Vespulakinins: New Carbohydrate-Containing Bradykinin Derivatives†

Hisanobu Yoshida,‡ Ronald G. Geller, and John J. Pisano*

ABSTRACT: Two structurally similar and highly active glycopeptides have been isolated from extracts of yellow jacket venom sacs by ion-exchange and droplet countercurrent chromatography procedures. Vespulakinin 1 (heptadecapeptide) and vespulakinin 2 (pentadecapeptide) are both highly basic and contain the nonapeptide bradykinin at their carboxy termini. Most unique is the presence of carbohydrate. Vespulakinins are the first reported naturally occurring glycopeptide derivatives of bradykinin and the first reported vasoactive glycopeptides.

Venoms of common stinging insects such as bees, wasps, hornets, and yellow jackets contain a variety of potent, biologically active substances (Habermann, 1968). They include amines and polypeptides, which act directly to produce pharmacological effects, and enzymes which produce active substances from substrates in the victim's tissues. Notable enzymes are phospholipase A, hyaluronidase, and histidine decarboxylase. Our interest in naturally occurring vasoactive polypeptides in venoms led us to extend the original observations of kininlike peptides in hornet venom (Schachter and Thain, 1954). We have studied the venom of wasps belonging to the genus *Polistes* and discovered polisteskinin (Prado et al., 1966), an octadecapeptide which contains the nonapeptide bradykinin at the carboxy-terminal end: <Glu-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Nakajima et al., 1967, 1970; Pisano, 1968). We now report on two new glycopeptides, vespulakinins 1 and 2, isolated from extracts of venom sacs of the yellow jacket *Vespula* (*Vespula*) *maculifrons*, perhaps the most offensive stinging insect in the United States.

Methods

Bioassay. Extracts and purified peptides were assayed with three smooth muscle preparations: the isolated rat uterus and duodenum and the guinea pig ileum (Trautschold, 1970). The effect on arterial blood pressure was determined in 250–300 g Sprague-Dawley rats anesthetized with sodium pentobarbital (30–40 mg/kg ip). A cannula was inserted in the femoral artery and pressure determined with a Statham P23DB transducer. Samples were administered through a cannula in the femoral vein; bradykinin (Sandoz BSR-640 batch no. 69055) was used as standard.

Chromatography. Two columns were packed with SP-Sephadex C-25. The large column, 0.9 × 55 cm, was used in the early purification (Figure 1) and the small column, 0.9 × 15 cm (no figure), was used to separate traces of vespulakinin 1 from vespulakinin 2 obtained in the droplet countercurrent chromatography step (Figure 2). The small column was eluted with a linear gradient made with 60 ml of 0.3 *M* ammonium formate (pH 6.5) and 60 ml of 1.0 *M* ammonium formate (pH 6.5). Active fractions were freeze-dried to remove buffer. It was necessary to repeat the freeze-drying step several times (by addition of distilled water to the dried sample) to effectively remove the ammonium formate.

Droplet countercurrent chromatography (Tanimura et al., 1970) was performed in an all-Teflon unit (Yoshida et al., 1971, 1976), consisting of 200 columns 20 cm long made of standard wall 14 gauge (1.68 mm i.d.) tubing. The columns were connected with standard wall 24 gauge (0.56 mm i.d.) tubing. Operating conditions are given in Figure 2.

Enzymatic Hydrolysis. Enzymes purchased from Worthington Biochemical Corp. were trypsin-Tos-PheCH$_2$Cl,[1] 185 μ/mg; α-chymotrypsin, 45 U/mg; carboxypeptidase A-iPr$_2$FP, 35 U/mg; carboxypeptidase B-iPr$_2$FP, 95 U/mg; and leucine aminopeptidase, 100 U/mg. Usually 1 μg of enzyme was incubated at 25°C with 1–10 nmol of peptide for 2 hr in 100 μl of triethylamine-HCO$_3$ buffer (pH 8.5).

