzyme pronase added to the trans side abolished the conductance with a negative trans potential but not without and it was proposed that the orientation of melittin within the membrane changed under the influence of the trans negative potential so that the N-terminal glycine spanned the membrane as in Figure 6B. Similar observations on the voltage dependence of conductance changes induced by melittin added to one side of a lecithin bilayer were reported by Tosteson and Tosteson a year earlier (1981). The conductance changes at fixed voltage were shown to depend on the fourth power of the melittin concentration, implicating tetrameric melittin in contrast to the models considered by Kempf and by Vogel, but the concentrations of melittin used were rather higher (100 ng ml^{-1}) than in the work of Kempf with black lipid membranes and phospholipid vesicles. A further short report by Tosteson and Tosteson appeared in 1984 favouring the view that melittin forms tetrameric channels through lipid bilayers.

The study, by numerous physico-chemical techniques, of the interaction between melittin and lipids has shown that the lipid influences the structure of the peptide, the peptide influences the structure of the lipid and a potential across a lipid bilayer influences the orientation of the peptide with respect to the lipid. The peptide is extremely potent in its effects, molar ratios of 1 melittin to 1000 lipid molecules giving measureable effects, so one would expect the peptide to influence both the structure and the function of cell membranes *in vivo*.

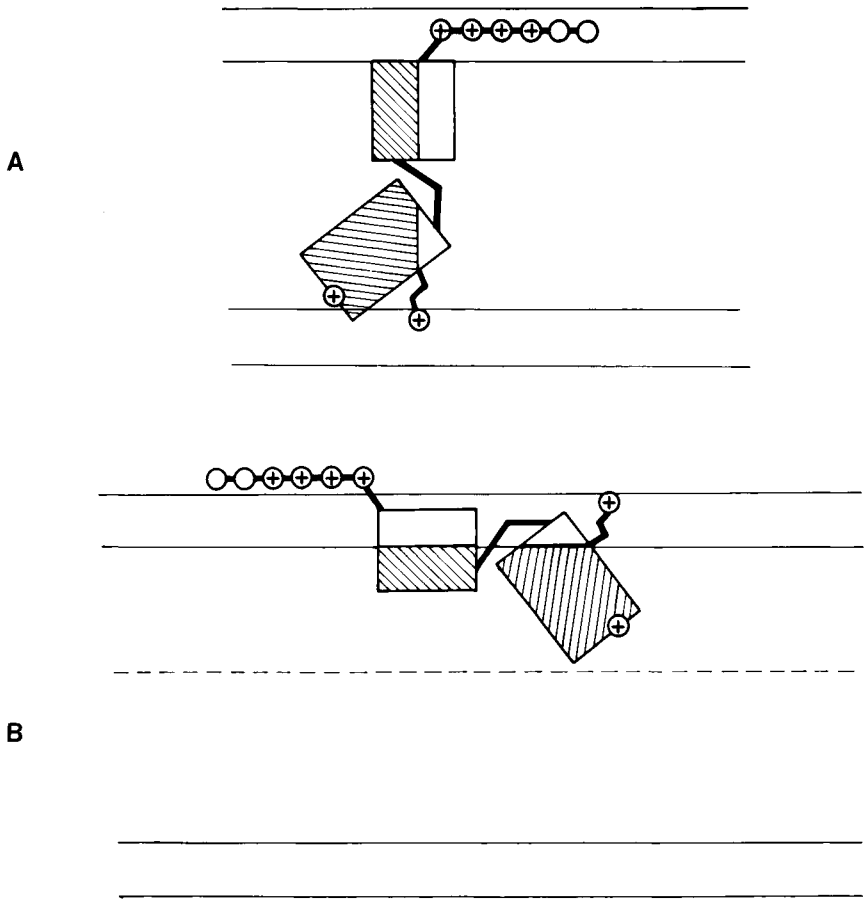


Fig. 6 Orientations of melittin in a membrane environment possibly corresponding to the biphasic leakage of cell contents. The molecule is barely long enough to span the membrane (B) but application of a voltage across the membrane would favour the extended orientation (A). Hatched areas are essentially hydrophobic.

8. The Effect of Melittin on Membrane Function

a. Haemolysis. The surfactant properties of melittin are consistent with its direct lytic effect, most readily observed on red blood cells. The early work, reviewed by Habermann in 1972 and later in 1980 can be summarised briefly as follows.

Native melittin lyses red blood cells causing 100% haemolysis at 10 μg ml^{-1}. Based on the results of chemical modification of the indole of tryptophan 19 and of the primary amino groups, Habermann and Kowallek (1970) concluded that no simple correlation existed between lytic activity and surface activity. The effect of phosphate ions on the lytic activity was reported to be negligible whereas the surface activity was considerably greater in phosphate buffer than in barbiturate buffers, which is of interest in view of the influence of phosphate on the self-aggregation of melittin. It would appear that the surface activity of the tetramer is greater than that of the monomer, whereas the lytic activity does not depend on aggregation. The lack of correlation of lytic and surface activity has been confirmed by studies with synthetic fragments (Schröder *et al.*, 1971). Overall, the surface activity seems to be associated more with the C-terminal part of the molecule, whereas haemolytic activity is typical of the total structure. Formylation of the N-terminal glycine lowers the haemolytic activity to 80% of that of melittin itself, a finding that was made as a result of identification of the formylated compound as a component of the natural venom (Lübke *et al.*, 1971). The existence of the formyl derivative was first suggested by Habermann and Jentsch (1967) and independently in the same year by Kreil and Kreil-Kiss (1967). Without the first six amino acid residues, 70% of surface activity is still observed but the haemolytic activity is reduced to 3% (Schröder *et al.*, 1971). From Hider's group (Dawson *et al.*, 1978) a consistent observation was reported for the naturally occurring fragment of melittin, melittin-F, which is lacking the first seven amino acid residues (Gauldie *et al.*, 1978). This fragment was found to be devoid of lytic activity up to 100 μg ml^{-1}. What influence the loss of the first six or seven residues has on the interaction of the remainder with lipid bilayers or synthetic phospholipid vesicles is a matter for speculation (Dawson *et al.*, 1978), but it could be that its lack of haemolytic activity argues in favour of models in which the melittin actually spans the erythrocyte membrane (see previous section). A shorter peptide might be unable to achieve this.

Much light has recently been shed upon the relation between the structure of melittin and its lytic activity by work from the laboratories of Kaiser and Kezdy based on their interest in the interaction between essentially water-soluble peptides and proteins with amphiphilic surfaces such as those of cell membranes (Kaiser and Kezdy, 1984). Given the α-helical structure of melittin in the tetrameric state and as induced by association with phospholipid vesicles or bilayers, DeGrado *et al.* (1981) pointed out that if the lytic activity depended on one surface of the α-helical bent rod being hydrophobic and the other hydrophilic, so that the molecule could form a stable relation with the cell surface and that the C-terminal polar, cationic residues could then

have a disruptive effect on the order of the phospholipid bilayer structure, it should be possible to make a synthetic peptide with the same properties. The sequence of the first 20 residues was deliberately chosen to give an amphiphilic α helix while the C-terminal hexapeptide was the same as in melittin. Hydrophobic leucine residues were interspersed with gluatmines and serines, and a tryptophan was incorporated at position 19, as in melittin, to facilitate spectroscopic studies. The synthetic peptide analogue had the sequence given in Fig. 7(A), showing that it has no homologies with melittin itself. Projections of melittin and of the analogue are shown in Figs. 7B [(1) and (2)] illustrating the disposition of the leucines on one surface and the hydrophilic residues on the other. The analogue was found to be more effective than melittin as a lytic agent.

The mechanism of haemolysis by melittin and by the synthetic analogue has been studied by Degrado and co-workers (1982), who have shown that, at least at low temperatures, at which, of course, the structure of the red cell membrane will not be the same as under physiological conditions, the kinetics of lysis can be interpreted as reflecting rapid binding of melittin to the cell surface and a transient leakage of some haemoglobin, followed by penetration of melittin into the red cell, where it dimerises and again causes leakage of cell contents in a diffusion-controlled process. The synthetic analogue binds to the red cells as effectively as melittin but apparently dimerises more readily after internalisation, perhaps because it has a higher hydrophobic:hydrophilic ratio in the α-helical segment than does melittin itself. At higher temperatures, also, the interaction of melittin with the red cell membrane is biphasic. These experiments were carried out at relatively high ratios of melittin:red cells (about 10^4–10^5 molecules per cell). It is possible that the mechanism proposed may not hold at lower melittin concentrations but it is reasonable to suppose that the lytic mechanism will be complex.

There is still argument about whether haemolysis is caused by monomeric or tetrameric melittin or both. A report by Knöppel *et al.* (1979) suggested that the tetramer, cross-linked by reaction with dimethylsuberimidate, was equipotent as a lytic agent with the native peptide. The tetramer certainly binds to lipid micelles and membranes (De Bony *et al.*, 1979; Faucon *et al.*, 1979). Habermann's early finding that phosphate buffer increased the surface activity of melittin, particularly at higher concentrations (10 μg ml^{-1}), without altering lytic activity (Habermann and Kowallek, 1970) was made at 0.1 *M* phosphate, which is below the levels used by Hider and co-workers (Drake and Hider, 1979; Hider *et al.*, 1983) of 0.15 to 0.5 *M* to stabilise the tetrameric form. In the later paper, Hider reports that the tetrameric form of melittin is not lytic, having shown that haemolysis did not occur in the

(A)

(1) f-Met-Ala-Gln-Asp-Ile-Ile-Ser-Thr-Leu-Gly-Asp-Leu-Val-

(2) Ala -Ile-Val-Glu-Phe-

(1) -Lys-Trp-Ile-Ile-Asp-Thr-Val-Asn-Lys-Phe-Thr-Lys-Lys

(2) Leu -Ala-Glu- Glu Ile

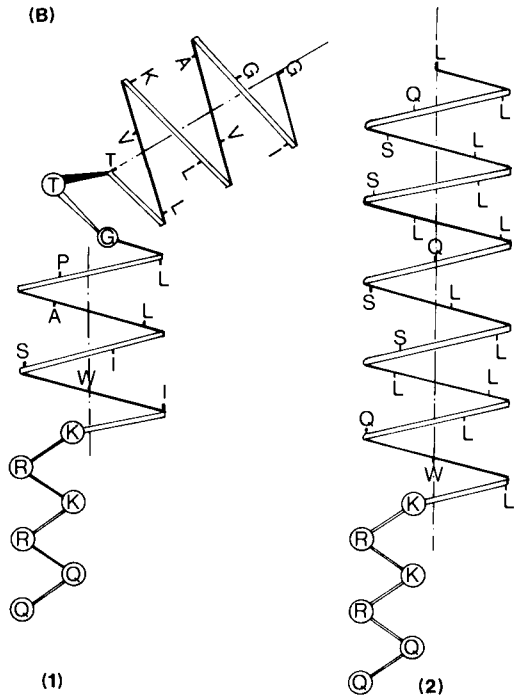
δ- Haemolysin from ***Staphylococcus aureus***

Leu-Leu-Gln-Ser-Leu-Leu-Ser-Leu-Leu- Gln-Ser-Leu-Leu-

-Ser-Leu-Leu-Leu-Gln-Trp-Leu-LysArg-Lys-Arg-Gln-Gln-NH_2

Melittin analogue

(B)



presence of high phosphate concentrations (0.5 *M*). However, he confirmed Knöppel's finding that tetrameric melittin, cross-linked by reaction with suberimidate, was as lytic as native melittin in isotonic saline, but the lytic activity of the cross-linked melittin was not reduced by high phosphate concentrations. Hider has interpreted these observations by suggesting that the suberimidate cross-linked tetramer may be a linearly aggregated entity rather than the tightly packed aggregate that is the native tetrameric form. Other cross-linking agents were found to give mixed products, from none of which could a clear tetrameric entity be isolated (Hider *et al.*, 1983).

In summary, melittin causes lysis of red cells at concentrations of the order of 10^{-6} *M*, but at lower levels (10^{-8} *M*), since it opens ion channels in synthetic lipid membranes (Tosteson and Tosteson, 1981, 1984) and alters ion permeation of black lipid membranes (Kempf *et al.*, 1982), it may well have a disruptive influence on red cell membranes, releasing ions without releasing the larger haemoglobin molecules.

b. Enzyme Activities. As early as 1957, Habermann observed that there was an intimate relation between the actions of melittin and of the enzyme phospholipase A_2. In the presence of melittin, phospholipase A_2 appears to have better access to phospholipid substrates and consistently, first Vogt *et al.* (1970) and then Mollay and Kreil (1974) established that melittin and phospholipase A_2 acted synergistically on erythrocytes and on synthetic phospholipid vesicles or liposomes. The enhancement of phospholipase A_2 activity depended on the fluidity of the phospholipid substrate, on the ratio of melittin to phospholipid up to a value of ~1:150 and on the formation of a hydrophobic complex between the melittin and the substrate. The maximum rate enhancement was about five-fold. With *Escherichia coli* membranes having a very low endogenous phospholipase A_2 activity, addition of melittin (0.13 nmol to 40 nmol membrane phospholipid) caused a four-fold increase in the rate of release of labelled oleic acid from membrane lipid and it was also found that the effect of the melittin was the same for attack by added phospholipase A_2 on the natural *E. coli* membranes and on synthetic phosphatidyl ethanolamine liposomes (Mollay *et al.*, 1976). Melittin also enhances endogenous phospholipase A_2 activity in cultured mammalian cells (Shier, 1979) and it has been suggested that interference with the normal regulatory role of phospholipase A_2, with its catalytic effect on a rate-

Fig. 7 (A) Primary sequences of the haemolysins from *Staphylococcus aureus* isolated from (1) noncanine and (2) a canine source and of the analogue of melittin synthesised by DeGrado *et al.* (1981). From Tu (1984), by permission of Marcel Dekker, Inc. (B) Side views of the disposition of hydrophobic and neutral or hydrophilic residues in the helical segments of (1) melittin and of (2) the melittin analogue.

limiting step in prostaglandin synthesis, may be the underlying cause of the toxic effects of melittin.

Shier has summarised his theory of the action of cytolytic toxins in the publication of the proceedings of a symposium on toxinology held in 1979 in Uppsala (Shier, 1980). Melittin has been found to cause the hydrolysis of up to 60% of labelled lipids in a mouse fibroblast cell line (3T3) by activating the endogenous phospholipase A_2. The activation is critically dependent on melittin concentration, the product (prostaglandins from the membrane-associated prostaglandin-synthesising complex) being detectable within minutes with melittin at $< 10^{-6}$ *M*, but at higher concentrations (3.5×10^{-6} *M*) a decreased rate of arachidonic acid conversion to prostaglandins is found, presumably because of inhibition of fatty acid cyclooxygenase by lysolecithin (Fig. 1). Higher levels of melittin totally inhibit prostaglandin synthesis in the cultured cells, presumably by affecting the lipoxygenase pathway as well as the cyclooxygenase. Melittin does not have these effects at low concentrations in the presence of EDTA, which complexes with calcium. The cytotoxicity of the melittin on these cells is correlated with the ability to activate phospholipase A_2.

That the action of melittin is influenced by calcium is not surprising in view of the involvement of extracellular calcium with general membrane stabilisation but there may be a more specific interaction, since it has been shown that melittin forms a 1:1 complex with the calcium regulatory protein, calmodulin, isolated from bovine brain (Comte *et al.*, 1983). The affinity between calmodulin and melittin is 10^3 times greater than that for other peptide hormones such as ACTH, glucagon or substance P and the intracellular association with calmodulin may be one of the causes of cell death if the melittin does traverse cell membranes. Functionally, melittin has been shown to block the normal activation of brain phosphodiesterase by calmodulin.

Other relevant lytic agents are the direct lytic factor from the venom of *Haemachatus haemachatus* (African Ringhals cobra) (Mollay and Kreil, 1974) and possibly the δ haemolysin from *Staphylococcus aureus*. The latter is a 26-residue peptide of known sequence (Fitton *et al.*, 1984) that is surface active and soluble both in water and in chloroform–methanol and penetrates plasma membranes, causing cytolysis (Colacicco *et al.*, 1977) (Fig. 7A). The ability of the δ haemolysin to form films at air–water interfaces is similar to that of apolipoprotein, the principal polypeptide component of high density lipoprotein that is responsible for stabilisation of lipid in an aqueous (physiological) environment. The δ haemolysin, melittin and apolipoprotein are all membrane active by virtue of their ability to form amphiphilic α helices, a property that is clearly highly significant in other peptide hormones/regulators (e.g. calcitonin, corticotropin-releasing hormone,

glucagon) that interact with cell surfaces. The subject has been reviewed by Kaiser and Kezdy (1984).

Not only is endogenous phospholipase A_2 activity enhanced by melittin but other membrane-bound enzymes have also been shown to be influenced. The adenylate cyclase in rat heart microsomes is activated at relatively low melittin concentrations (<45 μg ml^{-1}) but inhibited at higher levels (Lad and Shier, 1980), while an enzyme involved in the biosynthesis of melittin, the so-called signal peptidase (see next section) that converts prepromelittin to promelittin requires either a detergent, like deoxycholate, or melittin for enzyme activity to be manifest (Mollay *et al.*, 1982).

The toxicity of bee venom is largely due to the melittin it contains, together with the indirect lytic factor, phospholipase A_2, which is activated by the melittin. The approximate concentration of melittin in native venom is 17–21 m*M* and the amount of melittin in a sting is 10–12 nmol. When diluted in the intracellular fluids its concentration could be $\sim 10^{-4}$ *M*, high enough to be lytic and to cause local inflammation, particularly in association with endogenous and exogenous phospholipase A_2.

9. Biosynthesis

Because melittin has such potent membrane activity, it is intrinsically unlikely that it should be made and secreted in its active form since this would be likely to damage the venom apparatus of the bee. This was first pointed out by Kreil in 1971 and since then, work from his laboratories has lead to elucidation of the biosynthetic pathway for this, the major component of bee venom. A summarising article in *New Scientist* in 1978 by Kreil was entitled Biochemical Surprise of a Bee Sting because information about the way in which melittin is made in the form of a much larger precursor, presumably devoid of surface activity, and particularly the ways in which the precursor are processed, eventually producing melittin, are relevant to the biosynthesis of secretory proteins in general, for example, antibody light chains, insulin and albumin, all of which are made as larger, inactive precursors.

In the earliest experiments on the biosynthesis of melittin, radioactive amino acids were fed to worker bees at known times after hatching and, after varying periods of time, the bulk of the melittin in the venom gland was extracted with butanol (an unusual solvent for an unusual peptide) and the remaining aqueous phase was found to contain radioactivity in a component larger than melittin but obviously related to it as judged from the composition of proteolytic enzyme digests. The time course of incorporation of radioactivity into melittin and into the related but larger molecule supported the hypothesis that melittin is formed as a precursor. The precursor is labelled earlier than

melittin; it does not appear in the venom from mature bees but, taken from immature bees, it is converted to melittin by venom gland extracts. It is therefore called *promelittin*. The sequence was attempted in 1973 (Kreil, 1973a), but proved difficult. It was clearly unusual, with a high proportion of acidic amino acids and of proline residues. Attachment of a negatively charged polar segment to the *N*-terminus of melittin would obviously disturb the close association between melittin and phospholipids, thus neutralising its lytic activity.

