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CHARACTERIZATION OF KININS IN WASP VENOM

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In mammals, kinins are formed from inactive protein precursors by the action of specific proteases at or near the site of their action. Wasp and hornet venoms are in interesting contrast to the mammalian system in that they contain free kinins which act immediately upon injection (Jacques and Schachter, 1954; Schachter and Thain, 1954; Holdstock et al., 1957; Mathias and Schachter, 1958; and Bhoola et al., 1961). In *Vespa vulgaris* a single major and two minor components were resolved on columns of Amberlite (XE-64). All three kinin activities were completely destroyed by chymotrypsin and greatly reduced by trypsin. Bradykinin and kallidin are not inactivated by trypsin and could be distinguished chromatographically from the wasp kinins (Mathias and Schachter, 1958). Hornet venom contains a single, different kinin which is not inactivated by trypsin (Bhoola et al., 1961). Peptides from wasps and hornets have pharmacological actions very similar to bradykinin and kallidin; they contract isolated smooth muscle preparations, lower arterial blood pressure, increase capillary permeability, and cause pain when applied to an exposed blister base on human skin.

The present study was undertaken to characterize the potent venom kinins chemically. Their structural relationship to bradykinin and kallidin, if any, is of interest, since virtually any amino acid substitution in bradykinin causes an almost com-

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plete loss of biological activity (Boissonnas et al., 1960; Bodanszky et al., 1963; Nicolaides et al., 1963; Schröder and Hempel, 1964). It would be of equal interest to find the bradykinin structure in such a highly specialized fluid as venom which also contains serotonin, histamine (Jaques and Schachter, 1954), acetylcholine (Bhoola et al., 1961), and pharmacologically active enzymes (Beard, 1963). The discovery of mammalian kinins in venom should contribute to a better understanding of them and strengthen the prevailing opinion that these peptides are uniquely active substances of physiological importance in man.

MATERIALS AND METHODS

Three species, *Polistes exclamans* Viereck, *P. annularis* Linnaeus, and *P. fuscatus pallipes* Lepeletier, were brought to the laboratory and their venom mixed. When not used immediately, wasps were kept alive 1-3 days at 3-5°C or for as long as 2 weeks at room temperature when fed fresh or canned fruit. Most of the wasps, about 6,000, were frozen at −10°C upon arrival at the laboratory. The abdomens were dissected and the lower halves, which contain the venom glands, sacs, ducts, and lancets were stored up to 6 months at −10°C.

Extraction of peptides and amines

For purpose of comparison, several methods of extraction were employed. The venom sac, duct, and lancet were removed intact from anesthetized insects by enlarging the abdominal opening slightly with forceps, grasping the lancet, and pulling the entire apparatus through the opening (Schachter and Thain, 1954). The apparatus was homogenized in glacial acetic acid and the peptides precipitated from the extracts with diethyl ether as previously described (Holdstock et al., 1957). Extracts were also prepared with 0.15 M saline instead of acetic acid. These were bioassayed directly for their amine content, and precipitation with diethyl ether was omitted. The electric shock technique (O'Connor et al., 1963) was used to obtain pure venom.

Bioassays

Rat uteri, guinea pig ilea, and rat duodena were suspended in a 10-ml muscle chamber and the assays performed in the usual manner. Rat blood pressure was recorded with a Statham strain gauge and a Sanborn recorder. Assays were based on responses to single and double doses of unknowns and standards. Chemical assays for histamine (Shore et al., 1959) and serotonin (Bogdanski et al., 1956) were also employed.

Chromatography and electrophoresis

Columns of CM (carboxymethyl)-cellulose, 0.8 x 23 cm, were eluted with ammonium formate essentially as described previously (Pierce and Webster, 1961). High-voltage paper electrophoresis* was performed on Whatman 3MM paper in 4% formic acid for 4 hours at 20°C with a potential of 5,000 V. Descending paper chromatograms were developed with 1-butanol:glacial acetic acid:water (60:15:25). Whatman 3MM paper was washed previously by the descending technique, with a solution of equal volumes of formic acid, ethanol, and water. After chromatography or electrophoresis, the papers were dried and either sprayed with a modified Sakaguchi reagent (F. Irreverre, personal communication) or cut into segments, eluted with Tyrode's buffer, and assayed directly for biological activity. Synthetic bradykinin and kallidin were used as standards.