Amino Acid Analysis. A 10-nmol norleucine internal standard was added to peptides prior to hydrolysis in 5.7 *N* HCl at 110°C for 24 hr. Amino acids were analyzed by the Moore and Stein method with a Beckman Model 120-C analyzer modified for rapid elution and high sensitivity (Hubbard and Kreman, 1965).

Carbohydrate Analysis. Internal standards of 10 nmol of xylose and glucosamine were added to the samples prior to hydrolysis in 2.5 *N* trifluoroacetic acid at 100°C for 6 hr. Internal standards of 10 nmol of norleucine and glucosamine were added to glycopeptide samples before hydrolysis in 5.7 *N* HCl at 110°C for 24 hr. Hexosamines (Tamura et al., 1968) and hexoses and pentoses (Imanari et al., 1969) were determined by gas chromatography; galactosamine was determined on the Beckman amino acid analyzer. Carbohydrate-containing amino acid thiazolinones were separated from the shortened peptide by the use of SP-Sephadex H$^+$, 0.3 ml bed volume, in a 1-ml tuberculin syringe (Figure 3).

A β-elimination reaction (Tanaka and Pigman, 1965) was also performed on the native peptide to determine serine and threonine O-glycosidic linkages. After β elimination the reaction mixture was adjusted to pH 4 with dilute acetic acid and passed through the 0.3-ml SP-Sephadex H$^+$ column. The nonadsorbed fraction was hydrolyzed in 2.5 *N*

† From the Section of Physiological Chemistry, Laboratory of Chemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received April 15, 1975.*

‡ Present address: Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Hiroshima, Japan.

[1] Abbreviations used are: Tos-PheCH$_2$Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; iPr$_2$FP, diisopropyl fluorophosphate; Pth, phenylthiohydantoin.

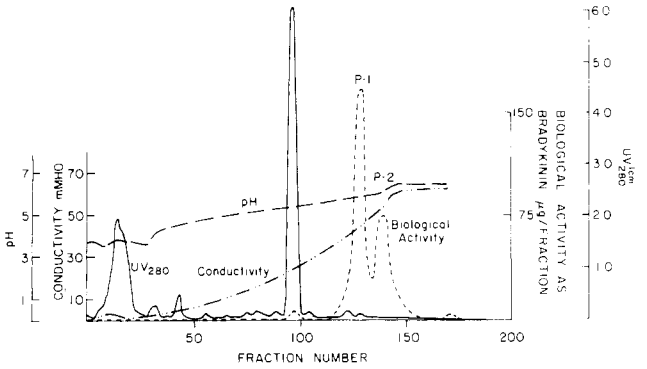

FIGURE 1: Separation of vespulakinins 1 and 2 (P-1 and P-2) on an SP-Sephadex column, 0.9 × 55 cm, equilibrated with 0.01 *M* ammonium formate (pH 3.6). The freeze-dried sample (250 mg of white powder with activity ≃ 1.1 mg of bradykinin or 1 μmol of bradykinin) was dissolved in starting buffer and eluted with a buffer gradient made with a four-chamber unit. Each chamber was filled with 100 ml of an ammonium formate buffer: 0.01 *M*, pH 3.6 (starting buffer), 0.05 *M*, pH 5.0; 0.2 *M*, pH 5.0, and 1.0 *M*, pH 6.6 (final buffer); flow rate, 20 ml/hr; fraction volume, 3.3 ml.