Only very small amounts of promelittin could be isolated from worker bee venom glands. In order to obtain larger quantities, Kreil turned to the much larger venom gland of the queen bee and from this isolated the messenger RNA, a high proportion of which coded for melittin as the major polypeptide product (Kindas-Mügge *et al.*, 1974, 1976). Injection of the queen bee venom mRNA into frog oocytes gave promelittin as a stable end product (Kindas-Mügge *et al.*, 1974) whereas the promelittin in worker bee venom glands is slowly processed *in situ*, causing considerable difficulty for sequence determination on a heterogeneous mixture of partly processed molecules. In cell-free systems derived from wheat germ, Kreil and co-workers have since shown that the queen bee venom mRNA does not give promelittin as the primary product but an even larger precursor called *prepromelittin* that is presumably too short-lived in the insect venom gland or frog oocytes to be detected. Most additional amino acids are attached to the N-terminal end of promelittin and constitute a so-called signal peptide that is believed to be the N-terminal, hydrophobic sequence of peptides that are made for export or secretion from their site of synthesis inside cells. Examples of these have already been noted. Apart from the signal peptide at the N-terminus of promelittin, (Suchanek *et al.*, 1978) the C-terminus of the melittin is also modified in what is called prepromelittin, there being an extra glycine residue attached whereas the C-terminus in melittin is glutaminamide (Suchanek and Kreil, 1977).

The total sequence of prepromelittin, with 70 residues, is given in Fig. 8. The structure starts with a methionine residue, the common initiating signal for peptide biosynthesis.

It has been estimated that the melittin mRNA represents more than 80% of the total mRNA from queen bee venom glands. Kreil has recently cloned the cDNA from total mRNA and found two cDNA clones that contain the information for synthesis of prepromelittin. The nucleotide sequence of the cloned DNA showed that prepromelittin was the largest polypeptide that could be made by the messenger RNA formed from this cloned DNA, so no larger preprepromelittin is to be expected. It also contained a stop codon or message immediately following the C-terminal glycine found in prepromelittin (Suchanek and Kreil, 1977). This finding is significant because it had been

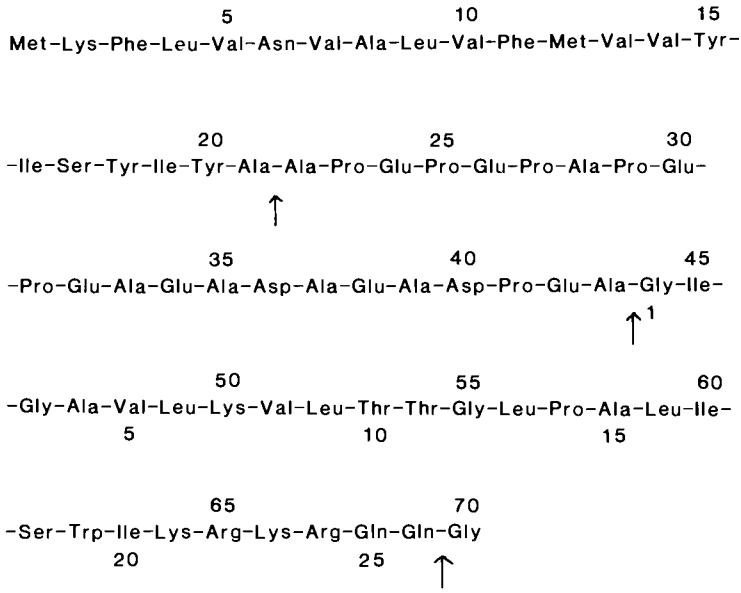


Fig. 8 Amino acid sequence of prepromelittin. The position of cleavage by the signal peptidase (-Ala^{21}-Ala^{22}-), the last cleavage by the dipeptidyl peptidase (-Ala^{43}-Gly^{44}) and removal of the C-terminal glycine to leave glutamine amide are indicated by arrows. The residues of melittin are numbered below the sequence.

suggested that for the formation of C-terminal amides, found quite commonly in functional mammalian peptides such as oxytocin and glucagon as well as in bee venom peptides apamin and 401, a glycine in a precursor peptide was followed by a basic amino acid possibly necessary for amidation of the new C-terminus on removal of glycine. This is clearly not possible for melittin. The question of the formation of C-terminal amides has been discussed by Bradbury *et al.* (1982) who have found a pig pituitary enzyme that converts a C-terminal -X-Gly sequence to X-$CONH_2$.

The question of how prepromelittin is processed after translation to give biologically active melittin has been examined by Kreil and co-workers and has led to other observations that were inconsistent with accepted ideas about the processing of polypeptide precursors in mammalian systems. Apart from the removal of glycine from the C-terminus and the formation of C-terminal glutaminamide, two other steps are involved, the first, presumably preceding C-terminal amidation (since promelittin has only C-terminal glutaminamide), is cleavage of the pre- or signal peptide by breaking the Ala^{21}-Ala^{22} bond and second, removal of the pro-sequence by cleavage between Ala^{43} and Gly^{44}. Most proenzymes and prohormones are converted in a single step to the active forms by enzymes with trypsin-like activity cleaving C-terminal

to lysine or arginine (see, for example, Steiner *et al.*, 1974). In the case of promelittin, the prosequence does not end with a basic residue but with alanine. No endopeptidase with the necessary specificity could be detected in queen bee venom glands and eventually, Kreil and co-workers found that promelittin is processed in an entirely novel way by a dipeptidyl peptidase IV (EC 3.4.14–) removing successive X-Pro or X-Ala dipeptides (X, Glu or Asp). Such enzymes are widely distributed in mammalian cells and that from hog kidney was found to convert promelittin to melittin (Kreil *et al.*, 1980a). Extracts of queen bee venom gland also contained dipeptidyl peptidase activity. Starting with promelittin labelled by feeding queen bees with radioactive proline, stepwise degradation was inferred from observations that the expected dipeptides Ala-Pro and Asp-Pro were released first and last from intact promelittin, that fragments of promelittin with odd numbers of residues preceeding the melittin sequence were not hydrolysed and that melittin itself was not attacked. Added to this, in large scale fractionations of crude bee venom, no peptide corresponding to the intact pro-sequence is found in amounts comparable to or even considerably less than the amount of melittin (Gauldie *et al.*, 1976). Dipeptides are not found in abundance either because the venom gland also contains a dipeptidase.

The significance of these findings in relation to the physiological role of mammalian dipeptidyl peptidases has been discussed by Kreil *et al.* (1980a).

Secreted polypeptides are synthesised with an N-terminal sequence that is known as the signal peptide. The amino acid composition of the signal peptide is hydrophobic and its function is presumably to enable transit of the propeptide across lipid cell membranes. In eukaryotic cells, the signal peptide is cleaved at an early stage in synthesis by a microsomal membrane-bound enzyme that is neither species nor tissue specific. The subject has been reviewed by Kreil (1981).

It has been shown that prepromelittin in the presence of detergents (deoxycholate or melittin) and a rat liver microsomal signal endopeptidase incorporated into phospholipid vesicles generates promelittin and the intact signal peptide (Kreil *et al.*, 1980b; Mollay *et al.*, 1982). The signal peptide was isolated by butanol extraction, a method that will probably be of general value in handling peptides with a high degree of hydrophobicity.

The finding of the intact signal peptide suggests that the signal peptidase is an endopeptidase and that the processing of signal peptides generally occurs in a single step, not by stepwise cleavage. Since other signal peptides have been reported to be very short-lived *in vivo*, (Habener *et al.*, 1979), Kreil's findings with prepromelittin are of considerable importance.

It is rare that work from a single group on one subject proves to be of such importance. Over a 12-year period, the major component of bee venom,

with some help from Kreil and his colleagues, has yielded information on peptide biosynthesis that has general implications. The results are considerably less ambiguous than those relating to the mechanism of action of this lytic agent and have been obtained from a series of extremely elegant experiments that have used the bee to best advantage. That melittin has proved a useful agent is undoubted but now we turn to components of venom that are present in much smaller quantities and for which the purification is annoyingly complicated by the dominant presence of this major detergent-like peptide.

B. Apamin

A dialysate of bee venom containing low molecular weight components was first shown by Hahn and Leditschke (1937) to cause convulsions on injection into mice. The constituent responsible for this activity is the neurotoxic peptide apamin, that was first separated from other components by paper electrophoresis (Neumann *et al.*, 1952) and some properties described (Neumann and Habermann, 1954), although separation on a large enough scale to permit a more complete study was not achieved until the 1960s (Habermann and Reiz, 1964, 1965a,b). At doses above 0.5 mg kg^{-1}, apamin in mice causes uncoordinated movement of skeletal muscles that increases to spasms and convulsions apparently originating from the spinal cord. The time of onset of these symptoms depends on the dose.

The peptide is only a minor constituent of the venom (~2%) (Table II) and with a molecular weight close to 2000 is the smallest of the group of pharmacologically active components of bee venom that have been investigated thoroughly since the 1960s. Since it was associated first with central nervous activities, most attention was paid to this aspect of the pharmacology of apamin, primarly by Habermann who isolated, named and first sequenced the peptide as well as recording its central effects. Since then, Russian workers have reported that apamin has interesting peripheral activities on isolated tissues, smooth muscle in particular (Vladimirova and Shuba, 1978; Shuba and Vladimirova, 1980) and this has led to a proliferation of papers on the pharmacology of apamin, on binding studies using radiolabelled derivatives and on structure-activity relations. The subject is still being actively studied since the mode of action of apamin is far from clear. One complicating factor in recent work is the use by pharmacologists of commercial samples of apamin, apparently without further purification. Freeing apamin from contaminating melittin is no easy task (Shipolini *et al.*, 1967; Banks *et al.*, 1981) and it should be clear from earlier sections that such contamination could well give rise to spurious results, since melittin not only has direct effects on membrane properties but will also activate

endogenous phospholipase A_2 enzymes and may cause indirect disruption of membrane structures. This will surely be particularly important in studies of excitable tissues (nerve and muscle).

1. Purification

Apamin can be isolated from bee venom by a combination of gel filtration and ion exchange chromatography making use of its size and its basic properties. Tandem columns of G-25 and G-50 Sephadex followed by SP-Sephadex C25 at pH 4.2 and repeated rechromatography on SP-Sephadex C25 at pH 4.6 eventually yields a homogeneous product (Gauldie *et al.*, 1976). A simpler procedure has been described (Banks *et al.*, 1981) that makes use of heparin–Sepharose from column chromatography. Melittin has a very strong affinity for this material and both apamin and the mast cell degranulating peptide can be freed of contaminating melittin by a single passage through a heparin–Sepharose column. In the same paper (Banks *et al.*, 1981) a sensitive fluorometric method is described for detecting melittin, which contains tryptophan, in samples of apamin and other peptides (except tertiapin), which do not.

The importance of using apamin free of melittin contamination for all pharmacological studies cannot be overemphasised.

2. Primary Structure and Synthesis

The amino acid sequence of apamin, an octadecapeptide, was determined independently in two laboratories (Haux *et al.*, 1967; Shipolini *et al.*, 1967), with the workers from London showing that the C-terminal histidine residue was amidated and later establishing the positions of the disulphide bridges (Callewaert *et al.*, 1968). The structure is given in Fig. 9. A minor, less basic variant with the same toxicity and amino acid composition precedes the main peak and can be isolated by ion exchange chromatography, though only with difficulty (Habermann and Reiz, 1965a; Gauldie *et al.*, 1976). This has been found to be formylated on the ϵ-amino group of lysine in position 4 (R. A. Shipolini, unpublished observations).

When the disulphide bridges are reduced and allowed to reform under mild oxidative conditions, they return to their original positions (Hartter and Weber, 1975). This observation encouraged attempts in several laboratories to synthesise the peptide, successfully in two cases, namely, by van Rietschoten and co-workers in Marseilles (1975) and Sandberg and Ragnarsson in Uppsala (1978), both groups using solid state synthesis methods. Variants of apamin have also been synthesised in order to identify

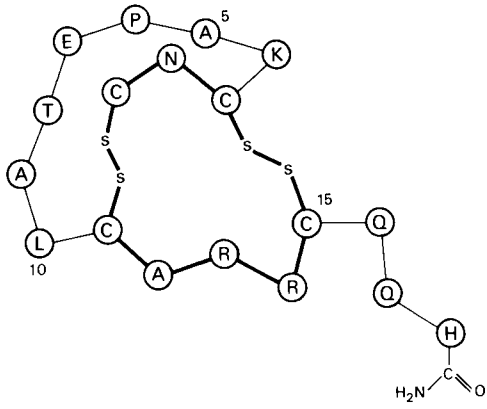


Fig. 9 Structure of apamin.

structurally important residues. Granier *et al.* (1978) made Lys^{13}, Lys^{14} apamin, Lys^{13} apamin and Lys^{14} apamin and Sandberg (1979) added the guanidinated forms of the last two, that is, $homoarginine^{13}$ apamin and $homoarginine^{14}$ apamin, while Cosand and Merrifield earlier prepared $ornithine^{13}$, $ornithine^{14}$ apamin and the guanidinated derivative thereof (1977).

3. Secondary and Tertiary Structure

It has not so far proved possible to prepare crystals of apamin suitable for X-ray diffraction studies. The only information at present available on the spatial conformation is based on spectroscopic studies. The circular dichroism spectra of apamin in solution are consistent with those of peptides with α-helical structure and show a high degree of stability over a wide range of pH and with change of solvent polarity (Miroshnikov *et al.*, 1978; Walde *et al.*, 1981). This contrasts with melittin, the structure of which is totally dependent on environment and presumably is a consequence of the disulphide bridges, which impose rigidity on large sections of the apamin molecule. The circular dichroism spectrum does not change over the concentration range 2.7×10^{-3} up to 4.6 m*M* nor is there any other evidence for aggregation of apamin. ^{1}H-NMR studies give more information about the conformation of apamin in solution (Bystrov *et al.*, 1978, 1980), indicating a rigid, folded structure stabilised not only by the two disulphide bridges but by at least seven intrachain hydrogen bonds, judged by slowness of deuterium exchange. The slowest deuterium exchange and low values of H-NCα-H proton coupling constants were Leu^{10}, Cys^{11}, Ala^{12} and Arg^{13}. Ala^{9} also had a very

low H-N Cα-H coupling constant and these residues were assumed to form an α-helical segment (right-handed), in agreement with both CD and Raman spectra (Efremov *et al.*, 1980). It was shown by model building that only a short section of α-helix could be constructed because of the constraints imposed by the disulphide bridges. A β turn at residues 1 to 3 would orientate the α helix correctly and β turns from residues 2–5 and 12–15 were also proposed (Bystrov *et al.*, 1980). Other weak intramolecular hydrogen bonds might occur, and overall the impression is of considerable rigidity with only the C-terminal glutamine-histidine amide dipeptide showing any degree of flexibility. The imidazole of His^{18} has a normal pK_a (6.5) and is reasonably distant from the guanidino groups of Arg^{13} and Arg^{14}, believed to be functionally significant (see later) (Bystrov *et al.*, 1980; Wemmer and Kallenbach, 1982).

The conformation proposed by Bystrov and his co-workers is based on experimental observations and model building. The small size of apamin had earlier encouraged speculation and calculation of the likely conformation of the molecule based on energy minimisation by residual representation, (Busetta, 1980) atomic representation (Mel'nikov and Popov, 1980) and by secondary structure prediction using Chou and Fasman's rules (Hider and Ragnarsson, 1980) or Levitt's parameters (Hider and Ragnarsson, 1981). The theoretically predicted structures do not fit the observed spectroscopic data as well as does the conformation proposed by Bystrov *et al.*, confirming that caution must be observed in attempting to interpret the CD spectra of small peptides in terms of periodic structure predictions derived for larger molecules that are not so heavily restricted by disulphide bridges (Walde *et al.*, 1981).

4. *Chemical Modification*

A considerable number of more or less well-defined chemical derivatives of apamin have been prepared with two objectives in mind: first, to identify those amino acid residues involved in the neurotoxic and peripheral effects of apamin and second, to facilitate the preparation of radioactive or photoaffinity-labelled derivatives. The early work from Lazdunski's laboratories, from Habermann's group and from Granier and co-workers is summarised by Granier *et al.* (1978). Most attention has been paid to the lysine in position 4, the α-amino group of Cys^1 and to the C-terminal Histidine amide, the last being the most likely site for labelling with ^{125}I in order to produce a derivative of high specific activity. To summarise briefly the results obtained by these workers, only guanidination of Lys^4 left the neurotoxicity unchanged. Reductive alkylation of Lys^4 and Cys^1 reduced the activity to about one-half; acetylation of the same groups lowered the neurotoxicity, the extent depending on the report, Cheng-Raude *et al.* (1976)

claiming a small (20%) reduction and Vincent *et al.* (1975) a rather larger drop (60%). Modification of the histidine amide either by iodination or ethoxyformylation gave a substantial loss of activity.

Derivatives of synthetic analogues were also prepared by Granier *et al.* (1978) and confirmation of the importance of the charged arginine side chains and the distance of the charges from the peptide backbone obtained. Banks *et al.* (1978) and in the following year Habermann and Fischer (1979b) reported the preparation of further derivatives including α-*N*-formyl-ϵ-formyl apamin, which, like the guanidinated Lys^4 derivative, was fully active as a neurotoxin in mice; both were able to displace ^{125}I-labelled apamin from brain homogenates as well as apamin itself. Substitution of the arginine residues in positions 13 and 14 or reduction and carboxamidomethylation of apamin abolished both neurotoxicity and ability to displace labelled apamin from brain homogenates. The importance of the two arginines and of the secondary structure is confirmed.

The question of the relevance of the C-terminal histidine to neurotoxicity is important for preparing the ^{125}I-labelled derivative. The earliest work by Habermann and Fischer (1979a) on the preparation of iodinated apamin did not really lead to a well-defined product though mono- and di-iodinated derivatives are separable by methods depending on the enhanced hydrophobicity induced by one or two iodine atoms. A method for preparing pure mono-iodinated apamin was described by Hugues *et al.* (1982a) involving separation of the mono-iodinated derivative from native apamin and the di-iodinated derivative on a column of SP-Sephadex-C25. The method has been used satisfactorily by other workers (Cook *et al.*, 1983). The iodinated apamin is less toxic than the native molecule, the LD_{50} being roughly doubled (Hugues *et al.*, 1982a) and the product is not very stable. It can, however, be prepared at high specific activity and used for binding studies with few problems of loss of label under conditions used *in vitro*. Some of the earliest studies on the fate of apamin *in vivo* depended on the use of ^{14}C-labelled acetyl derivatives (Vincent *et al.*, 1975); these were later criticized by Habermann (Cheng-Raude *et al.*, 1976) on the ground that the label did not stay on unchanged apamin *in vivo*. The finding of ^{14}C associated with spinal cord material was thought to be spurious.

In order to prepare a stable radiolabelled derivative that does not have a much changed hydrophobic character that may underlie the loss in neurotoxicity found with mono-iodapamin, we have prepared a series of α and ϵ-*N*-mono-formylated and bisformylated derivatives and the corresponding α-*N*,*N*- and ϵ-*N*,*N*-mono and bis-dimethylated compounds. Each has been carefully characterised and tested both for neurotoxicity and for activity in isolated smooth muscle preparations. As a result, α-*N*-formyl-ϵ-*N*,*N*-dimethylated apamin (Dempsey, 1982) was found to have the same

potency in the isolated smooth muscle preparation as native apamin and an LD_{50} within 70% of that of apamin. The tritium-labelled version of this derivative has been used for metabolic tracer studies, the label being introduced by sodium borotritiide in the reductive methylation of the ϵ-amino group of Lys^4. Consistently with Habermann's observations on the handling of polybasic drugs generally (Just *et al.*, 1977), apamin has been found to be concentrated very largely in the kidneys of mice treated with sub-lethal doses of labelled material. No evidence has been found for accumulation in the central nervous system (C. E. Dempsey and H. M. Parsons, unpublished observations).

The effects of various chemical modifications of the native molecule and the activities of synthetic analogues are summarised in Table III.