RESULTS AND DISCUSSION

Yields of active peptides and amines were essentially the same from samples obtained by electric shock or by acetic acid extraction of the venom apparatuses. It was usually necessary to shock wasps 3 to 4 times before their venom supply was depleted. In some cases venom could be obtained daily for as long as 5 days; however, mortality was higher than 50%. Although the electric shock technique was efficient, it was time-consuming and impractical for handling large numbers of insects. One important difference was noted in the various prepa-

*Electrophorator model D, Gilson Medical Electronics Co., Middletown, Wis.

rations: i.e. kinin activity of venom obtained by electroshock was more stable than saline and acetic acid extracts of the venom apparatus. The latter slowly lost their activity on standing at room temperature. Activity of the extracts could be stabilized by heating in a boiling water bath as noted earlier (Schachter and Thain, 1954).

Active amines

The histamine content of several venom preparations was determined both by bioassay and chemical techniques. An average value of 0.8 μg per insect was obtained with the guinea pig ileum (after Code's treatment; Code, 1937) while 0.6 μg per insect was the value obtained fluorometrically. All the contractile activity of these preparations was blocked by diphenhydramine.

The fluorometric procedure for serotonin gave an average of 1.2 μg per insect. All biological activity on the rat uterus of butanol extracts from the above procedure was inhibited by lysergic acid diethylamide. Acetylcholine-like activity was not observed in any of the venom preparations. However, if acetylcholine were present in amounts less than 0.15 μg per insect, it would have been undetected in the bioassays. The European wasp (Jaques and Schachter, 1954) and hornet (Bhoola et al., 1961) also contain high levels of these amines.

Active peptides

The estrus rat uterus pretreated with lysergic acid diethylamide (1 μg/ml muscle chamber) was used to detect and quantify peptides. Antihistaminics were usually omitted because the rat uterus is relatively insensitive to the amine. Contraction of this tissue was of the slow type (Fig. 1) characteristic of bradykinin and kallidin and, like these peptides, activity was abolished by previous incubation with chymotrypsin but not trypsin. Assays of several preparations gave a maximal value of activity per insect equivalent to 1.5 μg of synthetic bradykinin. However, a sample assayed on uteri of different rats frequently gave varying values, suggesting the presence of one or more peptides whose activity on this tissue did not always parallel that of the bradykinin standard.

When various crude extracts were examined by CM-cellulose chromatography, five peaks of activity were observed.