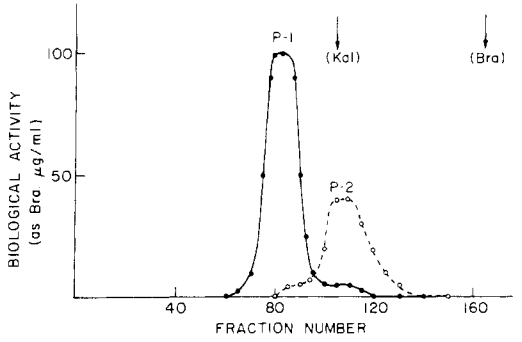

FIGURE 2: Droplet countercurrent chromatography of vespulakinins 1 and 2 (P-1 and P-2). Fractions 126-132 (P-1) and 133-143 (P-2) (Figure 1) were combined and analyzed separately. The freeze-dried fractions were dissolved in 100 μl of aqueous lower (moving) phase of the solvent system *sec*-butyl alcohol–trifluoroacetic acid–water (120:1:160); flow rate, 2.4 ml/hr; fraction volume, 0.6 ml; Kal denotes kallidin (Lys-bradykinin) and Bra denotes bradykinin whose elution volumes are indicated by arrows. The yields of P-1 and P-2 determined by amino acid analysis were 0.25 and 0.1 μmol, respectively.

trifluoroacetic acid and analyzed for carbohydrate (Imanari et al., 1969). The adsorbed fraction was eluted and analyzed for amino acids after hydrolysis with 5.7 *N* HCl (Figure 3).

Sequence Analysis. The amino acid sequence was determined twice, on 20 and on 50 nmol of peptide. The dansyl-Edman method (Gray, 1967) was used with the following modifications: (1) phenyl isothiocyanate and buffer were removed by directing a nitrogen stream into the tube heated at 70°C for 20 min, (2) *n*-butyl chloride was used instead of *n*-butyl acetate, and (3) the aqueous phase was frozen to facilitate removal of *n*-butyl chloride. Dansyl amino acids were identified by thin-layer chromatography using polyamide sheets (Woods and Wang, 1967).

Analysis of dansyl peptides (Tamura et al., 1973), subtractive Edman analysis, and Pth[1] amino acid analysis by gas chromatography (Pisano and Bronzert, 1969) were also employed.

Extraction. Live insects were stored up to 4 days at 5°C before being killed by immersion in methanol. Venom sacs were removed by pulling out the lancet which is connected

SCHEME FOR CARBOHYDRATE ASSIGNMENT

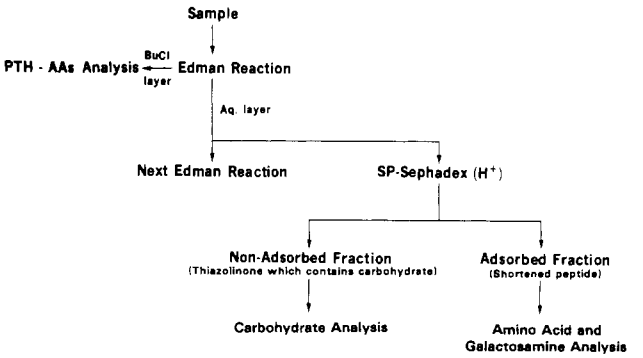

FIGURE 3: Scheme for carbohydrate assignment. Peptide (50 nmol) was degraded. The aliquots of aqueous layer taken were: step 1, 15%; step 2, 22%; step 3, 43%; step 4, the entire sample. The nonadsorbed fraction was obtained by washing the column (0.3 ml of SP-Sephadex H^+ in a 1-ml tuberculin syringe) with 1 ml of H_2O; the adsorbed fraction was obtained by elution with 1 ml of 1 *M* ammonium formate (pH 6.5). Xylose and glucosamine, 10 nmol each, were added as internal standards to the nonadsorbed fraction; 10 nmol of norleucine and glucosamine was added to the adsorbed fractions.

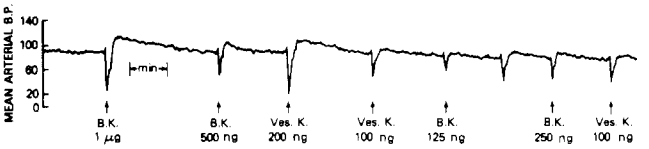

FIGURE 4: Hypotensive effects of vespulakinin 1 (Ves. K) and bradykinin (B.K.) in the anesthetized rat. Kinin was administered iv at the time indicated by arrows; pressure in millimeters of mercury.

to the sac by a strong duct. Sacs were stored 6 months at −10°C prior to extraction.