5. *Pharmacology*

The central effects of apamin, most studied in Habermann's laboratories, have long been well established and have led to the classification of apamin as the smallest known peptide neurotoxin. During the 1970s came a report that apamin has β-adrenergic and antiarrhythmic effects (Vick *et al.*, 1972) but this was hopelessly confused by a later paper from the same group (Vick *et al.*, 1974) given almost the same title but this time claiming that the β-adrenergic and antiarrhythmic properties were associated with a bee venom peptide called 'cardiopep' that was different from apamin according to amino acid composition. ('Cardiopep' has even been referred to in a Hollywood production about killer bees, such was the influence of the reported work.) However, of the very large quantities of *Apis mellifera* venom processed in our laboratories, we have at no time found evidence for a peptide component corresponding to 'cardiopep' and we have demonstrated quite conclusively that apamin does not have β-adrenergic effects, whether on isolated heart (Banks *et al.*, 1979b) or on rabbit papillary muscle (Parsons 1983 and unpublished observations). Although apamin at low concentrations ($10^{-8}M$) enhances the force of contraction of isolated rabbit hearts and, somewhat erratically, of isolated rat hearts and of rabbit papillary muscle, it has no discernible effects on the rates of contraction.

It is more than likely that Vick and Shipman's results were due to using impure fractions that may well have been contaminated with noradrenaline (Banks *et al.*, 1976), especially as the peptide fraction was obtained from bee venom by a single column chromatographic step.

Peripheral effects of apamin were first reported from Shuba's laboratories in 1978. It has long been known that there is an inhibitory nerve supply to the smooth muscle of the gut other than the sympathetic adrenergic ennervation. Russian workers (Vladimirova and Shuba, 1978; Baidan *et al.*,

Table III

Synthetic and Chemically Modified Forms of Apamin[a]

	Reference[b]	Central toxicity[c]	Binding assay[c]	Smooth muscle effects[c]
Synthetic apamin				
Lys^{13}	1	+[d] +[e]	+[d] −[e]	N[d]
Lys^{14}	1	+[d] +[e]	−[d] −[e]	N
$Har^{4}Har^{13}$	1	−	−	N
$Har^{4}Har^{14}$	1	−	−	N
$Lys^{13}Lys^{14}$	1	−	−	N
Tyr^{18}	1[e]	+	+	+
Natural apamin				
Diiodo-His^{18}	2	+	+	+
Monoiodo-His^{18}	3	+	+	+
Har^{4}	4,5	+	+	N
Reduced/reoxidised	1,5	+	+	N
Reduced and carboxamidomethylated	1,5	−	−	N
α-*N*-formyl-Cys^{1}	6	+	N	+
ε-*N*-formyl-Lys^{4}	6	+	N	+
α-*N*,ε-*N*-(bis)formyl-$Cys^{1}Lys^{4}$	6	+	N	+
α-*N*,*N*-dimethyl-Cys^{1}	6	N	N	−
ε-*N*,*N*-diemethyl-Lys^{4}	6	+	N	+
α-*N*,*N*-ε-*N*,*N*-(bis)dimethyl $Cys^{1}Lys^{4}$	6	−	N	−
α-*N*-formyl-Cys^{1}-ε-*N*,N-dimethyl-Lys^{4}	6	+	N	+

(continued)

Table III (*continued*)

Synthetic and Chemically Modified Forms of Apamin[a]

Synthetic apamin	Reference[b]	Central toxicity[c]	Binding assay[c]	Smooth muscle effect[c]
Glu^{7}mehtyl ester	6	N	N	+
ϵ-*N*-amidinated Lys^4	6	N	N	+
α-*N*,ϵ-*N*-(bis)amidinated	6	N	N	−

[a]Central toxicity, competition binding assays and ability to block inhibition of gut smooth muscle where known.

[b]Key to references: 1, Habermann and Fischer (1979b); 2, Habermann and Fischer (1979a); 3, Cook *et al.* (1983); 4, Vincent *et al.* (1975); 5, Banks *et al.* (1978); 6, Dempsey (1982, and to be published)

[c]Activity indicated as + if more than 25% of that of native molecule or − if less.

[d]N, Not known.

[d]Sample supplied by Dr Sandberg.

[e]Sample supplied by Dr Granier.

1978) reported that apamin blocked this nonadrenergic inhibitory nerve supply and the finding was greeted enthusiastically, particularly by those attempting to identify the transmitter in the nonadrenergic, noncholinergic peripheral nervous system (Burnstock, 1972). It has since been shown that apamin does indeed have very powerful effects at nanomolar levels in blocking the inhibitory effects of ATP superfusion on isolated gut smooth muscle preparations, but it also is equally effective in blocking perivascular (adrenergic) stimulated relaxation and acts as a noncompetitive blocker of α-adrenergic agonist inhibition of carbachol-contracted gut (Banks *et al.*, 1979a).

Although apamin is not a specific inhibitor of the noncholinergic nonadrenergic nervous system, it is still of considerable value as a pharmacological tool.

The fourth activity that has been attributed to apamin is as an antiinflammatory agent (Ovcharov *et al.*, 1976). Although confirmation of this effect is lacking, it is interesting to note that in the same paper Shkenderov records that at 10^{-7} *M* apamin caused some contractile response in isolated guinea pig ileum and he first suggested as a result that apamin might not solely by a neurotoxic agent because it appeared also to have 'myotropic' activity.

a. Neurotoxic Activity of Apamin. The neurotoxic effects of apamin can be elicited either by peripheral administration, in which case the LD_{50} for intravenous injection in mice is ~ 4 mg kg^{-1} and in rats about half this value, or by direct injection into the third ventricle in mice or rats, whereupon the same symptoms of uncoordinated movements and hyperactivity are observed and ~ 30 ng are sufficient to kill a mouse (Habermann and Cheng-Raude, 1975). The onset of symptoms following intraventricular administration of apamin is very rapid. Intrachordal injection of apamin, again in nanogram amounts, produces the same symptoms as by other routes and a distinction can be drawn between apamin and the much larger tetanus toxin, which produces its typical effects much more slowly than does apamin and for which peripheral and intrachordal injections produce different symptoms (Habermann, 1977).

From the results of microinjections of apamin into different parts of rat brain and intraperitoneal injection in rats, the spinal cord of which was transected between the sixth and seventh thoracic segments, it has been concluded that apamin poisoning involves both spinal and supraspinal neuronal activities (Habermann and Horvath, 1980). These workers have also studied the effects of apamin on isolated, perfused brain and have mapped the areas of rat brain that are most sensitive to apamin. According to Habermann, the primary influence of apamin is on motor function, rather

than on integrative centres, with the spinal cord and related areas in the brain stem being the primary targets.

That the spinal cord is directly affected by apamin was first demonstrated by Wellhöner (1969) who showed that apamin enhanced the polysynaptic spinal reflexes in the cat. Jurna and Habermann (1983) have shown that small amounts of apamin applied to the exposed surface of rat spinal cord enhanced the discharge of C fibres on stimulation of the sural nerve.

Although much has been established about the neurotoxic actions of apamin, it is still not known whether the peptide attacks particular cell types concentrated within the most sensitive central areas or even if the peptide has neurotoxic actions, in that it actually kills particular neurones. We have found no evidence of neurotoxicity in cultures of embryonic chick cerebral cortex or neonatal mouse cortical cells, even with apamin present in culture at levels far in excess of those eliciting symptoms of apaminism on intracerebral injection (T. E. Wholley, unpublished results). This lack of neurotoxicity can also be deduced from Seager *et al.*'s observations (1984) on ^{125}I-labelled apamin effects on rat embryonic brain cells (mixture of neurones and glia) in culture over a period of days. Spoerri *et al.* (1973), however, reported that apamin did cause biphasic cytological changes in embryonic mouse cortical neurones, neuronal processes first being lost or retracted and later reappearing and becoming longer than normal. The effects of apamin appeared to be primarily on subcellular organelles within the cultured neurones (Spoerri *et al.*, 1975). Spoerri (1983) has also reported that apamin produces morphological changes that resemble nonspecific cytotoxicity in neuroblastoma cells. At micromolar concentrations apamin causes hyperpolarisation of cultured rat embryonic central neurones and increases membrane resistance, but this concentration is considerably higher than that used in experiments on the peripheral effects of apamin, in which it acts at nanomolar concentrations.

In summary, apamin has profound effects on spinal cord function, causing hyperactivity, spasms and convulsions in skeletal muscles and, with lethal doses, death, commonly due to respiratory failure. The central activity is observed on systemic administration as well as on intraventricular injection, so the peptide is presumed to cross the blood–brain barrier. The symptoms of apamin poisoning are the same by either route. Some areas of the central system are more sensitive than others but an exact target has not yet been identified nor is there yet any evidence that apamin causes neuronal death as opposed to a malfunction of overall coordination.

b. Peripheral Actions. Vladimirova and Shuba (1978) and co-workers (Baidan *et al.*, 1978) first described the effects of apamin at nanomolar levels on the hyperpolarisation of smooth muscle of the gut that normally underlies

the inhibitory response of non-adrenergic, non-cholinergic nerve stimulation. The existence of a non-adrenergic inhibitory nervous system in the gut is long established but the identity of the transmitter is still in dispute. The strongest candidate at present for the role is the ubiquitous adenine nucleotide, ATP, first proposed by Burnstock and leading to the term 'purinergic' nervous system. Until 1978, a specific blocker of the purinergic nervous system had not been identified and this was a major stumbling block to the acceptance of Burnstock's hypothesis. With the Russians' report on the activity of apamin as a blocker of the purinergic nervous system at such very low levels came the hope that this problem had been solved but the hope was short-lived. Within 2 years, three separate groups had shown that apamin was not a specific antagonist of the purinergic nervous system but a blocker of inhibition by both adrenergic and purinergic stimulation of intestinal smooth muscle (Banks *et al.*, 1979a; Maas and den Hertog, 1979; Maas *et al.*, 1980; Muller and Baer, 1980). Using guinea pig taenia coli preparations, apamin was found to be a noncompetitive antagonist of α-adrenergic muscle relaxants such as amidephrine and to block the relaxation normally caused by both perivascular (adrenergic) and transmural (purinergic) electrical stimulation at nanomolar levels (Banks *et al.*, 1979a). Both these inhibitory effects are believed to be mediated by increased potassium permeability leading to hyperpolarisation of the smooth muscle membrane (Bennett *et al.*, 1966; den Hertog and Jager, 1975), whereas inhibition of intestinal smooth muscle by β-adrenergic agonists or AMP or adenosine (acting on so-called P_1 receptors as opposed to the P_2 receptors that are more responsive to ATP and ADP) do not involve increased potassium permeability and are not affected by apamin (Jenkinson, 1981; Brown and Burnstock, 1981). The finding of a lack of effect on adenosine relaxation is disputed by Muller and Baer (1980). Nonetheless, because attention was being drawn to changes in potassium permeability as being affected by apamin, Jenkinson and co-workers looked at the effects of apamin on potassium fluxes in systems other than intestinal smooth muscle. Guinea pig hepatocytes release potassium on treatment with α-adrenergic agonists or ATP and so do red blood cells, both changes in potassium flux being triggered by a rise in cytosolic calcium (Haylett, 1976; Burgess *et al.*, 1981), as is probably also the case in smooth muscle (Bulbring and Tomita, 1977). Apamin totally prevented the efflux of potassium from guinea pig hepatocytes in response to amidephrine, whereas the glucose release and calcium efflux that also occur were unaffected. With red blood cells, however, the increase in potassium permeability normally triggered by increased cytosolic calcium levels was unaffected by apamin even at 1 μM whereas 10 nM peptide blocks potassium fluxes from hepatocytes.

Apamin is thus a very potent but highly selective effector of the potassium permeabilities of certain cell membranes. In the case of smooth muscle of

the gut, it appears that apamin treatment at very low levels can convert the normal hyperpolarising response to adrenaline into a calcium-dependent depolarisation (Den Hertog, 1981), and atropine-resistant excitatory junction potentials have also been observed (Bywater *et al.*, 1981; Maas, 1981). Guinea pig colon preparations can be relaxed by the gut peptide, neurotensin. In publications from Lazdunski's laboratories it has been shown that apamin not only blocks this relaxing effect but may convert it to a contraction (Kitabgi and Vincent, 1981; Hugues *et al.*, 1982,a,b). The nonadrenergic, noncholinergic vasodilation following electrical stimulation in cat intestine is also either abolished or considerably diminished by apamin (Jodal *et al.*, 1983), possibly by depressing the release of vasoactive intestinal peptide (VIP) (Sjöquist *et al.*, 1983). The observed peripheral effects of apamin suggest that the central action may also be due to decreased potassium fluxes, since this would broadly reduce inhibitory tone and therefore increase excitability.

6. *Binding Studies*

It is currently accepted that pharmacologically active peptides and amino acid analogues interact with particular components of cell membranes that have been called 'receptors'. These, when they have been identified, are not single molecular entities but complex, aggregates or complexes of nonidentical protein subunits, the best characterised to date being the acetylcholine receptor from electric organs of aquatic species (Changeux, 1981). In order to identify receptors, it is common first to label either an inhibitor of the pharmacologically active substance or the active peptide and to determine the affinity with which the labelled material is bound to membranes prepared from responsive tissue. The first binding studies with labelled apamin and central nervous tissue were reported in 1979 from two laboratories (Habermann and Fischer, 1979a; Cavey *et al.*, 1979) but in neither case was a well-characterised derivative used. In 1982, from Lazdunski's laboratory came the publication of a method of preparing pure monoiodo-apamin and a study of its binding to rat brain synaptosomes, which were found to have a very high affinity for apamin ($K_D \sim 10$ p*M*) but also a low capacity (12 fmol/mg protein) (Hugues *et al.*, 1982a). This confirmed the earlier findings of Habermann with respect to high affinity and low capacity in rat forebrain homogenates, but there was some disagreement over the extent to which binding was dependent on potassium ions. Habermann had found a much more marked dependence with homogenates than appeared to be the case with synaptosomes.

Following the observation that apamin had peripheral as well as central actions, it was possible to make some sense of Habermann's observations that as well as binding to the grey matter of brain, apamin was also found

associated with the liver of rabbits and guinea pigs and with rabbit adrenal cortex. It has since been shown to bind to neuroblastoma cells (Hugues *et al., 1982a) rat myotubes (Hugues et al.*, 1982b) guinea pig colon (Hugues *et al.*, 1982c) guinea pig hepatocytes (Cook *et al.*, 1983) and cultured rat embryonic central neurones (Seagar *et al.*, 1984). Apamin is certainly not exclusively a neurotoxin. The tissues named all show very high affinities and low capacities for apamin, the binding being inhibited by sodium and largely enhanced by potassium up to physiological concentrations. Labelled apamin can be displaced by unlabelled analogues that retain central nervous toxicity (Bank *et al.*, 1978; Habermann and Fischer, 1979b) but not by any other well-established neurotransmitters (Cavey *et al.*, 1979). It was once thought that apamin and the gut peptide neurotensin shared a receptor site since neurotensin could displace labelled apamin from rat brain synaptosomes (Hugues *et al.*, 1982d) but the same workers later showed that the reverse was not true, that is, tritium-labelled neurotensin was not displaced by apamin (Hugues *et al.*, 1982a).

The relation between apamin and vasoactive intestinal peptide (VIP) is still not clear. Apamin is able to displace labelled VIP from some preparations (rat synaptosomes, muscle membranes, pig uterus) but not from others (pig liver membranes) (Fahrenkrug *et al.*, 1983) and it is not clear whether VIP can displace labelled apamin.

The analogues of apamin that have been used in binding studies and the activities of these, both central and peripheral, where known are given in Table III.

7. *Receptor Properties*

Since high-affinity sites in responsive cell membranes have been identified, attempts have been made to isolate the so-called receptor for apamin. Labelled apamin has been cross-linked to synaptic membranes by disuccinimidyl suberate and, following disruption, a labelled protein has been separated by disc gel electrophoresis (Hugues *et al.*, 1982c,d). This component is probably part of a more complex aggregate but it seems to be the primary recognition site for apamin.

It is possible that apamin may be a useful tool for research into cell membrane constituents associated with calcium-activated potassium channels but because its actions are not universal, it is unlikely to be as useful as the puffer fish poison, tetrodotoxin, in its action on sodium channels. Although a roughly parallel relation between effects on potassium flux and binding of apamin has been established for guinea pig hepatocytes (Cook *et al.*, 1983) and (using rubidium in place of potassium) for cultured embryonic rat neurones (Seagar *et al.*, 1984), a role for apamin as a blocker of calcium-

activated potassium channels remains to be universally accepted. It is always easier to publish positive results than negative ones and one wonders how many tissues apamin has been found not to affect apart from red blood cells (Burgess *et al.*, 1981) and pancreatic β-cells (Lebrun *et al.*, 1983), which could reasonably have been expected to be responsive. Likewise, the inotropic effects of apamin on the isolated heart are not readily explained simply by blocking of potassium channels (H. M. Parsons, to be published).

Overall, apamin, since Shuba's finding of peripheral effects, has proved to be an interesting peptide of great potency in certain situations but the mode of action is still not firmly established.

C. Mast Cell Degranulating Peptide (Peptide 401)

It has long been known that histamine is a major factor in the response to bee sting, the reddening, swelling and pain occurring locally being typical histamine-mediated responses. Bee venom itself contains too little histamine for this to be a major contributor (Markovic and Rexova, 1963) but both phospholipase A_2 and melittin, separately and together, will cause histamine release from skin mast cells as a result of cytolytic effects. Over and above the cytolysis of mast cells, another component of bee venom was first recognised by Fredholm (1966) as a potent degranulator of mast cells, that is, an agent that causes release of histamine from the mast cell granules, in which it is stored, by fusion of the granule membranes with the mast cell membranes and exocytosis of the granule contents without lysis of the mast cell. The peptide was first isolated by Breithaupt and Habermann (1968) and its action investigated both by Habermann and by Fredholm and Haegermark (1967, 1969). Its action on skin mast cells has been suggested by Higgenbotham and Karnella (1971) to be beneficial in that the heparin, that is also released on degranulation of the mast cells, may complex with and neutralize the toxic and basic proteins of the venom. Experiments *in vitro* on the effect of heparin on bee venom lend some support to this view but *in vivo* verification of the hypothesis would be difficult. It is of some interest that one of the now-preferred methods of separating the minor peptides from contaminating melittin and from each other uses immobilised heparin as a column chromatographic material (Banks *et al.*, 1981).

Most experimental work on the peptide, named by Habermann mast cell degranulating or MCD-peptide, has been done on rat peritoneal mast cells that are very sensitive to both MCD-peptide and to the synthetic histamine releaser called compound 48/80, the structure of which is given in Fig. 10. As will be seen later, the bee venom peptide and compound 48/80 have much in common.

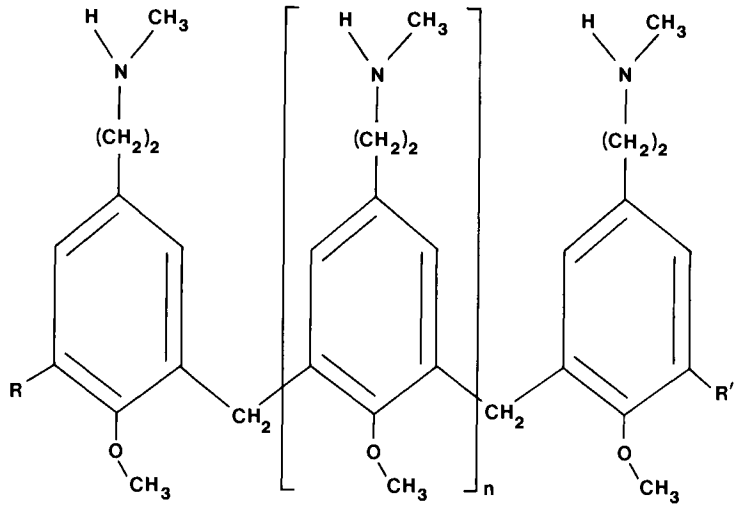


Fig. 10 Structure of compound 48/80.