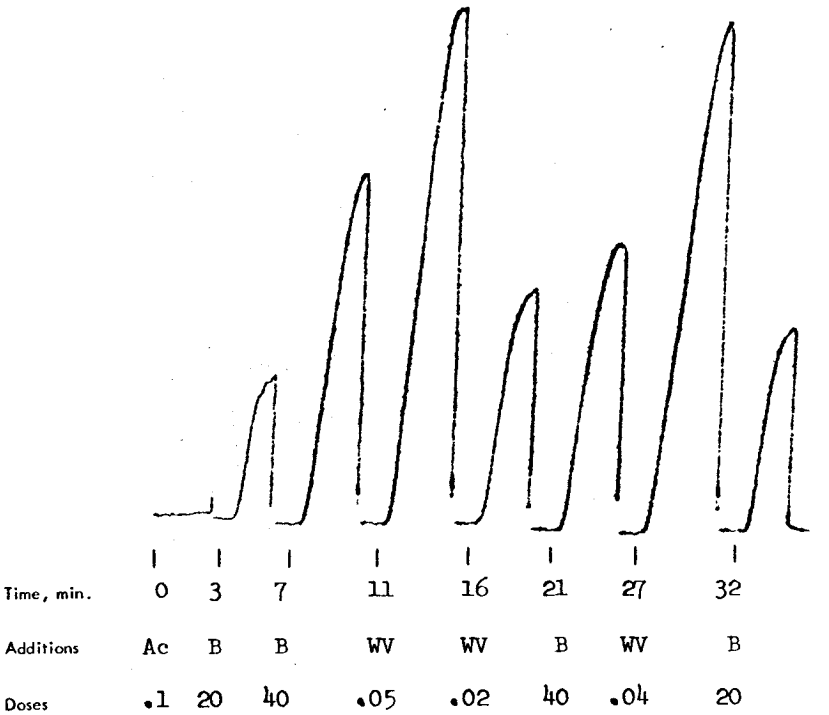

Fig. 1. The rat uterus was suspended in a 10-ml muscle chamber containing atropinized (0.010 μg/ml) de Jalon's solution maintained at 30°C. A 2-g load was applied to the muscle, and contractions (7 × magnification) were recorded for 60 sec. The drum was stopped when test solutions were washed out of the chamber. Acetylcholine, 0.10 μg, produced no contraction, and 0.020 ml of a 1:10 dilution of a wasp venom (WV) preparation (10 wasps/ml) gave a contraction equivalent to 0.03 μg bradykinin. Venom was obtained by the electric shock technique. Additions were: acetylcholine (Ac) in micrograms, bradykinin (B) in nanograms, and wasp venom solution in milliliters.

Peaks 1 and 2 were indistinguishable by this technique from bradykinin and kallidin. Further similarities in these peptides with regard to electrophoretic and chromatographic mobilities, susceptibility to inactivation by enzymes, and activity on various tissues were also noted (Table 1).

CM-Cellulose chromatography of several extracts always

TABLE 1
Properties of Bradykinin, Kallidin, and Wasp Kinins (Peaks 1, 2, and 3)

	Bradykinin	Peak 1	Kallidin	Peak 2	Peak 3
Active fraction numbers from CM-cellulose [a]	21-25	19-23	31-35	26-35	43-51
Paper electroph.[b]	47-51	40-47	55-58	45-51	44-54
Paper chromat.[c]	8-10	5-10	3-6	0-5	0-2.5
Chymotrypsin [d]	+	+	+	+	+
Trypsin, pepsin, collagenase [e]	–	–	–	–	–
LSD, atropine [e]	–	–	–	–	–
Guinea pig ileum and rat uterus	contr.	contr.	contr.	contr.	contr.
Rat duodenum	relax.	relax.	relax.	relax.	relax.
Rat blood pressure	hypot.	hypot.	hypot.	hypot.	hypot.

[a] Fractions of 2.4 ml were collected. Eluent was ammonium acetate (Pierce and Webster, 1961).

[b] Distance in centimeters from origin cathodically. The buffer was 4% formic acid.

[c] Distance in centimeters from the origin. The solvent front was 38 cm and the solvent 1 – butanol:acetic acid: water (60:15:25).

[d] The enzymes, 100 μg/ml, either inactivated (+) or did not inactivate (–) the kinins and the standards.

[e] Lysergic acid diethylamide (LSD), 1 μg/ml, and atropine, 0.01 μg/ml, did not affect (–) the activity on the uterus.

showed that peak 1 contained the least and peak 3 the most biological activity. The variability in assays of crude venom noted above was due largely to peak 3. Such variability was not noted with peaks 1 and 2. An example of the activity in each of the peaks is given in Table 2, which shows several different assays employed.

TABLE 2
Bioassay of Peaks 1, 2, and 3 from CM-Cellulose

Bioassay and standard	Peak 1	Peak 2	Peak 3
G × B	1	20	135
G × K	3	50	400
RU × B	3.5	52	48*
RU × K	4.5	100	56
BP × B		360	540
BP × B		270	540
D × B	1	40	20
D × K		40	20

Activities expressed as micrograms of either bradykinin (B) or kallidin (K). Activity (from 625 wasps) added to the column was equivalent to 900 μg bradykinin when assayed on a previous day with the rat uterus. G, guinea pig ileum; RU, rat uterus; BP, rat blood pressure; D, rat duodenum.