Five milliliters of 1% acetic acid was added to 1300 sacs and the mixture was homogenized at room temperature in a Potter-Elvehjem homogenizer. The residue obtained after centrifugation was similarly extracted five more times; the extracts were combined, bioassayed, freeze-dried, and chromatographed on SP-Sephadex.

Results

Purification. The acetic acid extract of 1300 venom sacs contained activity equivalent to 1.1 mg (1.1 μmol) of bradykinin when assayed with the rat uterus. When the freeze-dried material, 250 mg, was fractionated on the 0.9 × 55 cm column of SP-Sephadex, two major peaks of activity, P-1 and P-2, were observed (Figure 1). Fractions 126–132 (P-1) and 133–143 (P-2) were combined and freeze-dried.

P-1 and P-2 were separately dissolved in 100 μl of the lower (aqueous) phase of the solvent system 2-butanol–trifluoroacetic acid–water (120:1:160) and purified by droplet countercurrent chromatography (Figure 2). Pure P-1 was obtained by this step; pure P-2 was obtained after rechromatography on the 0.9 × 15 cm SP-Sephadex column (see Methods). Amino acid analysis gave 0.25 and 0.1 μmol of P-1 and P-2, respectively.

Characteristics of P-1 and P-2. Both P-1 and P-2 contracted the rat uterus and guinea pig ileum, relaxed the rat duodenum, and lowered rat blood pressure (Figure 4), actions characteristic of bradykinin, Lys-bradykinin (kallidin), and Met-Lys-bradykinin. Vespulakinin 1 is at least twice as potent on a weight basis as bradykinin in lowering rat blood pressure. Relative potencies in the other tests were

Table I: Composition of Vespulakinin 1 and Carbohydrate Assignment to Residues Thr[3] and Thr[4] (See Figure 3 for the Scheme Used to Analyze the Shortened Peptide).

	AA and Carbohydrate Anal. of Fractions Adsorbed to SP-Sephadex[a]									Carbohydrate Anal. of Fractions Not Adsorbed to SP-Sephadex		
	Thr	Ser	Pro	Gly	Ala	Phe	Arg	Galactosamine[b]	Galactose	Galactosamine[c]	Galactose	GC Anal. of AA Pth
Native peptide	2.8	0.95	3.0	2.2	1.0	2.0	4.9	4.8	2.6	*i*	*i*	*i*
After 1 Edman cleavage	*2.0*[h]	0.97	2.9	2.0	1.0	2.0	5.0	4.6	*i*	*f*	*g*	Thr
After 2 Edman cleavage	2.0	0.99	3.0	2.1	*0.2*	2.0	5.1	4.3	*i*	*f*	*g*	Ala (Thr)[f]
After 3 Edman cleavage	*1.2*	0.98	3.1	2.1	0.2	2.0	5.1	*3.5*	*i*	1.6	0.8	(Ala)[f]
After 4 Edman cleavage	*0.6*	0.96	3.0	2.2	0.2	2.0	4.6	*1.2*	*i*	3.0	1.8	*g*
After β elimination	1.6	1.1	3.0	2.5	1.1	2.0	4.8	2.4	*i*	1.1[d]	1.0[e]	*i*

[a] See Figure 3. [b] Hydrolysis in 5.7 *N* HCl, 110°C, 24 hr. Analysis by amino acid analyzer. [c] Hydrolysis in 2.5 *N* trifluoroacetic acid, 110°C, 6 hr. Analysis by gas chromatography. [d] Galactosaminol. [e] Galactose: galactitol $\simeq$ 2:1. [f] Trace. [g] Not detected. [h] Significant numbers are in italics. [i] Analysis not performed.

not measured. Like the known kinins, P-1 and P-2 were inactivated by chymotrypsin and carboxypeptidase B but not by trypsin, carboxypeptidase A, or leucine aminopeptidase. The amino acid and carbohydrate compositions are shown in Table I.