The peptide isolated and later sequenced by Habermann was simultaneously separated and sequenced in Vernon's laboratories in London (Vernon *et al.*, 1969) but the pharmacological activity established was not that of mast cell degranulation but of anti-inflammatory activity in two rat models of inflammation, namely the carrageenin-induced hind-paw oedema test and in developing and established adjuvant arthritis in rats (Billingham *et al.*, 1973). The London group called the peptide peptide 401 according to the system used in their laboratories, the numbers denoting the position of elution from three successive column chromatography steps with zero indicating elution in the void volume. The system is designed to avoid attributing a single activity to a multifunctional peptide and it seemed for some time in the case of this bee venom peptide to be a wise precaution. When it was realised that peptide 401 was identical to Habermann's MCD-peptide, it was necessary to establish whether the two contradictory activities were in fact independent of each other, that is, whether the systemic anti-inflammatory activity depended on the peptide's ability to cause a local inflammatory response due to histamine release from mast cells. Early results suggested that this was not the case, pretreatment of rats with mepyramine and methysergide to antagonise the effects of histamine and of the 5-hydroxytryptamine also present in rat mast cells being reported to have no effect on the anti-inflammatory activity of peptide 401 (Billingham *et al.*, 1973; Hanson *et al.*, 1974).

These early reports have led to the inclusion of peptide 401 as a possible nonsteroidal anti-inflammatory agent of animal origin in a standard work on inflammation (Smith and Ford Hutchinson, 1979; Billingham and Davis, 1979), and of course have relevance to the old wives' tale that bee venom treatment has therapeutic value for arthritic conditions (Maberley, 1910). The present situation will be examined in a later section.

1. *Purification*

The mast cell degranulating peptide was isolated from bee venom by Habermann using gel filtration on Sephadex G-50 and chromatography on two ion exchange columns (Breithaupt and Habermann, 1968). The peptide is very basic with an isoelectric point above pH 10.5, as is also true of apamin. Two fractions with differing isoelectric points but identical amino acid analyses and mast cell degranulating activity can be detected. The isolation procedures used in Vernon's laboratories were also first dialysis and then gel filtration, but on tandem columns of Sephadex G-25 and G-50 followed by repeated ion exchange chromatography at pH 4.2 and 4.6 on SP-Sephadex C25. Again, two forms of the peptide were detected and proved remarkably difficult to isolate in homogeneous form (Gauldie *et al.*, 1976).

Isolation of the peptide by high performance liquid chromatography has already been referred to in Section III. This also gives two peaks corresponding to peptide 401, but the difference in structure between these is not the same as the difference between the two forms detected by conventional chromatography. The latter was finally shown to be due to the presence of a formyl group on one or other of the lysine residues at positions 2 and 17 (Doonan *et al.*, 1978), whereas the two forms detected by HPLC are not formylated and the difference between them is probably due to differences in the hydrophobicity of conformational isomers.

The behaviour of peptide 401 on gel filtration would suggest a molar mass of 5000 to 6000, which would be consistent with a dimeric form, but there is no other evidence that this is the case.

2. *Primary Structure*

Peptide 401 is a 22-residue peptide, the sequence of which was determined independently and virtually simultaneously by Haux in Habermann's laboratory (1969) and by workers in Vernon's laboratories (1969). The C-terminal residue is amidated and the molecule contains two disulphide bridges, the positions of which were originally assigned by analogy with those found in apamin since the structures looked very similar though the activities were quite different. Eventually, the disulphide bridge positions were confirmed

by a cunning series of cleavages using tryptic digestion rather than the conventional partial acid hydrolysis, followed by Edman degradation under conditions such that disulphide interchange did not occur (Gauldie *et al.*, 1978).

Sequence determination on the reduced and carboxymethylated peptide was possible by Edman degradation up to residue 20. The primary sequence can be seen in Fig. 11, which is a two-dimensional representation showing the positions of the disulphide bridges and emphasising the similarities between this peptide and both apamin (Fig. 9) and the more minor component, tertiapin (Fig. 13).

3. Secondary and Tertiary Structure

As with apamin, the crystal structure of peptide 401 has not so far been reported, but calculations of what the secondary structure might be have been reported by Hider and Ragnarsson (1981), who arrived at a nearly spherical model with positive charges distributed evenly over the surface. A similar globular structure was predicted by conformational analysis using the residual representation method (Busetta, 1980).

Experimental evidence for the secondary structure of peptide 401 comes from circular dichroism and ^{1}H-NMR methods (Kudelin *et al.*, 1979; Walde *et al.*, 1981; Wemmer and Kallenbach, 1982). The circular dichroism (CD) spectra show a close resemblance to those associated with α-helical peptides with considerable stability over the pH range 2–8. Since the peptide does not have a free carboxyl group this was interpreted to mean that the ionisation of the two histidine residues (pK_a 5.7 and 6.1) is not involved in stabilising the secondary structure in solution. Intrachain hydrogen bonds were found by ^{1}H-NMR methods, suggesting the involvement of at least six amide protons. A short α-helical region, probably from positions 13 to 18 or 19, was proposed.

Change of polarity of the solvent gave only small variations in CD spectra, consistent with minimal conformational transitions, probably due to a cluster of side chains partially exposed to the solvent. Overall, hydrophobic interactions were not thought to contribute significantly to the stability of the conformation in solution though they might facilitate dimer formation. Since the CD spectra showed no concentration dependence in the range 2 μM–2 mM, dimerisation is unlikely to occur. A moderate temperature dependence of the CD spectra has been interpreted as indicating some degree of flexibility in the structure, rather more than is the case with apamin. The reduced peptide had the CD spectrum of a typical random coil conformation.

A doubling of the number of histidine proton peaks expected in the ^{1}H-NMR spectrum of the oxidised, but not the reduced, peptide was interpreted

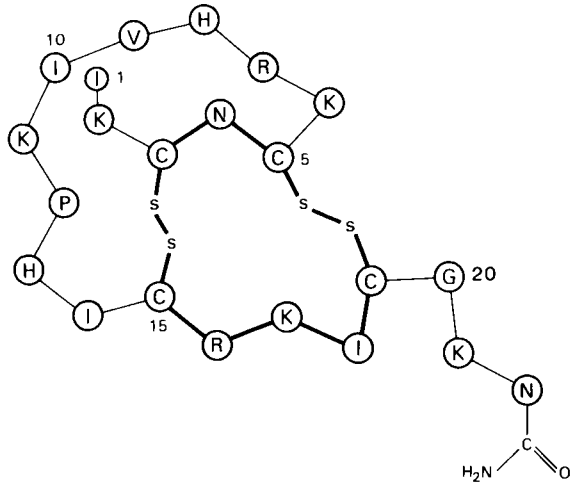


Fig. 11 Structure of peptide 401.

as meaning that the peptide has two closely related conformations in solution, possibly differing by *cis–trans* isomerism about the Pro^{12} His^{13} bond (Walde *et a.*, 1981). This was suggested by analogy with angiotensin and related peptides (Liakopoulou-Kryiakides and Galardy, 1979) in which satellite peaks associated with the histidine also occur. Cis–trans isomerism about the Pro^{12}-His^{13} bond would be required to induce two conformational states of the whole molecule or at least of residues 7–14 since both histidines show satellite peaks. Lauterwein and co-workers (1980) have reported similar observations for melittin, the γ-methyl groups of isoleucine showing satellite peaks attributed to two conformations of the monomer.

The major force in stabilising the conformation of peptide 401 in solution is the presence of two disulphide bridges. They form a 28-atom ring structure that is a common feature of apamin, peptide 401 and tertiapin. In order to form these bridges, residues 3–5 and 15–19 must adopt an appropriate secondary structure. The first of these sequences has a high tendency for a β turn and the second for α-helix formation.

4. *Synthesis*

The synthesis of peptide 401 was reported by Birr and Wengert-Müller (1979). The product had only 35% of the mast cell degranulating activity of the native peptide or its formylated derivative and was obtained in small quantities only. Other laboratories had experienced considerable difficulty in attempting the synthesis of this peptide (G. T. Young, personal

communication) and apamin also proved much harder to make than might have been predicted on the basis of its size (N. R. Rydon and U. Ragnarsson, personal communication). The problems presumably arise because of the disulphide bridges. These seem to form and reform more readily in apamin than in peptide 401, presumably due to the small differences in the size of the larger ring giving more flexibility in peptide 401 and so reducing the probability of disulphide bridge formation.

No synthetic analogues of peptide 401 have so far been reported.

5. Chemical Modification

Chemically modified forms of peptide 401 have been prepared in order to identify features of the structure associated with mast cell degranulating and anti-inflammatory activity. Care must be taken in such studies to work with well-defined species that are not contaminated with the original material and to ensure that the derivatives are stable under both, very different, test situations. Mast cell degranulation *in vitro* is rapid whereas in anti-flammatory testing *in vivo* there is obviously both more time and opportunity for alterations to the derivative to occur in the course of the hours or days of the test. The mast cell degranulating activities of derivatives of peptide 401 and of other basic peptides are given in Table IV with Compound 48/80 for comparison. Data on derivatives in which arginine and histidine residues have been modified have been excluded because the results are ambiguous. Garman, had reported that reaction of the arginines with 2,4-pentanedione reduced the mast cell degranulating activity 20-fold (Banks *et al.*, 1980) but Guschin *et al.* (1981) found only a slight reduction in activity following conversion of the arginines to pyrimidyl-ornithines. Reaction of imidazoles with diethylpyrocarbonate was reported by the Russians to suppress mast cell degranulation whereas treatment with bromacetone gave a derivative with reduced but still significant activity (Banks *et al.*, 1980). None of these derivatives was adequately characterised.

The amino groups have been formylated and reductively methylated and it can be concluded that the N-terminal amino group is not involved in mast cell degranulation. Removal of the N-terminal residue to give [des Ile^1]-peptide 401 does not reduce the peptide's activity (Banks *et al.*, 1983). The hexaformyl derivative, however, is totally inactive, while considerable activity is retained in the pentaformyl compounds. Hexa *N,N* dimethyl 401 retains significant degranulating activity (B. E. C. Banks & C. E. Dempsey, to be published).

Peptide 401 has been compared with other basic peptides that degranulate mast cells, though with less potency, such as protamine, somatostatin, polymixin and substance P and with polylysine, but beyond the basicity of all these agents, structural features needed for degranulating ability cannot

Table IV

Rat Peritoneal Mast Cell Degranulating Activities of Compound 48/80, Peptide 401, Both Native and Chemically Modified, and of Some Other Histamine-releasing Peptides

Substance	Concentration giving ~50% release (M)	Reference
Peptide 401	2×10^{-8}	Dempsey (1981)
Compound 48/80	6×10^{-7}	Dempsey (1981)
Protamine	5×10^{-7}	Jasani *et al.* (1979)
Polylysine	7×10^{-8}	Ennis *et al.* (1980)
[desIle^1]-401	2×10^{-8}	Dempsey (1981)
α-*N*-formyl-401	6×10^{-8}	Dempsey (1981)
Hexa-*N,N*-dimethyl-401	2×10^{-7}	Dempsey (1981)
Hexa-*N*-formyl-401	2×10^{-5}	Dempsey (1981)

be identified. In the case of peptide 401, the reduced and acetamidated peptide is inactive, so the primary sequence alone is insufficient for mast cell degranulation. The disulphide bridges may impose a juxtaposition of cationic side chain groups that is prevented by electrostatic repulsion in the unhindered, open chain form or in the polybasic synthetic peptide, polylysine, but on the whole the use of peptide 401 and its derivatives and other basic peptides has not led to definitive conclusions about structure–activity relations in this area. These agents do not appear to act as does the complement anaphylatoxin (C3a), also a polypeptide, which binds to a specific receptor on the mast cell surface (Jacobs *et al.*, 1978) in order to cause histamine release and involves considerable hydrophobic interaction between C3a and the cell surface. In the case of the other basic releasers hydrophobic forces appear not to be important.

6. *Pharmacology*

Although there is quite a strong similarity in the structures of apamin and peptide 401, their biological effects are quite different. Apamin has no effect on rat peritoneal mast cells and peptide 401 has no effect on the noncholinergic, nonadrenergic innervation of intestinal smooth muscle. When it comes to central activity, that of apamin occurs by whatever route the peptide is administered, indicating that it crosses the blood–brain barrier when given systemically. Peptide 401, on the other hand, does not produce neurotoxic symptoms unless it is given intraventricularly, in which case the LD_{50} is still ten times greater than that of apamin (330 ng/mouse cf. 20–40 ng for apamin) and the symptoms (coordinated hyperactivity) are not the

same as in apamin intoxication. Death from respiratory failure occurs abruptly, within 15 minutes of intraventricular administration (Habermann, 1977).

The toxicity of systemically administered peptide 401 varies markedly with species. In mice, an LD_{50} has not been determined though doses up to 40 mg kg^{-1} have been administered intravenously (Banks *et al.*, 1978). In rats, however, toxic symptoms are observed above 5 mg kg^{-1}, due to histamine release from mast cells, which, in the rat, are more responsive to degranulators than are mouse mast cells. Classic symptoms of histamine intoxication, such as lowering of blood pressure, are observed and cross-tachyphylaxis with the synthetic releaser, compound 48/80, has been established (Breithaupt and Habermann, 1968). Because the principal toxic effects of peptide 401 are due to its action on mast cells, only the two activities of mast cell degranulation and anti-inflammatory effects and the connection between them will be considered.

a. Mast Cell Degranulation. The rat peritoneal mast cell is much used by pharmacologists for the study of secretory mechanisms in which intracellular stores, in this case of histamine, 5-hydroxytryptamine and heparin, are released without lysis of the cell membrane. The process of degranulation depends on an intact cellular metabolism (Lawson *et al.*, 1977).

Mast cells are readily obtained from rats by peritoneal lavage whereas in other species (mouse, guinea pig, rabbit) the mast cells in the peritoneal cavity are more firmly associated with the mesenteric tissue and not readily removed. Consequently, much work has been done on the details of rat mast cell degranulation. Unfortunately, it is becoming increasingly clear that different mast cells have different sensitivities to releasing agents and, more particularly, to pharmacologically active substances believed to prevent degranulation (Pearce, 1982, 1983).

The importance of being able to prevent mast cell degranulation lies in the treatment of allergic conditions such as asthma, hay fever, psoriasis and Chrone's disease, all conditions in which mast cells in particular areas (lung, linings of the upper respiratory tract, skin and intestine) release histamine on contact with an allergen to which the cells have been sensitised. Since mast cells from the same area in different species and from different areas in a single species are variously responsive to releasing and blocking agents, the results obtained with a single mast cell type cannot be expected to have universal relevance. Nonetheless, much work has been done on the mechanism of rat peritoneal mast cell degranulation induced by allergens, by the synthetic histamine releaser compound 48/80 and also by peptide 401 or mast cell degranulating peptide from bee venom.

Many of the stimuli for histamine release are dependent on the availability

of extracellular calcium and are supposed to activate calcium gates in the membrane. The influx of calcium then triggers exocytosis (Foreman *et al.*, 1977). The coupling of stimulus to secretory response by means of a calcium flux appear to be common (Douglas, 1968). Examples of releasers that require extracellular calcium are dextran, ATP, the plant lectin, concanavalin A and the calcium ionophore A23187 while allergen-induced release, presumably involved in anaphylaxis, also depends on the availability of extracellular calcium. In contrast, the basic releasers, compound 48/80 and peptide 401, do not require extracellular calcium (Uvnäs and Thon, 1961; Assem and Atkinson, 1973) but are believed to act by mobilising intracellular calcium stores (Douglas and Ueda, 1973; Atkinson *et al.*, 1979). The dependence of release by peptide 401 and by compound 48/80 on calcium ions is complex, inhibition occurring at low concentrations of releaser and potentiation at high. There is no unambiguous interpretation of the available data and arguments about the mechanism of exocytosis continue in spite of much effort on this relatively simple system. How a rise in cytosolic calcium triggers the membrane fusion that precedes exocytosis is far more of a mystery than is the role of calcium fluxes in the contractile machinery of skeletal, smooth and cardiac muscle but that is perhaps because the mechanism of fusion of phospholipid bilayers is less well characterised than the mechanisms of actin–myosin interaction. It is not unreasonable that doubly charged cations should interact with negatively charged phospholipid nor that basic releasers should influence the interaction between calcium and cell membranes, but there is no evidence that an enzyme is involved in the fusion process, so investigation of the system is harder than in the case of muscle where the calcium is essentially involved in regulating an enzymic activity.

Whether further investigations with peptide 401 as a degranulator are of value remains to be seen. It may be useful in delineating the differences between different types of mast cell which is the area of most immediate importance since hay fever, asthma, psoriasis and Chrone's disease are unlikely to respond to the same treatment if this involves interference with the process of mast cell degranulation.

b. The Anti-inflammatory Activity of Peptide 401? The effectiveness of peptide 401 in reducing the swelling of rat hind paw in response to subplantar carrageenin was first established by Billingham (1969), working in Vernon's laboratories, and led Vernon to take out a patent on the peptide (Vernon *et al.*, 1969). Billingham went on to show that the peptide was also able largely to prevent the development of adjuvant arthritis in rats and to reduce the symptoms of the established condition (Billingham *et al.*, 1973). The level at which the peptide was active was typically 1 mg kg^{-1}, given by subcutaneous injection, a dose one hundredth of that of hydrocortisone in

the same tests and comparable to that of indomethacin, the classic nonsteroidal anti-inflammatory drug. Morley, working in the same area, (Hanson *et al.*, 1974) showed the peptide to be effective against other rat models of inflammation (e.g., turpentine oedema and arthritis) but suggested that the anti-inflammatory activity was confined to rats and was not apparent in guinea pigs, as judged by increases in vascular permeability in hypersensitivity and turpentine arthritis and skin reactions of allergic inflammation. In rats, extravasation in response to intradermal histamine, bradykinin, prostaglandin E, kallikrein and 5-hydroxytryptamine were also prevented by pretreatment of the rats with subcutaneously administered peptide 401 (1 mg kg^{-1}) given one hour before inflammatory challenge.

It appeared in the early 1970s that the old wives' tale that bee venom has therapeutic value in rheumatic conditions might have some scientific basis. However, lack of effectiveness in guinea pigs, which are immunologically closer to man than are rats, was a worrying finding, as was the problem of the quantity of peptide 401 in crude bee venom. There was too little to account for the alleged beneficial effects in terms of the measured potency in rats (Zurier *et al.*, 1973; Chang and Bliven, 1979). On the other hand, both Billingham and Morley independently reported that the apparent anti-inflammatory activity of peptide 401 in rats was not dependent on the ability to degranulate mast cells since (a) antagonists of histamine and 5-hydroxytryptamine did not abolish the anti-inflammatory activity in rats; (b) neither compound 48/80, the synthetic degranulator, nor melittin, a cytolytic agent, was significantly anti-inflammatory in the same test (Hanson *et al.*, 1974) and (c) although inflammatory in guinea pigs on intradermal injection, peptide 401 was not anti-inflammatory in this species (Hanson *et al.*, 1974). Based on indications that peptide 401 *might* be a genuine anti-inflammatory agent, considerable effort was expended on the preparation of chemically modified forms in the hope of dissociating the two paradoxical activities, that is of mast cell degranulation (inflammatory) on the one hand and suppression of oedemas, development of arthritis and extravasation in response to inflammatory challenge on the other. (Banks *et al.*, 1979, 1980, 1983). With the exception of partial dissociation of activities in an extensively formylated derivative prepared by Garman, both mast cell degranulating and anti-inflammatory activity changed in parallel. Guschin (1981), on the other hand, has reported that the two activities were dissociated by treatment of the peptide with tetraethoxypropane, an agent that is specific for guanidino groups. The mast cell degranulating activity was unaffected but ability to block increases in vascular permeability induced by histamine or 5-HT in rats was abolished, according to the Russian workers. One wishes to lose the *in*flammatory activity and retain the *anti*-inflammatory activity and this was only suggested in the formylated derivative prepared by Garman but,

unfortunately, this was a mixture of penta- and hexaformylated 401 and was ill-defined. It is essential in this type of study to use homogeneous samples of modified forms in order ot be able to interpret the results unambiguously. Where this has been done, the inflammatory and anti-inflammatory activities have always changed in parallel.