*Reassay of this material with several uteri gave higher results. Since no variability was ever noted in peaks 1 and 2, it is apparent most of the 900 μg of bradykinin activity assayed previously in the extract was due to peak 3.

Further characterization of peaks 1, 2, and 3

Peaks 1 and 3 from CM-cellulose chromatography also gave single peaks of kinin activity when chromatographed on columns of CM-Sephadex. However, peak 2 was resolved into two peaks. Amino acid analysis of peaks 1 and 2 revealed the presence of all twenty amino acids. These results were unexpected, since up to this time the wasp kinins had been indis-

tinguishable from bradykinin and kallidin. It became apparent that pure peptides could not be obtained by column chromatography alone. Consequently, the fluorescent derivative technique was introduced with the expectation that it could be used as both an analytical and preparative procedure.

A dramatic demonstration of the heterogeneity of peaks 1 and 2 was obtained when aliquots were reacted with 1-dimethylamino-naphthalene-5-sulfonyl chloride (Gray and Hartley, 1963) and the fluorescent peptide derivatives examined by thin-layer chromatography. With silica gel H (without binder) and the solvent system 1-butanol:acetic acid:water (5:1:5), seven fluorescent bands were observed on the plate. Furthermore, when eluted from the plate, three of the bands had biological activity. The less basic component of peak 2 from the CM-Sephadex step, when similarly treated, gave six fluorescent bands, of which five were biologically active. It is interesting that the fluorescent derivatives themselves were biologically active. The 1-dimethylamino-naphthalene-5-sulfonamides of bradykinin and kallidin have 1-2% the potency of the free peptides in the rat uterus assay. Because of the minute quantities of the subfractions of peaks 1 and 2, it was not possible to perform quantitative amino acid analyses on the fluorescent wasp kinin derivatives eluted from the thin-layer plates. However, qualitative results indicated relatively high levels of arginine, proline, and phenylalanine.

Peak 3, the most abundant peptide, appeared homogeneous in thin-layer chromatography. The amount isolated from 3,000 wasps was 1.3 mg. A portion, 0.15 mg, was hydrolyzed with HCl, and the liberated amino acids were determined by an ion-exchange procedure (Spackman et al., 1958). Preliminary results indicate that peak 3 is composed of Arg_2, Asp, Gly_2, Glu, Leu, Lys_2, Phe_2, Pro_3, Ser, and Thr. No tryptophan was found in alkaline hydrolysates.

Treatment of peak 3 with trypsin changed its chromatographic properties on columns of CM-Sephadex and on thin-layer plates. However, the biological activity was increased slightly. (As indicated earlier activity is variable because rat uteri differ in their sensitivity to peak 3.) The biological activity in the trypsin digest was recovered as a single peak from a column of CM-Sephadex, and one fluorescent band was obtained on thin-layer chromatography of the 1-dimethylamino-naphthalene-5-sulfonamide. Amino acid analysis of the free peptide obtained from the column step showed that it consisted of

Arg_2, Gly_2, Pro_3, Phe_2, Ser. The fluorescent peptide derivative eluted from the thin-layer plate had the identical composition less one glycine residue which was identified as the 1-dimethylamino-naphthalene-5-sulfonamide. Hence glycine was N-terminal, and the active tryptic peptide was glycine^1-kallidin (Fig. 2). Neither glycine nor any of the other constituent amino acids was N-terminal in the native peptide, suggesting the possibility that this position is occupied by pyroglutamic acid.

H—Gly—Arg—Pro—Pro—Gly—Phe—Ser—Pro—Phe—Arg—OH

Fig. 2. Structure of glycine^1-kallidin (glycylbradykinin), the active peptide released from peak 3 by trypsin.

Currently under study is the sequence of the other amino acids (Asp, Glu, Leu, Lys_2, and Thr) in peak 3 as well as the identification of the many other kinins found in peaks 1, 2, 4, and 5 obtained from the CM-cellulose step.