Amino Acid Sequence of P-1. Seven dansyl-Edman cycles indicated the partial sequence of P-1 as Thr-Ala-Thr-Thr-Arg-Arg-Arg plus a decapeptide with amino-terminal Gly composed of Arg-2, Ser-1, Pro-3, Gly-2, and Phe-2. Galactose and galactosamine were absent from the decapeptide, which was indistinguishable from authentic Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, i.e., Gly-bradykinin, when analyzed by thin-layer chromatography (Tamura et al., 1973). Furthermore, decapeptide from P-1 and Gly-bradykinin gave the same peptide degradation products when treated with chymotrypsin and carboxypeptidase B, i.e., the dansylated fragments from each had the same thin-layer mobilities and amino-terminal amino acids. Gly-bradykinin was also produced by trypsin digestion of 20 nmol of P-1.

The amino acid sequence deduced for native P-1 was: Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly-bradykinin.

Carbohydrate Assignment. The amino group of galactosamine is evidently blocked (acetylated?) in P-1, because only dansyl threonine and no dansyl galactosamine was detected in an acid hydrolysate of the dansylated native peptide. Since no carbohydrate was detected in the Gly-bradykinin (isolated from a trypsin digest of P-1 or after seven cycles of Edman degradation of P-1), it was surmised that the carbohydrates were linked through an O-glycosidic bond to one or more of the threonine residues (positions 1, 3, and 4).

Indirect evidence for attachment of carbohydrate at threonine residues in positions 3 and 4 was obtained from Pth analysis. Pth-alanine and -threonine were identified by gas chromatography after the first and second Edman cycles, but no Pth amino acid was found after the third and fourth cycles because they contained carbohydrate and were not volatile (Table I). Direct evidence for carbohydrate at Thr[3] and Thr[4] was obtained from the loss of carbohydrate in the shortened peptide (fraction adsorbed to SP-Sephadex) and its recovery in the cleaved residue (fraction not adsorbed to SP-Sephadex). The carbohydrate recovered from residues 3 and 4 accounted for all that found in the native peptide. Removal of carbohydrate from the native peptide by β elimination (Tanaka and Pigman, 1965) was apparently incomplete because only 1 residue of threonine should have remained; 1.6 were found. Also, the yields of carbohydrate were low. No loss of serine was observed which supports the earlier results where no carbohydrate was found in the Gly-bradykinin derived from P-1. The proposed structure for P-1 is shown in Figure 5.

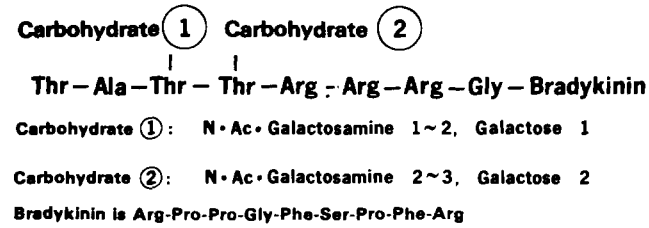

FIGURE 5: Structure of vespulakinin 1 (P-1). *N*-Acetylgalactosamine was not positively identified but deduced from the fact that the peptide does not contain free galactosamine. The pentadecapeptide vespulakinin 2 lacks the amino-terminal Thr-Ala. It has the same carbohydrate composition as vespulakinin 1.

The precise carbohydrate composition is not known. The methods employed would have detected hexosamines, hexoses, and pentoses; no effort was made to look for sialic acid.

Amino Acids Sequence of P-2. Analysis of P-2 by the procedures given above, including bioassays, amino acid and carbohydrate analyses, amino-terminal amino acid analyses, and the formation of Gly-bradykinin by trypsin digestion, indicated the amino acid sequence of P-2 as P-1 minus amino-terminal Ala-Thr, i.e., Thr-Thr-Arg-Arg-Arg-Gly-bradykinin. P-2 also contained as much galactose and galactosamine as P-1, presumably attached to the threonine residues.