Eventually, we decided to reexamine the pharmacological basis for believing that attempts to dissociate the activities might be successful. We found that, contrary to the earlier reports (Billingham *et al.*, 1973; Hanson *et al.*, 1974), pretreatment of rats with mepyramine and methysergide (2.5 mg kg^{-1}) largely abolished the anti-inflammatory activity of peptide 401 in the carrageenin oedema test. We also found that compound 48/80 at 1 to 2 mg kg^{-1}, like peptide 401, inhibited oedema development in this test, that histamine depletion or chromoglycate treatment prior to injection of 401 to block mast cell degranulation inhibited the apparent anti-inflammatory activity of the peptide and that in rats sensitised to the nematode *Nippostrongylus brasiliensis,* challenge with allergen in place of treatment with peptide 401 or compound 48/80 was also found to reduce the foot swelling caused by subplantar carrageenin. Compound 48/80 was also found to reduce the symptoms of adjuvant arthritis in rats and to relieve symptoms in the established condition (Banks *et al.*, 1980, 1983 and to be published).

We also examined the effects of injecting peptide 401 into the rat hind paw with the irritant since this has been recommended as a test to distinguish counterirritants from genuine anti-inflammatory agents. The latter are anti-inflammatory by whatever route they are administered. The former are not. They are either proinflammatory or without effect on subplantar injection (Atkinson and Hicks, 1975). Atkinson reported informally in the mid-1970s that he had found peptide 401 to be a counterirritant but his results were not published (D. C. Atkinson, private communication). We have since established that, given with carrageenin, compound 48/80 is strongly proinflammatory and peptide 401 less so; it certainly does not reduce the swelling of the injected foot. What is more, the mixture of hexa- and penta-formyl peptide 401 prepared by Garman and found to be virtually devoid of mast cell degranulating activity, though active still in the rat paw oedema test, was found to be strongly proinflammatory on local (subplantar) injection and must also be presumed to act as a counterirritant, though not necessarily by inducing histamine release (C. E. Dempsey and J. Yamey, to be published).

Peptide 401 or mast cell degranulating peptide is *not* a genuine anti-inflammatory agent. Its effectiveness in rat models of inflammation is due to its ability to release histamine from mast cells, which appears to act as a trigger for as yet unknown natural anti-inflammatory mechanisms.

The lack of activity in guinea pigs reported, though without data, by

Hanson *et al.* (1974) may well be due to the low responsiveness of guinea pig (mesenteric) mast cells as compared with cells from the rat peritoneum (Ennis and Pearce, 1980).

7. *Anti-inflammatory Activity of Crude Bee Venom?*

It has already been noted that there is an insufficient quantity of peptide 401 in whole venom to account for various claims that the mixture is anti-inflammatory (Couch and Benton, 1972; Vick and Shipman, 1972; Alfano *et al.*, 1973; Zurier *et al.*, 1973; Shkenderov, 1976a; Chang and Bliven, 1979). The possible involvement of the adrenocortical system was considered and conflicting conclusions drawn. Zurier *et al.* (1973) and Vick and Shipman (1972) found that bee venom injections increased plasma cortisol levels in rats and dogs, respectively, and Zurier and co-workers found adrenalectomised rats to be insensitive to bee venom treatment in the development of experimental arthritis. The early investigations of peptide 401, however, indicated that adrenalectomy in rats did not reduce the activity of peptide 401 either in hind paw oedema or adjuvant arthritis.

Although bee venom and its constituents may, on injection, increase the release of glucocorticoids from the adrenal gland, the anti-inflammatory activity of whole bee venom has not been firmly attributed to this effect. It is more than likely that whole venom, like peptide 401, acts by a counterirritant mechanism since the melittin and phospholipase A_2 both severally and together will cause local tissue mast cell damage to a greater extent than will the amount of mast cell degranulating peptide in crude venom.

In screening isolated fractions from bee venom for anti-inflammatory activity, Billingham in the late 1960s showed that a second anti-inflammatory substance other than 401 was present. It was eventually found (Banks *et al.*, 1976) to be due to a fraction that contained the catecholamines noradrenaline and dopamine, which are known to be present in bee venom (Owen, 1971) and active in the rat paw oedema test (Arntzen and Briseid, 1973).

The anti-inflammatory activity attributed to apamin (Ovcharov *et al.*, 1976) and later to a fraction from bee venom called first O_a (Habermann and Reiz, 1965a) and later termed 'adolapin' by Shkenderov and Koburova (1982) has not been confirmed (Banks *et al.*, 1983). The material isolated by Shkenderov's published procedure contained large amounts of phospholipase A_2, which probably accounted for the observed activity as a counterirritant.

The conclusion is that there is no hard scientific basis for believing that bee venom has potential therapeutic value for treating arthritic conditions except by means of counterirritation and possibly by stimulation of the adrenal cortex. Natural counterirritant mechanisms as yet unidentified may be triggered by envenomation just as they are following surgery or in

pregnancy when there may be marked relief from chronic arthritic symptoms. However, peptide 401 is not anti-inflammatory and the alleged activity of whole venom, though well substantiated in rats, is not at all understood. No other single well-characterised components with anti-inflammatory or counterirritant activity have been found.

D. Secapin

Secapin comprises only ~0.5% of crude bee venom. It was first isolated by Gauldie *et al.* (1976), who commented that it was surprising that it had not been detected previously, presumably because it is unusual with respect to amino acid composition, having a high proline content and, uniquely among the bee venom peptides, having an N-terminal tyrosine residue. It also has a single disulphide bridge. On the other hand, the peptide has no known physiological or pharmacological effect. It is remarkably nontoxic, so no LD_{50} has been determined, and the only symptoms reported after very high doses given intravenously to mice (80 mg kg^{-1}) were of hypothermia, piloerection and mild sedation. Since the isolation of components from a complex mixture is normally based on biological or enzymic assays for particular activities it is far from surprising that earlier fractionation procedures had not resulted in detection of this peptide that is devoid of a defined activity.

Two primary sequence determinations have been published (Gauldie *et al.*, 1978; Kudelin *et al.*, 1979) that agree up to the twenty-second residue (see Fig. 12), but then the C-terminal sequence proposed by the Russian workers on the basis of mass spectrometry was Leu-Val-Pro, making a total of 25 residues, while the British laboratory reported the sequence -Pro-Val, having used conventional sequence methods to give 24 residues. It is not at all clear why difficulties should have been experienced in establishing the C-terminal sequence but the discrepancy has only recently been resolved after a modified fractionation procedure from 20 g of bee venom, the last traces of melittin being removed by chromatography on heparin–Sepharose, (Liu and Vernon, 1984) after Phenyl-Sepharose and a reversed salt gradient, with increasing ethylene glycol, since secapin and melittin are both strongly hydrophobic and absorb tightly to this column from aqueous solution. The sequence determined by Liu and Vernon confirmed that the Russians were correct in finding 25 residues but the twenty-third was in fact isoleucine and not leucine. The correct sequence is given in Fig. 12.

Final confirmation that this is, in fact, the correct sequence comes from work in Kreil's laboratory on the isolation from queen bee venom glands of mRNA, translation of which in cell-free systems gave not only

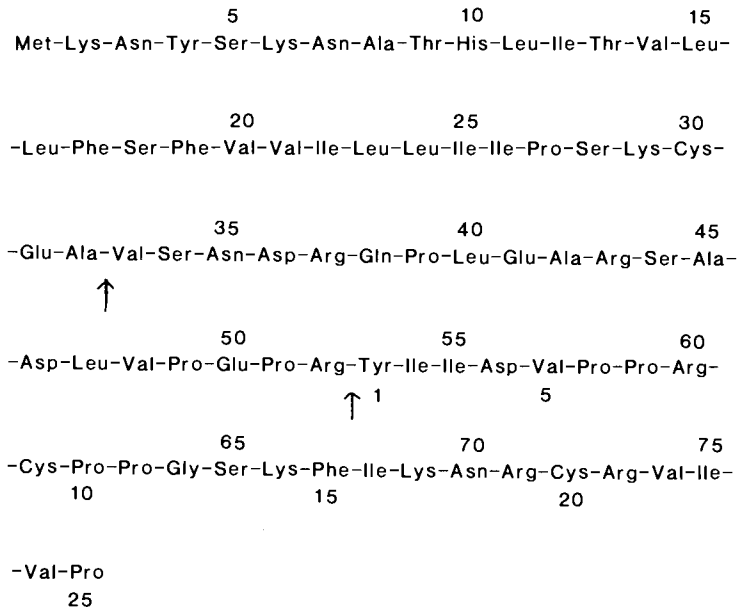


Fig. 12 Amino acid sequence of preprosecapin. The position of cleavage of the signal peptide and of the pro-sequence are indicated by arrows. The residues of secapin are numbered below the sequence. From Tu (1984), by permission of Marcel Dekker, Inc.

prepromelittin, the major product, but another component of ~20,000 molar mass detected by gel electrophoresis (Vlasak *et al.*, 1984). When translation was carried out in the presence of labelled histidine, an amino acid that does not occur in prepromelittin, a labelled polypeptide product comigrating with the melittin precursor was detected, and it was suggested that this might be preprosecapin. In looking for cDNA clones for melittin, two were found containing inserts coding for the precursor of secapin. These were sequenced and shown (Vlasak *et al.*, 1984) to differ only in the extension of one by ~100 nucleotides from the 3′ end. Both contain an open reading frame of 77 codons. Comparison with prepromellitin and the sequence of secapin suggests an unusually long signal peptide of 32 residues, cleavage of an Ala-Val bond releasing prosecapin with 45 residues. The C-terminal sequence of secapin (-Ile-Val-Pro) is confirmed by the cDNA sequence as shown.

Activation of prosecapin is unlikely to be by the same mechanism of endodipeptidase action as for promelittin since the odd sequence of the pro part of promelittin with proline or alanine residues in alternate positions is not found in prosecapin. Nor is there a requirement that processing be extracellular since, unlike melittin, secapin has no known cellular activity.

It is more likely that the prosecapin is processed intracellularly and since the liberation of secapin entails cleavage C-terminal to an arginine residue (Fig. 12), a tryptic-like specificity as for other prohormone cleaving proteases seems likely. Although cleavage between two basic amino acids is most common in the processing of prohormones, somatostatin (Goodman *et al.*, 1982) and vasoactive intestinal peptide (Itoh *et al.*, 1983) are both formed by cleavage after single arginine residues.

The sequence of secapin in unusual since it has two double prolines. The structural features should make it of considerable interest to those working on the conformation of small peptides in solution by NMR spectroscopy but the difficulty experienced in isolating pure material makes the product less than readily available. The absence of biological properties to date makes the peptide of scant interest though the recent finding by Kreil (Vlasak *et al.*, 1984) that the amount of mRNA coding for preprosecapin isolated from queen bee venom glands is greater relative to the melittin coding than would be predicted from the composition of worker bee venom hints at a role for secapin specific to the queen. It would be of interest to test secapin for toxicity in queen bees, which are the major victims of queen bee envenomation in the early days after eclosion.

E. Tertiapin

Tertiapin is a very minor component of bee venom, comprising $<0.1\%$ of the dry weight. A single sting would deliver ~ 70 nanograms. The isolation and characterisation were first reported by Gauldie *et al.* (1976) and the primary structure was determined by Russian workers some years later (Ovchinnikov *et al.*, 1980) and confirmed by Liu (1982). It is presumed that as with apamin and peptide 401, to which the molecule is very similar, the C-terminal residue is amidated since carboxypeptidases A and B do not liberate a free amino acid from the reduced and carboxymethylated peptide. The positions of two disulphide bridges are assumed to be the same as in apamin and peptide 401 since cyanogen bromide cleavage yields only one fragment with two N-terminal residues, alanine and cystine. The structure of tertiapin in two-dimensional representation is given in Fig. 13.

There is a substantial degree of homology with the sequence of peptide 401, 13 of 21 residues being identical and similarities also in the circular dichroism and Raman spectra. Using Levitt's parameters for secondary structure prediction, a three-dimensional model has been proposed by Hider and Ragnarsson (1981) with close similarities to the models for apamin and for peptide 401.

The information about the biological activity of tertiapin is limited. The peptide is not particularly toxic, the LD_{50} in mice being in excess of 40 mg

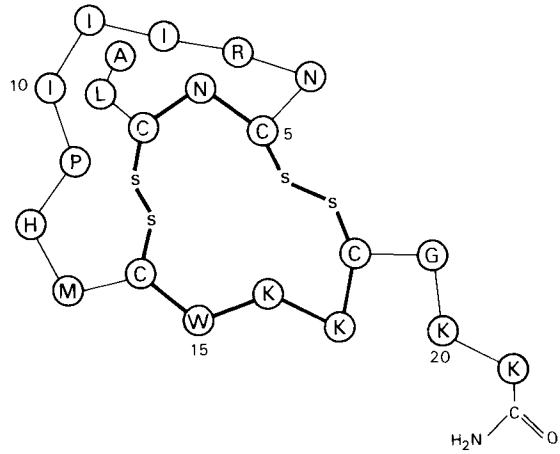


Fig. 13 Structure of tertiapin.

kg^{-1} on intravenous injection. It degranulates rat peritoneal mast cells but is somewhat less effective than peptide 401. Ovchinnikov and colleagues (1980) have attributed a presynaptic action to the peptide at concentrations in organ baths of 25 to 50 $\mu g\ ml^{-1}$, the frequency of miniature end plate potentials being reduced four-fold in nerve-muscle preparations, possibly due to a fall in spontaneous transmitter release. It was said that the action of tertiapin in this test resembled the effects of prostaglandins under the same conditions. It has also been suggested that the strong affinity that exists between tertiapin and the calcium-regulating protein, calmodulin (Miroshnikov *et al.*, 1983), may underlie the effect of tertiapin on transmitter release (de Lorenzo, 1982). However, the dissociation constant for the complex of calmodulin with two molecules of tertiapin is high (2 μM) and it is unlikely that, given the very small amounts of tertiapin present in the venom, the peptide will have any effects on envenomation.

F. Protease Inhibitor

Shkenderov's group first reported that bee venom from a number of sources displays marked ability to inhibit the proteolytic activity of trypsin (Shkenderov, 1973). A complex purification procedure was reported in 1976 and the product with protease inhibitory activity was said to comprise ~0.8% of crude venom, to have a molar mass of ~9000, 63–65 amino acid residues, to be thermostable and stable at acid pH, to have no free -SH groups and to be devoid of threonine, methionine and histidine and to have lysine as the N-terminal residue.

H_2N-Gly-Gly-Phe-Gly-Gly-Leu-Gly-Gly-Arg-Gly-Lys-Cys-Pro-

-Ser-Asx-Glx-Ile-

Peptide-H1

H_2N-Phe-Gly-Gly-Phe-Gly-Gly-Phe-Gly-Gly-Leu-Gly-Gly-Arg-

-Gly-Lys-Cys-Pro-Ser-Asn-Glu-Ile-Phe-Ser-Arg-Cys-Asp-Gly-Arg-

-Cys-Gln-Arg-Phe-Cys-Pro-Asn- Peptide-H3

Fig. 14 N-terminal sequences of peptides H1 and H3 that *may* be identified with the protease inhibitor of Shkenderov. (L. K. Liu and R. A. Shipolini, unpublished results).

Inhibitory activity was established against a wide spectrum of proteolytic enzymes but the material was inactive towards kallikrein and urokinase (Shkenderov, 1976b). Despite such 'polyvalent' activity, the inhibition of trypsin was not complete, even with a 10-fold molar excess of inhibitor to enzyme. At an equimolar ratio, inhibition was only 50% with chymotrypsin and trypsin, and esterolytic enzymes were even less sensitive.

Two components with similar amino acid compositions have been isolated in Vernon's laboratories but have not been identified with Shkenderov's protease inhibitor in terms of activity. They differ from each other only in that one lacks the first four residues at the N-terminus and the sequences have only been established up to residue 35 of the longer component. The sequence shows no homology with other proteolytic enzyme inhibitors (Fig. 14) from other animal tissues or snake venoms and so far has not been connected with any of the cDNA sequences in Kreil's library of bee venom clones. These peptides may be fragments from larger molecules but so far there is no evidence that they have any interesting biological properties (L. K. Liu and C. A. Vernon, to be published).

G. Histamine-containing Peptides

Two or three homologous peptides with histamine present as the C-terminal residue have been isolated from Canadian bee venom by Nelson and O'Connor (1968). Their sequences are given in Fig. 15. These are the first

Nelson's Peptide Ala-Gly-Pro-Ala-Gln-Histamine

Procamine A Ala-Gly-Pro-Gln-Histamine

Procamine B Ala-Gly-Gln-Gly-Histamine

Fig. 15 Histamine-containing peptides.

histamine-containing peptides to have been found in a natural source and are of considerable interest from the pharmacological standpoint since the properties of histamine should be modified when it is part of a peptide sequence. However, in spite of intensive effort, these peptides have not been detected in the Bulgarian bee venom that has been examined in Vernon's laboratories.

It is possible that these histamine peptides might be associated with the radioprotective properties attributed to bee venom by Shipman and Cole (1967). Procamine (see Fig. 15) and the simpler dipeptide glycylhistamine were synthesised and shown to be equi-potent against exposure of mice to high energy radiation (Peck *et al.*, 1978). A similar effect has been found with small doses of whole bee venom, the histamine content of which, whether free or incorporated as a C-terminal residue of peptides, might act as a free radical scavenger. The release of histamine by phospholipase A_2, melittin and peptide 401 would contribute to the radioprotective effect of whole bee venom.

VI. NONPEPTIDE COMPONENTS OF LOW MOLECULAR MASS

Insects have an open circulatory system and most small components of the plasma would be expected to appear in the venom. Those that are used in the synthesis of venom constituents would be expected to be present at the lowest concentrations. Constituents not present in the haemolymph (Florkin and Jeuniaux, 1974) but found in the venom could properly be classed as venom components.

A. Biogenic Amines

A number of members of this group of compounds are common constituents of natural venomous secretions from snakes and insects. Their

effects in mammals have been well studied but there is a scarcity of information on their effects in insects. Some are known to act as neurotransmitters. Noradrenaline, for instance, is involved in light production in the firefly under nervous control and 5-hydroxytryptamine has excitatory effects on the giant nerve in cockroaches. Tryptamine inhibits neuromuscular transmission in the locust but is excitatory in insect hearts and guts. The early work in this area was summarised by Pichon (1974). A sudden increase in the concentration of any of the pharmacologically active amines or their precursors, as would occur on injection of venom into the haemolymph of an insect victim, would interfere with the fine control of physiological function and have a profound effect. The contribution of these substances to the toxicity of bee venom in insects does not appear to have been properly examined. It must, however, be recalled that insect nerves are not as susceptible to active substances such as acetylcholine, when these are applied as bathing solutions, as are mammalian nerves. There certainly is an analogue of the blood-brain barrier in higher animals, but probably at the level of the protective nerve sheath (see Pichon, 1974, for summary).

B. Histamine

This is the most common biogenic amine in Hymenoptera venoms (see Chapter 5, and Chapter 6, Table I) and the only one in bee venom. Histamine levels in the venom are age-related, being absent immediately following eclosion and increasing to a maximum of ~2000 ng per sac or 18 nmol in bees of 35 to 45 days of age (Owen *et al.*, 1977; Owen, 1978). The slow rise in content of histamine with age will mean that pooled venom samples as well as individual ones will be expected to show variable amounts of histamine depending on the ages of individuals supplying the venom. On a molar basis, histamine is one of the major components of bee venom but the amount present in a single sting is too small (~6 nmol) to have a significant effect in most mammals although mammals do vary greatly in their reaction to histamine. It might cause local pain and increased capillary permeability but the amount of endogenous histamine release would exceed that delivered in a sting.

Histamine toxicity in worker bees corresponds to an LD_{50} of ~27 mg kg^{-1} (~ 30 nmol per insect) when injected into the haemocoel. The maximum histamine content of a worker bee's venom sac is therefore less than half the LD_{50} for another worker bee but there may well be synergism between histamine and other venom components in the overall toxicity to invertebrates.