If the biosynthesis of kinins in wasps is similar to the mammalian system, kininogens might also be present in these insects. This does not seem to be the case, however, since preliminary experiments indicate the absence of kininogens in venom and protein extracts of the entire wasp.

It is of interest that peptides containing the bradykinin structure are found in such a highly specialized fluid as wasp venom with histamine, serotonin and probably other pharmacologically active substances. Probably all these substances play a role in the pain, swelling, and inflammation caused by stings from these insects. Not only might these substances have individual roles but their combination could be particularly effective. For example, it has recently been shown that serotonin has a remarkable potentiating action on bradykinin-induced pain in man (Sicuteri et al., 1965).

From an evolutionary viewpoint there may be a correlation between the venom composition and the behavior of *Hymenopteri*. For example, the more primitive solitary wasp, *Sceliphron caementarium* (mud-dauber), preys primarily on insects. Its venom, which is less effective on mammals, lacks histamine, serotonin, acetylcholine, and presumably kinins (O'Connor and Rosenbrook, 1963). The more advanced social wasps and hornets, on the other hand, are not known to use their stings to capture insects; instead they sting only in defense of the colony, and their venom contains potent, pharmacologically active sub-

stances such as histamine, serotonin, kinins, phospholipase A, hyaluronidase, and histidine decarboxylase (Beard, 1963), which are native to mammals. Bees are very advanced in the evolutionary scheme; their venom is similar to that of wasps and hornets but lacks kinins. However, bee venom does contain a substance, presumably an enzyme, which produces a delayed, rapidly desensitized contraction in the guinea pig ileum (Schachter and Thain, 1954). This could be a kallikrein which liberates kinins from the host's substrate.

Regarding peak 3 isolated from wasps, it will be recalled that this is the most abundant kinin and that only after trypsin treatment and the formation of glycine^1-kallidin was there uniformly high activity on the rat uterus. Conceivably, the native peptide is a kininogen which after injection is hydrolyzed by the host's enzymes to form glycine^1-kallidin. This could provide a prolonged kinin action which, when considered with the synergistic action of serotonin on bradykinin, makes wasp venom a rather potent and exquisitely active toxin.

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DISCUSSION

Austen: What is the potential sensitivity of chemical assay with the dansyl reagent?

Pisano: It has been reported (Gray and Hartley, 1963) that 10^{-10} to 10^{-10} mole of dansyl derivative can be seen on paper when irradiated with ultraviolet light. In solution we can detect 10^{-10} mole/ml with the spectrophotofluorimeter, and with microcells it should be possible to measure 10^{-11} mole in 0.1 ml of solution. This would be approximately 0.01 μg of bradykinin or 0.005 μg of kallidin, since 2 equivalents of reagent react with this peptide (α and ϵ amino groups of lysine). In an actual application higher quantities would be required, since unavoidable losses would be encountered in the preparation and isolation of the dansyl derivative.

Schachter: There are many similarities in the results of the elegant chemistry of Drs. Prado and Pisano and their colleagues and our own simple chemistry carried out some years ago. Nevertheless, there are also some possibly significant differences. For example, none of our three kinin-like activities in wasp venom (or the one in hornet venom) corresponded to either kallidin or bradykinin, and all were inactivated by trypsin as well as by chymotrypsin. It may well be that there are species differences in the kinins in insect venoms and that the ones in *Vespa vulgaris*, *polistis* and *crabro* are different. If this is so, there is an abundance of work in this sphere for the organic and synthetic chemist.

Gladner: How reliable is the certainty of the amino acid analysis that relates to the molecular weight and purity?

Pisano: There is some question, since the analysis was not done by us.

Sicuteri: We have found a potentiation between bradykinin and 5-HT in pain receptors. This may be the reason for the association of these two substances in wasp venom.

Prado: We would agree with Dr. Sicuteri that this is quite possible. Serotonin and bradykinin in wasp venom have a potentiating effect on the pain produced by the sting.