Discussion

Venoms from bees, wasps, hornets, and yellow jackets contain a remarkable variety of potent pharmacologic agents. Substances which act directly include some well-known biogenic amines, acetylcholine, serotonin, catecholamines, histamine, and the polypeptides mellitin, apamin, mast cell degranulation peptide (MCD-peptide), and kinins. Substances which act indirectly such as the enzymes phospholipase A, hyaluronidase, and histidine decarboxylase probably produce active agents from substrates present in the victim's tissues. Interesting species differences are found in the distribution of amines and peptides. For example, bee venom is notable for its content of mellitin, apamin,

and MCD-peptide, but apparently it does not contain kinins, which are found in some hornet and wasp venoms (Schachter and Thain, 1954; Prado et al., 1966). Hornet and wasp venoms do not contain mellitin, apamin, or MCD-peptide. However, the variety of pharmacologic agents in the venom of any one species is apparently sufficient to contribute substantially to the pain, inflammation, and swelling which usually occur after a sting.

Yellow jacket venom sac extracts contain, in addition to vespulakinins 1 and 2, histamine, dopamine, noradrenaline, and histidine decarboxylase. Acetylcholine and 5-hydroxytryptamine, commonly found in venom of other insects such as wasps and hornets, were not found. Upon iv injection of a saline extract of one yellow jacket venom sac, an immediate and severe fall in blood pressure, a decrease in heart rate, and high incidence of death occurs in rats (Geller et al., 1976). It is probably that no single substance is responsible for the total effect, nor can the harmful reaction be the sum of individual activities. Rather, it is likely that the agents act synergistically. From a clinical viewpoint, it is probable that the antigen proteins of venoms are of greatest concern. Phospholipase A may be the major antigen in bee venom (Sobotka et al., 1974).

Polisteskinin, <Glu-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly-bradykinin, isolated from a mixture of wasps, *Polistes annularis, P. fuscatus,* and *P. exalamans,* has several notable actions which distinguish it from the mammalian kinins, bradykinin, Lys-bradykinin, and Met-Lys-bradykinin. Unlike these kinins it is not inactivated by passage through the rat pulmonary vascular bed (Ryan et al., 1970), it has a longer acting hypotensive effect, and it is the most potent naturally occurring releaser of histamine from rat mast cells (Johnson and Erdos, 1973). Vespulakinins are similar to polisteskinin. Bradykinin is at the carboxy-terminal end of the peptides, their amino-terminal portions are very basic, and they are about the same size. Vespulakinins contain three arginine residues and a free amino group at the amino terminus. Polisteskinin contains amino-terminal pyroglutamic acid, three lysines, and one arginine. The most distinguishing features of vespulakinins are the carbohydrate prosthetic groups. Vespulakinins may be the first known vasoactive glycopeptides. The extent to which the carbohydrate contributes to the biological activity remains to be determined. Vespulakinin 1 is at least twice as potent as bradykinin (on a weight basis) in lowering rat blood pressure, but the duration of the response is not significantly longer. Presumably, vespulakinin 1, like bradykinin, is rapidly inactivated by lung. The unusually high basicity of vespulakinins and their unique carbohydrate content invite further pharmacologic testing. Especially interesting would be a determination of their potency in releasing histamine from mast cells and leucocytes and their ability to produce pain.