C. Catecholamines

Dopamine and noradrenalin were identified in the venom reservoirs of honey-bees by fluorescence microscopy but no catecholamines could be detected in commercial (Canadian?) samples of venom (Owen, 1971). Later, the isolation of noradrenalin and dopamine from Bulgarian bee venom was reported (Banks *et al.*, 1976) amounting to 0.16% of noradrenalin (w/w in crude venom) and 0.13% of dopamine, although Owen had earlier estimated a higher proportion of dopamine in extracts of venom sacs. There may well be a difference in the ratios in tissues of invertebrates as suggested by other workers, ratios between 6:1 and 10:1 having been reported previously, but in the venom, there appears to be roughly a 1:1 ratio of dopamine to noradrenalin. Dopamine has been shown to produce hyperactivity in the central nervous system of insects and accelerates the insect heartbeat (Owen, 1971; for review, see Pichon, 1974) at the sort of concentrations that might be achieved were venom to be delivered into the haemolymph. The function of dopamine might, therefore, be to accelerate haemolymph circulation, so that other toxic substances from the venom are carried rapidly to their sites of action.

1. *Dopamine*

Following eclosion, dopamine levels in venom sacs are very low ($\sim$30 ng/sac) but rise rapidly to $\sim$1500 ng in the sacs of 20 to 25-day-old bees. Superimposed on the increase with age, there also appears to be some seasonal variation, very high levels (4000 ng/sac) being found in older bees at the height of August. The significance of this is not understood (Owen and Bridges, 1982; Owen, 1983).

Dopamine has a number of functions in insect physiology. It is an intermediate in the synthesis of quinones, used for cross-linking proteins in the formation and hardening of the cuticle. It also has a direct action on the circulatory and central nervous systems of insect victims.

2. *Noradrenalin*

This is a common neurotransmitter in vertebrates and, as a venom constituent, would be predicted to affect also the nervous system of an insect victim. Its level in worker bee venom is age related, being low on eclosion and peaking at $\sim$2000 ng/sac at about 40 days (Owen, 1983). The concentration of adrenergic substances in the haemolymph of an insect could be between 2×10^{-5} and 2×10^{-4} M, which is certainly significant.

D. Free Amino Acids

Nineteen free amino acids have been detected in bee venom (Nelson and O'Connor, 1968) but in amounts consistent with their having been derived from haemolymph (Florkin and Jeuniaux, 1974). The lower proportions of glutamic acid, proline and lysine would be consistent with the usage of these in the synthesis of large quantities of melittin.α-Aminobutyric acid (0.04%) and β-aminoisobutyric acid (0.02%), which are not significant constituents of haemolymph, might be genuine venom components.

Other carbohydrate and lipid constituents of venom almost certainly derive directly from the haemolymph and are not typical venom components.

VII. CONCLUSION

Bee venom is a mixture of proteins, peptides and small organic molecules that has been separated into constituents comprising from 50% to $<0.1\%$ of the venom dry weight. For man as victim, the most important and potentially dangerous constituents are the strongly antigenic, high molecule weight enzymes phospholipase A_2 and hyaluronidase to which individuals may become sensitised and therefore be at risk of an anaphylactic response to a single bee sting. For insects as victims, less is known of the toxicity of individual components though the venom volume:victim size ratio is so enormously different and the injection of venom directly into the haemolymph makes bee envenomation a more hazardous prospect for an insect victim.

The phospholipase A_2component, in addition to being a powerful allergen, is also an indirect lytic agent, that is, a substance that can lyse cell membranes given the assistance of another membrane-active (or rather membrane-disruptive) component that renders the membrane phospholipids susceptible to enzymatic cleavage. The phospholipase A_2 of bee venom is subject to allosteric activation by fatty acid products of its initial activity so that a cascade response is triggered. Bee venom contains in large quantities a peptide that is highly membrane active or disruptive, the direct lytic factor called melittin. Melittin renders cell membranes susceptible to attack by phospholipase A_2, both from bee venom and also from endogenous stores. Hyaluronidase, the other allergenic enzyme, also has an immediate function in that it attacks the intracellular ground substance to facilitate the spreading of toxic components.

Melittin is lytic in its own right and will assist the specific mast cell degranulator, peptide 401, to release histamine and cause increased capillary blood flow and permeability, again enhancing the spread of toxic components. Controlled histamine release is not necessarily disadvantageous, as natural

anti-inflammatory mechanisms may be triggered by some elevation of systemic histamine levels. This may be why therapeutic properties have long been attributed to bee venom, especially in arthritic conditions. In species that are very sensitive to histamine, bee sting could be lethal because of its high histamine-releasing potential.

Apamin, the centrally acting neurotoxic peptide with potent peripheral effects on mammalian tissue, blocks some calcium-activated potassium conductance changes, and may well be more effective against insects than mammals but, at present, has proved of more use in fundamental scientific investigations of potassium fluxes across membranes than of the effects of envenomation. The functions of the more minor peptides and organic molecules remain to be established.

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REFERENCES

Alfano, J. A., Elliot, W. B. and Brownie, A. C. (1973). The effect of bee venom on corticosterone levels and adrenal mitochondrial cytochrome *P*-450 in intact and hypophysectomised rats. *Toxicon* **11**, 101–102.

Anderson, D., Terwilliger, T. C., Wickner, L. V. and Eisenberg, D. (1980). Melittin forms crystals which are suitable for high resolution X-ray structural analysis and which reveal a molecular 2-fold axis of symmetry. *J. Biol. Chem.* **255**, 2578–2582.

Alexiev, B. and Shipolini, R. A. (1971). Weitere Unterschunger zur Fractionierung und Reinigung der toxischen Protein aus dem Gift der bulgarischen Viper (*Vipera ammodytes ammodytes*). *Z. Phys. Chem.* **352**, 1183–1187.

Arntzen, F. C. and Briseid, K. (1973). Inhibition of carrageenin-induced rat paw oedema by catecholamines and amine-depleting drugs. *Acta. Pharmacol. Toxicol.* **32**, 179–192.

Assem, E. S. K. and Atkinson, G. (1973). Histamine release by MCDP(401), a peptide from the venom of the honey bee. *Br. Pharmacol.* **48**, 337–338.

Atkinson, D. C. and Hicks, R. (1975). The anti-inflammatory activity of irritants. *Agents Actions* **5**, 239–249.

Atkinson, G., Ennis, M. and Pearce, F. L. (1979). The effect of alkaline-earth cations on the release of histamine from rat peritoneal mast cells treated with compound 48/80 and peptide 401. *Br. J. Pharmacol.* **65**, 395–402.

Bachmayer, H., Kreil, G. and Suchanek, G. (1972). Synthesis of promelittin and melittin in the venom gland of queen and worker bees: patterns observed during maturation. *J. Insect Physiol.* **18**, 1515–1522.

Baidan, L. B., Vladimirova, I. A., Miroshnikov, A. I. and Tarah, G. A. (1978). The action of apamin on sympathetic transmission in various types of synapses. *Dokl. Akad. Nauk. SSSR* **241**, 1224–1227.

Banks, B. E. C., Hanson, J. M. and Sinclair, N. M. (1976). The isolation and identification of noradrenaline and dopamine from the venom of the honey bee, *Apis mellifica*. *Toxicon* **14**, 117–125.

Banks, B. E. C., Garman, A. J. and Habermann, E. (1978). Structure-activity studies on apamin and mast cell degranulating peptide (MCDP)-401. *J. Physiol. (London)* **284**, 160–161P.

Banks, B. E. C., Brown, C., Burgess, G. M., Burnstock, G., Claret, M., Cocks, T. M. and Jenkinson, D. H. (1979a). Apamin blocks certain neurotransmitter-induced increases in potassium permeability. *Nature (London)* **282**, 415–417.

Banks, B. E. C., Brown, C., Burgess, G. M., Burnstock, G., Claret, M., Cocks, T., Jenkinson, D. H. and Parsons, H. (1979b). Some peripheral activities of apamin. *Toxicon, Suppl. 4,* **17**, 4.

Banks, B. E. C., Sinclair, N. M. and Vernon, C. A. (1979c). The polypeptide components of bee venom. *Proc. 3rd Symp. Plant, Animal Microbial Toxins*, pp. 65–78.

Banks, B. E. C., Dempsey, C. E., Vernon, C. A. and Yamey, J. (1980). The mast cell degranulating peptide from bee venom. *J. Physiol. (London)* **308**, 95–96P.

Banks, B. E. C., Dempsey, C. E., Pearce, F. L., Vernon, C. A. and Wholley, T. E. (1981). New methods of isolating bee venom peptides. *Anal. Biochem.* **116**, 48–52.

Banks, B. E. C., Dempsey, C. E. and Barboni, E. (1983). Anti-inflammatory activity in the venom of *Apis mellifera*. *Toxicon, Suppl. 3*, 29–32.

Barker, S. A., Bayyuk, S. H. I., Brimacombe, J. S. and Palmer, D. J., (1963). Characterisation of the products of the action of bee venom hyaluronidase. *Nature (London)* **199**, 693–694.

Barker, S. A., Mitchell, A. W., Walton, K. W. and Weston, P. D. (1966). Separation and isolation of the hyaluronidase and phospholipase components of bee venom and investigation of bee venom-human serum interactions. *Clin. Chim. Acta* **13**, 582–596.

Barnard, J. H. (1973). Allergic and pathologic findings in fifty insect sting fatalities. *J. Allergy Clin. Immunol.* **52**, 259–264.

Beck, B. (1935). 'Bee Venom Therapy'. Appleton, New York.

Bello, J., Bello, H. R. and Granados, E. (1982). Conformation and aggregation of melittin: dependence on pH and concentration. *Biochemistry* **21**, 461–465.

Bennett, M. R., Burnstock, G. and Holman, M. E. (1966). Transmission from intramural inhibitory nerves to the smooth muscle of the guinea pig taenia coli. *J. Physiol. (London)* **182**, 541–558.

Benton, A. W. (1967). Esterases and phosphatases of honey bee venom. *J. Apic. Res.* **6**, 91–94.

Benton, A. W., Morse, R. A. and Stewart, J. B. (1963). A method of collecting honey bee venom. *Science* **142**, 228–230.

Billingham, M. E. J. and Davies, G. E. (1979). Experimental models of arthritis in animals. *Heffter's Handb. Exp. Pharmacol.* **50**, 108–144.

Billingham, M. E. J., Morley, J., Hanson, J. M., Shipolini, R. A. and Vernon, C. A. (1973). An anti-inflammatory peptide from bee venom. *Nature (London)* **245**, 163–164.

Birr, C. and Wengert-Müller, M. (1979). Synthesis of the mast cell degranulating (MCD) peptide from bee venom. *Angew. Chem. Int. Ed. Engl.* **18**, 147–148.

Bradbury, A. F., Finnie, M. D. A. and Smyth, D. G. (1982). Mechanism of C-terminal amide formation by pituitary enzymes. *Nature (London)* **298**, 686–688.

Breithaupt, H. and Habermann, E. (1968). Mast zell degranulierendes Peptide (MCD-Peptide) aus Bienegift: isolierung, biochemische und pharmakologische Eigenschaften. *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.* **261**, 252–270.

Brown, C. M. and Burnstock, G. (1981). Evidence in support of the P_1/P_2 purinoceptor hypothesis in the guinea-pig taenia coli. *Br. J. Pharmacol.* **73**, 617–624.

Brown, L. R., Lauterwein, J. and Wüthrich, K. (1981). High resolution ^{1}H-NMR studies of self-aggregation of melittin in aqueous solution. *Biochim. Biophys. Acta* **622**, 231–244.

Brown, L. R., Braun, W., Kumar, A and Wüthrich, K. (1982). High resolution nuclear magnetic resonance studies of the conformation and orientation of melittin bound to a lipid–water interface. *Biophys. J.* **37**, 319–328.

Bulbring, E. and Tomita, T. (1977). Calcium requirement for the α-action of catecholamines on guinea-pig taenia coli. *Proc. R. Soc. London, Ser. B* **197**, 271–284.

Burgess, G. M., Claret, M. and Jenkinson, D. H. (1981). Effects of quinine and apamin on the calcium-dependent potassium permeability of mammalian hepatocytes and red cells. *J. Physiol. (London)* **317**, 67–90.

Burnstock, G. (1972). Purinergic nerves. *Pharmacol. Rev.* **24**, 509–581.

Busetta, B. (1980). Conformational analysis of apamin using the residual representation. *FEBS Lett.* **112**, 138–142.

Bystrov, V. F., Arseniev, A. S. and Gavrilov, Y. D. (1978). NMR of peptides and proteins. *J. Magn. Reson.* **30**, 157–184.

Bystrov, V. F., Okhanov, V. F., Miroshnikov, A. I., and Ochinnikov, Y. A. (1980). Solution spatial structure of apamin as derived from NMR studies. *FEBS Lett.* **119**, 113–117.

Bywater, R. A. R., Holman, M. E. and Taylor, G. S. (1981). Atropine-resistant depolarisation in the guinea-pig small intestine. *J. Physiol. (London)* **316**, 369–378.

Callewaert, G. L., Shipolini, R. A. and Vernon, C. A. (1968). The disulphide bridges of apamin. *FEBS Lett.* **1**, 111–113.

Cavey, D., Vincent, J.-P. and Lazdunski, M. (1979). A search for the apamin receptor in the central nervous system. *Toxicon* **17**, 176–179.

Chang, Y.-H. and Bliven, M. L. (1979). Anti-arthritic effect of bee venom. *Agents Actions* **9**, 205–211.

Changeux, J.-P. (1981). The acetylcholine receptor. *Harvey Lect.* **75**, 85–254.

Cheng-Raude, D., Treloar, M. and Habermann, E. (1976). Preparation and pharmacokinetics of labelled derivatives of apamin. *Toxicon* **14**, 467–476.

Colacicco, G., Basu, M. K., Buckelew, A. R. and Bernheimer, A. W. (1977). Surface properties of membrane systems. Transport of staphylococcal δ-toxin from aqueous to membrane phase. *Biochem. Biophys. Acta* **465**, 378–390.

Comte, M., Maulet, Y. and Cox, J. A. (1983). Ca^{++} dependent high affinity complex formation between calmodulin and melittin. *Biochem. J.* **209**, 269–272.

Cook, N. S., Haylett, D. G. and Strong, P. N. (1983). High affinity binding of (^{125}I)monoiodoapamin to isolated guinea-pig hepatocytes. *FEBS Lett.* **152**, 265–269.

Cosand, W. L. and Merrifield, R. B. (1977). Concept of internal structural controls for evaluation of inactive synthetic peptide analogs: synthesis of Orn^{13,14} apamin and its guanidination to an apamin derivative with full neurotoxic activity. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2771–2775.

Couch, T. L. and Benton, A. W. (1972). The effect of the venom of the honey bee, *Apis mellifera*, L., on the adrenocortical response of the adult, male rat. *Toxicon* **10**, 55–62.

Curtis, A. S. G., Campbell, J. and Shaw, F. M. (1975). Cell surface lipids and adhesion. I. The effects of lysophosphatidyl compounds, phospholipase A_2 and aggregation inhibiting protein. *J. Cell. Sci.* **18**, 347–356.

Dawson, C. R., Drake, A. F., Helliwell, J. and Hider, R. C. (1978). The interaction of bee melittin with lipid bilayer membranes. *Biochim. Biosphys. Acta* **510**, 75–86.

De Bony, J., Dufourcq, J. and Clin, B. (1979). Lipid–protein interactions: NMR study of melittin and its binding to lysophosphatidyl choline. *Biochim. Biophys. Acta* **552**, 531–534.

DeGrado, W. F., Kezdy, F. J. and Kaiser, E. T. (1981). Design, synthesis and characterisation of a cytolytic peptide with melittin like activity. *J. Am. Chem. Soc.* **103**, 679–681.

DeGrado, W. F., Musso, G. F., Lieber, M., Kaiser, E. T. and Kezdy, F. J. (1982). Kinetics and mechanism of haemolysis induced by melittin and by a synthetic melittin analogue. *Biophys. J.* **37**, 329–338.

de Haas, G. H., Postema, M. M., Nienwenhuizen, W. and van Deenan, L. L. M. (1968). Purification and properties of phospholipase A and its zymogen from porcine pancreas. *Bull. Soc. Chim. Biol.* **50**, 1383.

de Lorenzo, R. J. (1982). Calmodulin in neurotransmitter release and synaptic function. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 2265–2272.

Dempsey, C. E. (1981). PhD. thesis, University of London.

Dempsey, C. E. (1982). Selective formylation of α- or ϵ-amino groups of peptides. *J. Chem. Soc., Perkin Trans. 1* pp. 2625–2629.

den Hertog, A. (1981). Calcium and the α-action of catecholamines on guinea-pig taenia coli. *J. Physiol. (London)* **316**, 109–125.

den Hertog, A., and Jager, L. P. (1975). Ion fluxes during the inhibitory junction potential in the guinea-pig taenia coli. *J. Physiol (London)* **250**, 681–691.

Dennis, E. A. (1983). Phospholipases. *In* 'The Enzymes' (P. D. Boyer, ed.), 3rd ed., Vol. XVI, pp. 307–353. Academic Press, New York.

Dimitrov, S. D. and Natchev, L. A. (1977). Fractionation of some bee venom components on a new type of modified cellulose. *Toxicon* **15**, 447–448.

Doery, H. M. and Pearson, J. E. (1964). Phospholipase B in snake venoms and bee venom. *Biochem. J.* **92**, 599–605.

Doonan, S., Garman, A. J., Hanson, J. M., Loudon, A. G. and Vernon, C. A. (1978). Identification by mass spectrometry of *N*-formyl lysine residues in a peptide from bee venom. *J. Chem. Soc., Perkin Trans. 1* pp. 1157–1160.

Douglas, W. W. (1968). Stimulus–secretion coupling: The concept and clues from chromaffin and other cells. *Br. J. Pharmacol. Chemother.* **34**, 457–474.

Douglas, W. W. and Ueda, Y. (1973). Mast cell secretion (histamine release induced by 48/80: calcium-dependent exocytosis inhibited strongly by cytocholasin only when glycolysis is limiting. *J. Physiol. (London)* **234**, 97P–98P.

Drainas, D. and Lawrence, A. J. (1978). Activation of bee venom phospholipase A_2 by oleoyl-imidazolide. *Eur. J. Biochem.* **91**, 131–138.

Drainas, D., Moores, G. R. and Lawrence, A. J. (1978). The preparation of activated bee venom phospholipase A_2. *FEBS Lett.* **86**, 49–52.

Drainas, D., Harvey, E., Lawrence, A. J. and Thomas, A. (1981). Mechanisms for albumin-mediated membrane damage. *Eur. J. Biochem.* **114**, 239–245.

Drake, A. F. and Hider, R. C. (1979). The structure of melittin in lipid bilayer membranes. *Biochem. Biophys. Acta* **555**, 371–373.

Dufourcq, J. and Faucon, J.-F. (1977). Intrinsic fluorescence study of lipid–protein interactions in membrane models. Binding of melittin, an amphipathic peptide, to phospholipid vesicles. *Biochim. Biophys. Acta* **467**, 1–11.

Efremov, E. S., Nabiev, I. R. and Nurkhametov, A. Kh. (1980). *IX Natl. Conf. Mol. Spectrosc., Albena, Bulgaria* (Abstr.).

Ennis, M. and Pearce, F. L. (1980). Differential reactivity of isolated mast cells from the rat and guinea-pig. *Eur. J. Pharmacol.* **66**, 339–345.

Ennis, M., Pearce, F. L. and Weston, P. M. (1980). Some studies on the release of histamine from mast cells stimulated with polylysine. *Br. J. Pharmacol.* **70**, 329–334.

Fahrenkrug, A., Gammeltoft, S., Staun-Olsen, P., Ottesen, P. and Sjöquist, A. (1983). Multiplicity of receptors for vasoactive intestinal peptide (VIP): Differential effects of apamin on binding in brain, uterus and liver. *Peptides* **4**, 133–136.

Faucon, J.-F., Dufourcq, J. and Lussan, C. (1979). The self-association of melittin and its binding to lipids. *FEBS Lett.* **102**, 187–190.

Fitton, J. E., Dell, A., Hunt, D. F., Marasco, J., Shabanowitz, J. and Winston, S. (1984). The amino acid sequence of delta-haemolysin purified from a canine isolate of *S. aureus*. *FEBS Lett.* **169**, 25–29.

Florkin, M. and Jeuniaux, C. (1974). Haemolymph: Composition. *In* 'The Physiology of Insecta' (M. Rockstein, ed.), pp. 255–307. Academic Press.

Foreman, J. C., Hallett, M. B. and Mongar, J. L. (1977). Movement of strontium ions into mast cells and its relationship to the secretory response. *J. Physiol. (London)* **271**, 193–214.

Fredholm, B. (1966). Studies on a mast cell degranulating factor in bee venom. *Biochem. Pharmacol.* **15**, 2037–2042.

Fredholm, B. and Haegermark (1967). Histamine release from mast cells induced by a mast cell degranulating fraction from bee venom. *Acta Physiol. Scand.* **69**, 304–312.

Fredholm, B. and Haegermark (1969). Studies on the histamine releasing effect of bee venom fractions and compound 48/80 on skin and lung tissue in the rat. *Acta Physiol. Scand.* **76**, 288–298.

Free, R. B. (1977). 'The Social Organisation of Honeybees'. Arnold, London.

Fryklund, L. and Eaker, D. (1973). Complete aminoacid sequence of a nonneurotoxic haemolytic protein from the venom of *Haemachatus haemachates*, (African Ringhals cobra). *Biochemistry* **12**, 661–667.

Gary, N. E. (1974). Pheromones that affect the behaviour and physiology of honey bees. *In* 'Pheromones' (M. C. Birch, ed.), pp. 200–221. North-Holland Publ., Amsterdam.

Gauldie, J., Hanson, J. M., Rumjanek, F. D., Shipolini, R. A. and Vernon, C. A. (1976). The peptide components of bee venom. *Eur. J. Biochem.* **61**, 369–376.

Gauldie, J., Hanson, J. M., Shipolini, R. A. and Vernon, C. A. (1978). The structures of some peptides from bee venom. *Eur. J. Biochem.* **83**, 405–410.

Golden, D. B. K., Meyers, D. A., Kagey-Sobotka, A., Valentine, M. D. and Lichtenstein, L. M. (1982). Clinical relevance of the venom-specific immunoglobulin G antibody level during immunotherapy. *J. Allergy Clin. Immunol.* **69**, 489–493.

Goodman, R. H., Jacobs, J. W., Dee, P. C. and Habener, J. F. (1982). Somatostatin-28 encoded in a cloned cDNA obtained from a rat medullary thyroid carcinoma. *J. Biol. Chem.* **257**, 1156–1159.

Granier, C., Pedroso-Muller, E. and van Rietschoten, J. (1978). Use of synthetic analogs for study on the structure-activity relationship of apamin. *Eur. J. Biochem.* **82**, 293–299.

Guschin, I. S., Miroshnikov, A. I., Martynov, V. I. and Sviridov, V. V. (1981). Histamine releasing and anti-inflammatory activities of MCD-peptide and its modified forms. *Agents Actions* **11**, 69–71.

Gushchin, L. S., Miroshnikov, A. I., Martynov, V. I. (1977). Character of the histamine liberating action of MCD peptide from bee venom. *Bull. Exp. Biol. Med. (Engl. Transl.)* **84**, 1013–1018.

Habener, J. F., Rosenblatt, M., Dee, P. C. and Potts, J. T. (1979). Cellular processing of pre-proparathyroid hormone involves rapid hydrolysis of the leader sequence. *J. Biol. Chem.* **254**, 10596–10599.

Habermann, E. (1957). Manometrische bestimmung von phospholipase A. *Biochem. Z.* **328**, 474–484.

Habermann, E. (1958). Zur Wirkung tierischer Gifte und von Lysolecithin auf Grenzflachen. *Z. Gesamte Exp. Med.* **130**, 19-23.

Habermann, E. (1972). Bee and wasp venoms: The biochemistry and pharmacology of their peptides and enzymes are reviewed. *Science* **177**, 314-322.

Habermann, E. (1977). Neurotoxicity of apamin and MCD peptide upon central application. *Arch. Pharmacol.* **300**, 189-191.

Habermann, E. (1980). Melittin-structure and activity *In* 'Natural Toxins' (D. Eaker and T. Wadström, eds.), pp. 173-181. Pergamon, Oxford.

Habermann, E. and Cheng-Raude, D. (1975). Central neurotoxicity of apamin, crotamin, phospholipase A_2 and α-amanitin. *Toxicon* **13**, 465-467.

Habermann, E. and El Karemi, M. M. A. (1956). Antibody formation by protein components of bee venom. *Nature (London)* **178**, 1349.

Habermann, E. and Fischer, K. (1979a). Bee venom neurotoxin (apamin): Iodine labelling and characterisation of binding sites. *Eur. J. Biochem.* **94**, 355-364.

Habermann, E. and Fischer, K. (1979b). Apamin, a centrally acting neuropeptide: Binding and actions. *Adv. Cytopharmacol.* **3**, 387-394.

Habermann, E. and Horvath, E. (1980). Localisation and effects of apamin after application to the central nervous system. *Toxicon* **18**, 549-560.

Habermann, E. and Jentsch, J. (1967). Sequential analysis of melittin from tryptic and peptic fragments. *Biochem. Z.* **348**, 37-52.

Habermann, E. and Kowallek, H. (1970). Modification der Amino gruppen und des Tryptophans in Melittin als Mittel zur Erkennung von Struktur-Wirkungs Beziehungen. *Z. Physiol. Chem.* **351**, 884-890.

Habermann, E. and Krusche, H. (1962). Wirkung der Phospholipasen A und C auf Plasmalipoide und Erythrozyten. *Biochem. Pharmacol.* **11**, 400-412.

Habermann, E. and Neumann, W. P. (1957). Reinigung der Phospholipase A des Bienengiftes. *Biochem. Z.* **328**, 465-473.

Habermann, E. and Reiz, K. G. (1964). Apamin, ein basisches zentral erregendes Polypeptide aus Bienengift. *Naturwissenschafter* **51**, 61.

Habermann, E. and Reiz, K. G. (1965a). Ein neues Verfahren zur Gewinnung der Komponenten von Bienengift, inbesondere des zentralwirsamen Peptids Apamin. *Biochem. Z.* **341**, 451-466.

Habermann, E. and Reiz, K. G. (1965b). Zur Biochemie der Bienengift peptide Melittin und Apamin. *Biochem. Z.* **343**, 192-203.

Hahn, G. and Leditschke, H. (1937). Uber das Bienengift. IV. Mitt. Gewinnung beider Giftkomponenten durch Dialyse. *Ber. Dtsch. Chem. Ges.* **70**, 1637-1644.

Hanson, J. M., Morley, J. and Sorria-Herrera, C. (1974). Anti-inflammatory property of 401 (MCD-peptide), a peptide from the venom of the bee, *Apis mellifera*, L. *Br. J. Pharmacol.* **50**, 383-392.

Hartter, P. and Weber, U. (1975). Isolierung, Reduction und Reoxidation von Apamin und MCD-peptide. *Z. Physiol. Chem.* **356**, 393-399.

Haux, P. (1969). Die aminosaurensequens von MCD Peptid, einem specifisch Mastzellen degranulierenden Peptid aus Bienengift. *Z. Physiol. Chem.* **350**, 536-541.

Haux, P., Sowenthal, H. and Habermann, E. (1967). Sequenzanalyse des Bienengift-neurotoxins (Apamin) aus seinen tryptischen und chymotryptischen Spaltstücke. *Z. Physiol. Chem.* **348**, 737.

Hax, W. M. A., Demel, R. A., Spies, F., Vossenberg, J. B. J. and Linnemans, W. A. M. (1974). Increased phospholipase A activity and formation of communicative contact between *Acanthamoeba castellanii* cells. *Exp. Cell Res.* **89**, 311-319.

Hayashi, O. and Kornberg, A. (1954). Metabolism of phospholipids by bacterial enzymes. *J. Biol. Chem.* **206**, 647-653.

Haylett, D. G. (1976). Effects of sympathomimetic amines on ^{45}Ca efflux from liver slices. *Br. J. Pharmacol.* **57**, 158-160.

Hider, R. C. and Ragnarsson, U. (1980). A proposal for the structure of apamin. *FEBS Lett.* **111**, 189-193.

Hider, R. C. and Ragnarsson, U. (1981). A comparative structural study of apamin and related bee venom peptides. *Biochim. Biophys. Acta* **667**, 197-208.

Hider, R. C., Khader, F. and Tatham, A. S. (1983). Lytic activity of monomeric and oligomeric melittin. *Biochem. Biophys. Acta* **728**, 206-214.

Higginbotham, R. D. and Karnella, S. (1971). The significance of the mast cell response to bee venom. *J. Immunol.* **106**, 233-240.

Hoffman, D. R., Shipman, W. H. and Babin, D. (1977). Allergens in bee venom. Two new high molecular weight allergenic specificities. *J. Allergy Clin. Immunol.* **59**, 147-153.

Hol, W. G. J., Duijnen van, P. T. and Berendsen, H. J. C. (1978). The α-helix dipole and the properties of proteins. *Nature (London)* **273**, 443-446.

Hong, S.-C. L. and Levine, L. (1976). Stimulation of prostaglandin synthesis by bradykinin and thrombin and their mechanism of action on MC5-5 fibroblasts. *J. Biol. Chem.* **251**, 5814-5816.

Hugues, M., Duval, D., Kitabgi, P., Lazdunski, M. and Vincent, J.-P. (1982a). Preparation of pure monoiodo derivative of bee venom neurotoxin apamin and its binding properties to rat brain synaptosomes. *J. Biol. Chem.* **257**, 2762-2769.

Hugues, M., Duval, D., Schmid, H., Kitabgi, P., Lazdunski, M. and Vincent, J.-P. (1982b). Specific binding and pharmacological interactions of apamin, the neurotoxin from bee venom. *Life Sci.* **31**, 437-443.

Hugues, M., Romey, G., Duval, D., Vincent, J.-P. and Lazdunski, M. (1982c). Apamin as a selective blocker of the calcium-dependent potassium channel in neuroblastoma cells: voltage-clamp and biochemical characterisation of the toxin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1308-1312.

Hugues, M., Schmid, H. and Lazdunski, M. (1982d). Identification of a protein component of the Ca^{++}-dependent K^+ channel by affinity labelling with apamin. *Biochem. Biophys. Res. Commun.* **107**, 1577-1582.

Itoh, N., Obata, K., Yanaihara, N., and Okamoto, H. (1983). Human preprovasoactive intestinal polypeptide contains a novel PHI-27-like peptide, PHM-27. *Nature (London)* **304**, 547-550.

Ivanov, C. P., Shkenderov, S. and Krysteva, M. A. (1972). Isolation and purification of hyaluronidase from bee venom. *C. R. L'Acad. Bulg. Sci.* **25**, 229-232.

Ivanova, I. and Shkenderov, S. (1982). A newly isolated enzyme with lysophospholipase activity from bee venom. *Toxicon* **20**, 333-335.

Jacobs, J. W., Rubin, J. S., Hugli, T. E., Bogardt, R. A., Mariz, I. K., Daniels, J. S., Daughaday, W. H. and Bradshaw, R. A. (1978). Purification, characterisation and aminoacid sequence of rat anaphylatoxin ($C3_a$). *Biochemistry* **17**, 5031-5038.

Jasani, B., Kreil, G., Mackler, B. F. and Stanworth, D. R. (1979). Further studies on the structural requirements for polypeptide-mediated histamine release from rat mast cells. *Biochem. J.* **181**, 623-632.

Jenkinson, D. H. (1981). Peripheral actions of apamin. *Trends Pharmacol. Sci.* **2**, 318-320.

Jodal, M., Lundgren, O. and Sjöquist, A. (1983). The effects of apamin on the non-adrenergic, non-cholinergic vasodilator mechanisms in the intestine of the cat. *J. Physiol. (London)* **338**, 207-219.

Jurna, I. and Habermann, E. (1983). Intrathecal apamin selectively facilitates activity in ascending axons of rat spinal cord evoked by stimulation of afferent C fibres in sural nerve. *Brain Res.* **280**, 186–189.

Just, M., Erdmann, G. and Habermann, E. (1977). The renal handling of polybasic drugs. I. Gentamicin and aprotinin in intact animals. *Naunyn-Schmiedebergs Arch. Pharmacol.* **300**, 57–66.

Kaiser, E. T. and Kezdy, F. J. (1984). Amphiphilic secondary structure design of peptide hormones. *Science* **223**, 249–255.

Kemeny, D. M. and Vernon, C. A. (1983). Hyaluronidase from the venom of the honey bee, *Apis mellifera*. *Toxicon, Suppl. 3*, 227–228.

Kemeny, D. M., Banks, B. E. C., Lawrence, A. J., Pearce, F. L. and Vernon, C. A. (1981). The purification of hyaluronidase from the venom of the honey bee. *Biochem. Int.* **2**, 145–151.

Kemeny, D. M., Migachi, S., Platts-Mills, T. A. E., Wilkins, S. and Lessof, M. H. (1982). The immune response to bee venom. Comparison of the antibody response to phospholipase A_2 with the response to inhalant antigens. *Int. Arch. Allergy Appl. Immunol.* **68**, 268–274.

Kemeny, D. M., Harries, M. G., Youlten, L. J. F., Mackenzie-Mills, M. and Lessof, M. H. (1983a). Antibodies to purified bee venom proteins and peptides I. Development of a highly specific RAST for bee venom antigens and its application to bee sting allergy. *J. Allergy Clin. Immunol.* **71**, 505–514.

Kemeny, D. M., Mackenzie-Mills, M., Harries, M. G., Youlter, L. J. F. and Lessof, M. H. (1983b). Antibodies to purified bee venom proteins and peptides. II. A detailed study of changes in IgE and IgG antibodies to individual bee antigens. *J. Allergy Clin. Immunol.* **72**, 376–385.

Kemeny, D. M., Dalton, N., Lawrence, A. J., Pearce, F. L. and Vernon, C. A. (1984). The purification and characterisation of hyaluronidase from the venom of the honey bee. *Eur. J. Biochem.* **139**, 217–223.

Kempf, C., Klausner, R. D., Weinstein, J. N., van Rensworde, J. and Pincus, M., (1982). Voltage dependent transbilayer orientation of melittin. *J. Biol. Chem.* **257**, 2469–2476.

Kindas-Mügge, I., Lane, C. D. and Kreil, G. (1974). Insect protein synthesis in frog cells: The translation of honey bee promelittin messenger RNA in Xenopus oocytes. *J. Mol. Biol.* **87**, 451–462.

Kindas-Mügge, I., Frasel, L. and Diggelman, H. (1976). Characterisation of promelittin messenger RNA from the venom gland of young queen bees. *J. Mol. Biol.* **105**, 177–181.

King, T. P., Sobotka, A. K., Kochoumian, L. and Lichtenstein, L. M. (1976). Allergens of honeybee venom. *Arch. Biochem. Biophys.* **172**, 661–671.

Kitabgi, P. and Vincent, J.-P. (1981). Neurotensin is a potent inhibitor of guinea pig colon contractile activity. *Eur. J. Pharmacol.* **74**, 311–318.

Knöppel, E., Eisenberg, D. and Wickner, W. (1979). Interactions of melittin, a preprotein model, with detergents. *Biochemistry* **18**, 4177–4181.

Kreil, G. (1973a). Biosynthesis of melittin, a toxic peptide from bee venom, amino acid sequence of the precursor. *Eur. J. Biochem.* **33**, 558–566.

Kreil, G. (1973b). Structure of melittin isolated from two species of honey bees. *FEBS Lett.* **33**, 241–244.

Kreil, G. (1975). The structure of *Apis dorsata* melittin: Phylogenetic relationship between honey bees as deduced from sequence data. *FEBS Lett.* **54**, 100–102.

Kreil, G. (1978). Biochemical surprise of a bee sting. *New Sci.* **79**, 618–620.

Kreil, G. (1981). Transfer of proteins across membranes. *Annu. Rev. Biochem.* **50**, 317–348.

Kreil, G. and Bachmayer, H. (1971). Biosynthesis of melittin, a toxic peptide from bee venom, detection of a possible precursor. *Eur. J. Biochem.* **20**, 344–350.

Kreil, G. and Kreil-Kiss, G. (1967). The isolation of *N*-formyl glycine from a polypeptide present in bee venom. *Biochem. Biophys. Res. Commun.* **27**, 275–280.

Kreil, G., Haiml, L. and Suchanek, G. (1980a). Stepwise cleavage of the pro part of promelittin by dipeptidylpeptidase IV. Evidence for a new type of precursor-product conversion. *Eur. J. Biochem.* **111**, 49–58.

Kreil, G., Mollay, C., Kaschnitz, R., Haiml, L. and Vilas, U. (1980b). Prepromelittin: specific cleavage of the pre and pro-peptide *in vivo Ann. N. Y. Acad. Sci.* **343**, 338–346.

Krysteva, M. A., Mesrob, B. K., Ivanov, C. P. and Shkenderov, S. (1973). Partial characterisation of bee venom hyaluronidase. *C. R. L'Acad. Bulg. Sci.* **26**, 917–918.

Kudelin, A. B., Martinov, V. I., Kudelina, I. A. and Miroshnikov, A. I. (1979). *Abstr. 15th, Meet. Eur. Pept. Symp.* p. 84.

Lad, P. L. and Shier, W. T. (1980). Effect of melittin-induced membrane alterations on rat heart adenylate cyclase activity. *Arch. Biochem. Biophys.* **204**, 418–424.

Langer, J. (1897). Uber das Gift unserer Honigbiene. *Arch. Exp. Pathol. Pharmakol. Leipzig*, 38.

Lauterwein, J., Bosch, Ch., Brown, L. R. and Wuthrich, K. (1979). Physicochemical studies of the protein–lipid interactions in melittin-containing micelles. *Biochim. Biophys. Acta* **556**, 244–264.

Lauterwein, J., Brown, L. R. and Wuthrich, K. (1980). High resolution ^{1}H-NMR studies of monomeric melittin in aqueous solution. *Biochim. Biophys. Acta* **622**, 219–230.

Lawrence, A. J. (1975). Lysolecithin inhibits an action of bee venom phospholipase A_2 in erythrocyte membranes. *FEBS Lett.* **58**, 186–189.

Lawrence, A. J. and Moores, G. R. (1975). Activation of bee venom phospholipase A_2 by fatty acids, aliphatic anhydrides and glutaraldehyde. *FEBS Lett.* **49**, 287–291.

Lawrence, A. J., Moores, G. R. and Steele, J. (1974). A conductimetric study of erythrocyte lysis by lysolecithin and linoleic acid. *Eur. J. Biochem.* **48**, 277–286.

Lawson, D., Raff, M. C., Gomperts, B., Fewtrell, C. and Gilula, N. B. (1977). Molecular events during membrane fusion. A study of exocytosis in rat peritoneal mast cells. *J. Cell Biol.* **72**, 242–259.

Lebrun, P., Atwater, I., Claret, M., Malaisse, W. and Herchuelz, A. (1983). Resistance to apamin of the Ca^{++}-activated K^+-permeability in pancreatic B-cells. *FEBS Lett.* **161**, 41–44.

Levin, I. W., Lavialle, F. and Mollay, C. (1982). Comparative effects of melittin and its hydrophobic and hydrophilic fragments on membrane organisation by Raman spectroscopy. *Biophys. J.* **37**, 339–349.

Lewis, J. L., Day, A. J. and Lande, I. S. (1968). Phospholipase A in the venom of the Australian bulldog ant, *Myrmecia pyriformis*. *Toxicon* **6**, 109–112.