It may be of interest to note that MCD-peptide of bee venom is also a potent histamine releaser and that this structurally different, highly basic, cysteine-containing, 22-residue peptide (Habermann, 1968) has an unexpected and remarkable antiinflammatory action (Hanson et al., 1974). This action of MCD-peptide may shed light on the controversy concerning the alleged beneficial effect of bee venom in the treatment of human arthritis. Yellow jacket venom is also suspected by some of having a similar beneficial effect. While the therapeutic effect of yellow jacket venom is conjectural, many can attest to the harmful effects from personal experience. In the U.S., venomous Hymenoptera cause more deaths than snakes, spiders, or scorpions (Christy, 1967). One allergist practicing in the Washington, D.C. area has reported that in 87 cases of insect-sting allergy in which the offending insect was positively identified, 47% were yellow jackets, 27% honey bees, 14% wasps, 6% bumble bees, and 6% hornets (Barr, 1974). The yellow jacket may be the most offensive venomous animal in the United States.

References

Barr, S. E. (1974), *J. Am. Med. Assoc. 228,* 718.

Christy, N. P. (1967), *Am. J. Med. 42,* 107.

Geller, R. G., Yoshida, H., Beaven, M. A., Horakova, Z., Atkins, F. L., Yamabe, H., and Pisano, J. J. (1976), *Toxicon* (in press).

Gray, W. R. (1967), *Methods Enzymol. 11,* 139.

Habermann, E. (1968), *Ergben. Physiol. 60,* 220.

Hanson, M. J., Morley, J., and Soria-Herrera, C. (1974), *Br. J. Pharmacol. 50,* 383.

Hubbard, R., and Kreman, D. M. (1965), *Anal. Biochem. 12,* 592.

Imanari, T., Arakawa, Y., and Tamura, Z. (1969), *Chem. Pharm. Bull. 17,* 1967.

Johnson, A. R., and Erdos, E. G. (1973), *Proc. Soc. Exp. Biol. Med. 142,* 1252.

Nakajima, T., Pisano, J. J., and Udenfriend, S. (1967), *Proc. Int. Congr. Biochem. 7th Col. VIII-4.*

Nakajima, T., Tanimura, T., and Pisano, J. J. (1970), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29,* 282 Abstr.

Pisano, J. J. (1968), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 27,* 58.

Pisano, J. J., and Bronzert, T. (1969), *J. Biol. Chem. 244,* 45597.

Prado, J. L., Tamura, Z., Furano, E., Pisano, J. J., and Udenfriend, S. (1966), in Hypotensive Peptides, New York, N.Y., Springer-Verlag New York, p 93.

Ryan, J. W., Roblero, J., and Stewart, J. M. (1970), in Bradykinin and Related Kinins, Cardiovascular, Biochemical and Neural Actions, Sicuteri, F., Rocha e Silva, M., and Back, N., Ed., New York, N.Y., Plenum Press.

Schachter, M., and Thain, E. M. (1954), *Br. J. Pharmacol. 9,* 352.

Sobotka, A., Franklin, R., Valentine, M., Adkinson, F. N., Jr., and Lichenstein, L. (1974), *J. Allergy Clin. Immunol. 53,* 103 Abstr.

Tamura, Z., Imanari, T., and Arakawa, Y. (1968), *Chem. Pharm. Bull. 16,* 1964.

Tamura, Z., Nakajima, T., Nakayama, T., Pisano, J. J., and Udenfriend, S. (1973), *Anal. Biochem. 52,* 595.

Tanaka, K., and Pigman, W. (1965), *J. Biol. Chem. 240,* 1487.

Tanimura, T., Pisano, J. J., Ito, Y., and Bowman, R. L. (1970), *Science 169,* 54.

Trautschold, I. (1970), *Handb. Exp. Pharmakol. 25,* 52.

Woods, E. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 14133,* 369.

Yoshida, H., Tanimura, T., and Pisano, J. J. (1971), *Fed. Proc., 30,* 1182 Abstr.

Yoshida, H., Zimmerman, C. L., and Pisano, J. J. (1976), in Peptides: Chemistry, Structure and Biology (Proceedings of the Fourth American Peptide Symposium), Walter, R., and Meienhofer, J., Eds., Ann Arbor, Mich., Ann Arbor Science Publishers Inc., in press.