Liakopoulou-Kyriakides, M. and Galardy, R. E. (1979). s-Cis and s-trans isomerism of the His-Pro peptide bond in angiotension and thyroliberin analogues. *Biochemistry* **18**, 1952–1957.

Lichtenstein, L. M. (1977). Allergic responses to airborne allergens and insect venoms. *Fed. Proc., Fed. Am Soc. Exp. Biol.* **361**727–1731.

Lichtenstein, L. M., Sobotka, A. K. and Valentine, M. D. (1974). A case for venom treatment in anaphylactic sensitivity to hymenoptera sting. *N. Engl. J. Med.* **290**, 1223–1227.

Liu, L. K. (1982). PhD. thesis, University of London.

Liu, L. K. and Vernon, C. A. (1984). The structure of secapin: a peptide from bee venom. *J. Chem. Res. (S), 1984*, pp. 10–11.

Lübke, K., Matthes, S. and Kloss, G. (1971). Isolation and structure of *N*-formyl melittin. *Experientia* **27**, 765–766.

Maas, A. J. J. (1981). The effect of apamin on responses evoked by field stimulation in guinea-pig *Taenia* caeci. *Eur. J. Pharmacol.* **73**, 1–9.

Maas, A. J. J. (1981). The effect of apamin on responses evoked by field stimulation in guinea-pig *Taenia* caeci. *Eur. J. Pharmacol.* **73**, 1–9.

Maas, A. J. J., den Hertog, A., Ras, R. and Van Den Akker, J. (1980). The action of apamin on guinea-pig *Taenia caeci*. *Eur. J. Pharmacol.* **67**, 265–270.

Maberly, F. H. (1910). Brief notes on the treatment of rheumatism by bee stings. *Lancet* **2**, 235.

Markovic, O. and Rexova, L. (1963). The components of various types of honeybee venoms. *Chem. Invest.* **17**, 767–784.

März, L., Kühne, C. and Michl, H. (1983). The glycoprotein nature of phospholipase A_2, hyaluronidase and acid phosphatase from honey bee venom. *Toxicon* **21**, 893–896.

Marz, R., Mollay, C., Kreil, G. and Zelger, J. (1981). Queen bee venom contains much less phospholipase than worker bee venom. *Insect Biochem.* **11**, 685–690.

Mel'nikov, P. N. and Popov, E. M. (1980). *Bioorg. Khim.* **6**, 21–30.

Meyer, K. (1971). Hyaluronidases. 'The Enzymes' (P. D. Boyer, ed.), 3rd ed., Vol. 5, pp. 307–320. Academic Press, New York.

Meyer, K., Hoffman, P. and Linker, A. (1960). Hyaluronidases. *In* 'The Enzymes', Vol. 4, 2nd ed. (P. D. Boyer, H. Lardy and K. Myrback, eds.). Academic Press, New York and London. pp. 447–460.

Miroshnikov, A. I., Elyakova, E. G., Kudelin, A. B. and Senyavina, L. B. (1978). Physicochemical characteristics of apamin, a neurotoxin from honey-bee, *Apis mellifera*. *Bioorg. Khim.* **4**, 1022–1028.

Miroshnikov, A. I., Boikov, V. A. Snezhkova, L. G., Severin, S. E., Shvets, V. I. and Dudkin, S. M. (1983). Interactions between tertiapin, a neurotoxin from bee venom, and calmodulin. *Bioorg. Khim.* **9**, 26–32.

Mohammed, A. H. and El Karemi M. M. A. (1961). Immunity of bee keepers to some constituents of bee venom. *Nature (London)* **189**, 837–838.

Mohammed, A. H., Kamel, A. and Ayobe, M. H. (1968). Studies of phospholipase A and B activities of Egyptian snake venoms and scorpion toxins. *Toxicon* **6**, 293–298.

Mollay, C. (1976). Effects of melittin and melittin fragments on the thermotropic phase transition of dipalmitoyl lecithin and on the amount of lipid-bound water. *FEBS Lett.* **64**, 65–68.

Mollay, C. and Kreil G. (1973). Fluorimetric measurements on the interaction of melittin with lecithin. *Biochem. Biophys. Acta* **316**, 196–203.

Mollay, C. and Kreil G. (1974). Enhancement of bee venom phospholipase A_2 activity of melittin, direct lytic factor from cobra venom and polymixin B. *FEBS Lett.* **46**, 141–144.

Mollay, C., Kreil, G. and Berger, H. (1976). Action of phospholipase A_2 on the cytoplasmic membrane of *E. coli*: stimulation by melittin. *Biochim. Biophys. Acta* **426**, 317–324.

Mollay, D., Vilas, U. and Kreil, G. (1982). Cleavage of honey bee prepromelittin by an endoprotease from rat liver microsomes; identification of intact signal peptide. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2260–2264.

Montreuil, J. (1980). Primary structure of glycoprotein glycans. *Adv. Carbohydr. Chem. Biochem.* **37**, 157–160.

Moores, G. R. and Lawrence, A. J. (1972). Conductimetric assay of phospholipids and phospholipase A_2. *FEBS Lett.* **28**, 201–204.

Muller, J. and Baer, H. P. (1980). Apamin, a non-specific antagonist of smooth muscle relaxants. *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.* **311**, 105–107.

Munjal, D. and Elliot, W. B. (1971). A simple method for the isolation of a phospholipase from honey bee. *Apis mellifera*, venom. *Toxicon* **9**, 403–410.

Munjal, D. and Elliot, W. B. (1972). Further studies on the properties of phospholipase A from honey bee (*Apis mellifera*) venom. *Toxicon* **10**, 152–160.

Nelson, D. A. and O'Connor, R. (1968). The venom of the honey bee (*Apis mellifera*); free amino acids and peptides. *Can. J. Biochem.* **46**, 1221–1226.

Neumann, W. and Habermann, E. (1954). Beiträge zur Characterisierung der Wirkstoffe des Bienengiftes. *Naunyn-Schmiedebergs Arch. Pharmakol.* **222**, 367–387.

Neumann, W., Habermann, E. and Amend, G. (1952). Zur papierelektrophoretischen Fraktionierung tierischer Gifte. *Naturwissenschaften* **39**, 286–287.

O'Connor, R. and Peck, M. L. (1978). Venoms of the Apidae. *Handb. Exp. Pharmacol.* **48**, 613–659.

O'Connor, R., Rosenbrook, W., Jr. and Erickson, R. (1963). Pure venom from wasps, bees and hornets. *Science* **139**, 420–421.

O'Connor, R., Henderson, G., Nelson, D., Parker, R. and Peck, M. L. (1967). The venom of the honey bee (*Apis mellifera*). I. General character. *In* 'Animal Toxins' (F. E. Russell and P. R. Saunders, eds.), pp. 17–22. Pergamon, Oxford.

Ovcharov, R., Shkenderov, S. and Mihailova, S. (1976). Anti-inflammatory effects of apamin. *Toxicon* **14**, 441–447.

Ovchinnikov, Y. A., Miroshnikov, A. I., Kudelin, A. B., Kostina, M. B., Boikov, V. A., Magazanik, L. G. and Gotgilf, I. M. (1980). Structure and presynaptic activity of tertiapin, a neurotoxin from bee venom, (*Apis mellifera*). *Bioorg. Khim.* **6**, 359–365.

Owen, M. D. (1971). Insect venoms: identification of noradrenaline and dopamine in wasp and bee stings. *Experientia* **27**, 544–546.

Owen, M. D. (1978). Venom replenishment as indicated by histamine in honey bee, (*Apis mellifera*), venom. *J. Insect Physiol.* **24**, 433–437.

Owen, M. D. (1979). Relationship between age and hyaluronidase activity in the venom of queen and worker bees, (*Apis mellifera*). *Toxicon* **17**, 94–98.

Owen, M. D. (1983). Quantitative and temporal changes in honey bee venom—a review. *Toxicon Suppl. 3*, 329–332.

Owen, M. D. and Bridges, A. R. (1976). Ageing in the venom of queen and worker honey bees, (*Apis mellifera*): some morphological and chemical observations. *Toxicon* **14**, 1–5.

Owen, M. D. and Bridges, A. R. (1982). Catecholamines in honey bee (*Apis mellifera*) and various vespid (hymenoptera) venoms. *Toxicon* **20**, 1075–1084.

Owen, M. D., Braidwood, J. L. and Bridges, A. R. (1977). Age-dependent changes in histamine content of venom of queen and worker bees. *J. Insect Physiol.* **23**, 1031–1036.

Parsons, H. M. (1984). PhD. thesis, University of London.

Pearce, F. L. (1982). Functional heterogeneity of mast cells from different species and tissues. *Klin. Wochenschr.* **60**, 954–957.

Pearce, F. L. (1983). Mast cell heterogeneity. *Trends Pharmacol. Sci.* **4**, 165–167.

Peck, M. L. and O'Connor, R. (1974). Procamine and other basic peptides in the venom of the honey bee (*Apis mellifera*). *J. Agric. Food Chem.* **22**, 51–53.

Peck, M. L., O'Connor, R., Johnson, T. J., Isbell, A. F., Martell, A. E., McLenden, G., Neff, R. D. and Wright, D. A. (1978). Radioprotective potential and related properties of glycylhistamine, an analogue of histamine terminal peptides found in bee venoms. *Toxicon* **16**, 690.

Pichon, Y. (1974). Pharmacology of the insect nervous system. *In* 'The Physiology of the Insecta' (M. Rockstein, ed.), 2nd ed., Vol. 4, pp. 101–174. Academic Press, London.

Posch, M., Rabusch, U., Mollay, C. and Laggner, P. (1983). Cooperative effects in the interaction between melittin and phosphatidyl choline model membranes. Studies by temperature scanning densitometry. *J. Biol. Chem.* **258**, 1761–1766.

Rao, R. H. and Subramanian, D. (1970). Distribution and properties of phospholipase A of *Culex pipiens fatigans*. *Arch. Biochem. Biophys.* **140**, 443–449.

Rosenberg, P. (1979). Pharmacology of phospholipase A_2 from snake venoms. *In* 'Snake Venoms' (C. Y. Lee, ed.), pp. 403–447. Springer-Verlag, Berlin and New York.

Rosenberg, P., Condrea, E., Fletcher, J. E., Rapisano, B. E. and Yang, C.-C. (1983). Dissociation between enzymatic activity and pharmacological properties of snake venom phospholipase A_2. *Toxicon, Suppl. 3*, 371–375.

Sandberg, B. E. B. (1979). Solid phase synthesis of 13-lysine apamin, 14-lysine apamin and the corresponding guanidinated derivatives. *Int. J. Pept. Protein Res.* **13**, 327–333.

Sandberg, B. E. B. and Ragnarsson, U. (1978). *Int. J. Pept. Protein Res.* **11**, 238–245.

Schoch, P. and Sargent, D. F. (1980). Quantitative analysis of the binding of melittin to planar lipid bilayers allowing for the discrete charge effect. *Biochim. Biophys. Acta* **602**, 234–247.

Schröder, E., Lübke, K., Lehmann, M. and Beetz, I. (1971). Haemolytic activity and action on the surface tension of aqueous solutions of synthetic melittin and its derivatives. *Experienta* **27**, 764–766.

Seagar, M. J., Granier, C. and Couraud, F. (1984). Interactions of the neurotoxin, apamin, with a Ca^{++}-activated K^+ channel in primary neuronal cultures. *J. Biol. Chem.* **259**, 1491–1495.

Sessa, G., Freer, J. H., Colacicco, G. and Weissmann, G. (1969). Interaction of a lytic polypeptide, melittin, with lipid membrane systems. *J. Biol. Chem.* **244**, 3575–3582.

Shepherd, D. W., Elliot, W. B. and Arbesman, C. E. (1974). Fractionation of bee venom. I. Preparation and characterisation of four antigenic components. *Biochem. Prep.* **4**, 71–88.

Shier, W. T. (1979). Activation of high levels of phospholipase A_2 in cultured cells. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 195–199.

Shier, W. T. (1980). Activation of self-destruction as a mechanism of action for cytolytic toxins. *In* 'Natural Toxins' (D. Eaker and T. Wadström, eds.), pp. 193–200. Pergamon, Oxford.

Shipman, W. H. and Cole, L. J. (1967). Increased resistance of mice to X-irradiation after the injection of bee venom. *Nature (London)* **215**, 311–312.

Shipolini, R. A., Bradbury, A. F., Callewaert, G. L. and Vernon, C. A. (1967). The structure of apamin. *Chem. Commun. 1967*, p. 679.

Shipolini, R. A., Callewaert, G. L., Cottrell, R. S., Doonan, S., Vernon, C. A. and Banks, B. E. C. (1971). Phospholipase A_2 from bee venom. *Eur. J. Biochem.* **20**, 459–468.

Shipolini, R. A., Callewaert, G. L., Cottrell, R. C. and Vernon, C. A. (1974a). The amino acid sequence and carbohydrate content of phospholipase A_2 from bee venom. *Eur. J. Biochem.* **48**, 465–476.

Shipolini, R. A., Doonan, S. and Vernon, C. A. (1974b). The disulphide bridges of phospholipase A_2 from bee venom. *Eur. J. Biochem.* **48**, 477–483.

Shkenderov, S. (1973). A protease inhibitor in bee venom. Identification, partial purification and some properties. *FEBS Lett.* **33**, 343–347.

Shkenderov, S. (1974). Anaphylactogenic properties of bee venom and its fractions. *Toxicon* **12**, 529–534.

Shkenderov, S. (1976a). New pharmacobiochemical data on the anti-inflammatory effect of bee venom. *In* 'Animal, Plant and Microbial Toxins' (A. Ohsaka, K. Hayashi and Y. Sawai, eds.), Vol. 2, pp. 319–336. Plenum, New York.

Shkenderov, S. (1976b). Further purification, inhibitory spectrum and some kinetic properties of the protease inhibitor in bee venom. *In* 'Animal, Plant and Microbial Toxins' (A. Ohsaka, K. Hayashi and Y. Sawai, eds.), Vol. 1, pp. 263–272. Plenum, New York.

Shkenderov, S. and Koburova, K. (1982). Adolapin—a newly isolated analgetic and anti-inflammatory polypeptide from bee venom. *Toxicon* **20**, 317–321.

Shkenderov, S., Ivanova, I. and Grigorova, K. (1979). An acid monophosphatase and α-glucosidase enzymes newly isolated from bee venom. *Toxicon Suppl. 1*, **17**, 169–170.

Shuba, M. F. and Vladimirova, I. A. (1980). Effect of apamin on the electrical responses of smooth muscle to adenosine-5′-triphosphate and to non-adrenergic, non-cholinergic nerve stimulation. *Neuroscience* **5**, 853–859.

Sjöquist, A., Fahrenkrug, J., Jodal, M., and Lundgren, O. (1983). Effect of apamin on the release of vasoactive intestinal polypeptide (VIP) from the cat intestines. *Acta Physiol. Scand.* **119**, 69–76.

Smith, M. J. H. and Ford-Hutchinson, A. W. (1979). Anti-inflammatory agents of animal origin. Heffter's, *Handb. Exp. Pharmacol.* **50**, 661–697.

Sobotka, A. K., Franklin, R. M., Adkinson, N. F., Valentine, M. D., Baer, H. and Lichtenstein, L. M. (1976). Allergy to insect stings. II. Phospholipase A_2: The major allergen in honey bee venom. *J. Allergy Clin. Immunol.* **57**, 29–40.

Spoerri, P. E. (1983). Changes induced by apamin from bee venom on differentiated neuroblastoma cells in culture. *Acta Anat.* **117**, 346–354.

Spoerri, P. E., Jentsch, J. and Glees, P. (1973). Apamin from bee venom: Effects of the neurotoxin on cultures of the embryonic mouse cortex. *Neurobiology* **3**, 207-214.

Spoerri, P. E., Jentsch, J. and Glees, P. (1975). Apamin from bee venom. Effects of the neurotoxin on subcellular particles of neural cultures. *FEBS Lett.* **53**, 142-147.

Steiner, D. F., Kemmler, W., Tager, H. S. and Peterson, J. D. (1974). Proteolytic processing in the biosynthesis of insulin and other proteins. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33**, 2105-2115.

Strom, R., Crifo, C., Viti, V., Guidoni, L. and Podo, F. (1978). Variations in circular dichroism and proton nmr relaxation properties of melittin upon interaction with phospholipids. *FEBS Lett.* **96**, 45-50.

Suchanek, G. and Kreil, G. (1977). Translation of melittin messenger RNA *in vitro* yields a product terminating with glutaminyl glycine rather than with glutaminamide. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 975-978.

Suchanek, G., Kreil, G. and Hermodson, M. A. (1978). Amino acid sequence of honey bee prepromelittin synthesised *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 701-704.

Talbot, J. C., Dufourcq, J., de Bony, J., Faucon, F. C. and Lussan, C. (1979). Conformational change and self-association of monomeric melittin. *FEBS Lett.* **102**, 191-193.

Tatham, A. S., Hider, R. C. and Drake, A. F. (1983). The effect of counterions on melittin aggregation. *Biochem. J.* **211**, 683-686.

Terwilliger, T. C. and Eisenberg, D. (1982a). The structure of melittin: I. Structure determination and partial refinement. *J. Biol. Chem.* **257**, 6010-6014.

Terwilliger, T. C. and Eisenberg, D. (1982b). The structure of melittin: II. Interpretation of the structure. *J. Biol. Chem.* **257**, 6015-6022.

Terwilliger, T. C., Weissman, L. and Eisenberg, D. (1982). The structure of melittin in the form I crystals and its implication for melittin's lytic and surface activities. *Biophys. J.* **37**, 353-361.

Tosteson, M. T. and Tosteson, D. C. (1981). The sting. Melittin forms channels in lipid bilayers. *Biophys. J.* **36**, 109-116.

Tosteson, M. T. and Tosteson, D. C. (1984). Activation and inactivation of melittin channels. *Biophys. J.* **45**, 112-114.

Tu, A. T., ed. (1977). 'Venoms: Chemistry and Molecular Biology,' pp. 502-515. Wiley, New York.

Tu, A. T., ed. (1984). 'Handbook of Natural Toxins,' Vol. 2. Marcel Dekker, Inc., New York.

Uvnäs, B. and Thon, I.-L. (1961). Evidence for enzymatic histamine release from isolated mast cells. *Exp. Cell Res.* **23**, 45-57.

Van der Bosch, (1974). Phosphoglyceride metabolism. *Annu. Rev. Biochem.* **43**, 243-277.

Van Golde, L. M. G. and van den Bergh, S. G. (1977). General pathways in the metabolism of lipids in mammalian tissues. *In* 'Lipid Metabolism in Mammals' (F. Snyder ed.), Vol. 1, pp. 1-33. Plenum, New York.

van Rietschoten, J., Granier, C., Rochat, H., Lissitzky, S. and Miranda, F. (1975). Synthesis of apamin, a neurotoxic peptide from bee venom. *Eur. J. Biochem.* **56**, 35-40.

Verma, S. P. and Wollach, D. F. H. (1976). Effect of melittin on the thermotropic lipid state transitions in phosphatidylcholine liposomes. *Biochim. Biophys. Acta* **426**, 616-623.

Vernon, C. A., Hanson, J. M. and Brimblecombe, R. W. (1969). Peptides, British Patent No. 1324823.

Vick, J. A. and Shipman, W. H. (1972). Effects of whole bee venom and its fractions (apamin and melittin) on plasma cortisol levels in dogs. *Toxicon* **10**, 377-380.

Vick, J. A., Shipman, W. H., Brooks, R. B. and Hassett, C. C. (1972). Beta adrengeric and anti-arrhythmic effects of apamin, a component of bee venom. *Am. Bee J.* **112**, 339-340.

Vick, J. A., Shipman, W. H. and Brooks, R. B. (1974). Beta-adrenergic and anti-arrhythmic effects of cardiopep, a newly isolated substance from whole bee venom. *Toxicon* **12**, 139-144.

Vincent, J.-P., Schweitz, H. and Lazdunski, M. (1975). Structure-function relationships and