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Bradykinin-Potentiating Peptides from the Venom of *Agkistrodon halys blomhoffii*. Isolation of Five Bradykinin Potentiators and the Amino Acid Sequences of Two of Them, Potentiators B and C*

Hisao Kato and Tomoji Suzuki

ABSTRACT: Five bradykinin-potentiating peptides (potentiators A, B, C, D, and E) were isolated from the venom of the Japanese poisonous snake, *Agkistrodon halys blomhoffii*, using gel filtrations on columns of Sephadex G-100, Sephadex G-10, and Sephadex G-25 and ion-exchange chromatography on a column of CM-Sephadex C-50. These five bradykinin-potentiating peptides were characteristic in having a high proline content, 5 or 6 of the 11 amino acid residues being proline residues. The biological activities of these five peptides were measured using isolated guinea pig ileum and rat uterus. Of the five potentiators, B and C had the strongest bradykinin-potentiating activity on guinea pig ileum. On the other hand, only potentiator E showed high bradykinin-potentiating activity on rat uterus, the other four peptides having very slight activity. These peptides did not cause contraction of smooth muscles in the absence of bradykinin. The amino acid sequences of two bradykinin-potentiating peptides, B and C, were determined to be as follows, and these were confirmed by chemical synthesis of the peptides: potentiator B: Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro; potentiator C: Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro.

Bradykinin, which has the amino acid sequence of Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, is a typical plasma kinin, causing contraction of isolated smooth muscles, vasodilatation, and permeability increasing of capillaries. Kinin is liberated into the plasma from its precursor protein, kininogen, by kinin-releasing enzymes, and inactivated by kininase. There is much evidence that plasma kinins are important in various physiological and pathological conditions, although there are still many complex problems regarding their actions.

The contractile action of bradykinin on isolated smooth muscles is known to be potentiated by several types of compounds. Sulfhydryl compounds (Ferreira and Rocha e Silva, 1962; Auerswald and Doleschel, 1967; Cirstea, 1965), such as cysteine, 2,3-dimercaptopropanol (BAL), or thioglycolic acid and reduced glutathione (Edery and Grunfeld, 1969), increase the size of the contraction of isolated guinea pig ileum induced by bradykinin. ϵ-Aminocaproic acid (Doleschel and Auerswald, 1966), a tryptic hydrolysate of denatured human plasma (Aarsen, 1968), an extract of pig liver (Tewksbury and Stahmann, 1965), and products of degradation of fibrinogen by plasmin (Buluk and Malofiejew, 1969) also have bradykinin-potentiating activity. Hamberg *et al.* (1969) attempted to isolate bradykinin-potentiating peptides from a tryptic hydrolysate of human plasma and a plasmic hydrolysate of human fibrinogen, and found that the bradykinin-potentiating activity of the peptides seemed to depend on the presence of C-terminal arginine or lysine. Bovine fibrinopeptide B (Gladner *et al.*, 1963) and human fibrinopeptide A-β (Osbahr *et al.*, 1964) had bradykinin-potentiating activity on rat uterus. Thus, many peptide-like substances were found to have bradykinin-potentiating activity. However, none of their amino acid sequences except those of fibrinopeptides are known yet.

In 1965, Ferreira found that the venom of the snake, *Bothrops jararaca*, contains an alcohol-soluble factor which potentiates the effects of bradykinin both *in vitro* and *in vivo* (Ferreira, 1965). We found that the venom of the Japanese poisonous snake, *Agkistrodon halys blomhoffii* contains two or more bradykinin-potentiating peptides (Suzuki *et al.*, 1966), and later we purified five bradykinin-potentiating peptides from the venom. Preliminary reports have appeared on their amino acid compositions (Kato and Suzuki, 1969) and the amino acid sequence of one of them, potentiator B (Kato and Suzuki, 1970a). This paper describes the purification and biological activities of five bradykinin-potentiating peptides and results of studies on the amino acid sequences of two of them, potentiators B and C. Recently, Ferreira *et al.* (1970a; Greene *et al.*, 1970) purified a bradykinin-potentiating peptide from the venom of *Bothrops jararaca* and determined its amino acid sequence as Pyr[1]-Lys-Trp-Ala-Pro. No similarity was found between this pentapeptide and the undecapeptide potentiator B or C. However, the amino-terminal part of potentiator E (Kato and Suzuki, 1970c), one of the five bradykinin-potentiating peptides described in this paper, is strikingly similar to the above pentapeptide in its amino acid sequence.

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[1] The abbreviation used is: Pyr, pyrrolidonecarboxylic acid.

Materials and Methods

Carboxypeptidase B, collagenase Type III (clostridiopeptidase A) from *Clostridium histolyticum*, a protease from *Streptomyces griseus* type VI, and twice-crystallized trypsin were purchased from Sigma Chemicals Co., Ltd. Aminopeptidase M was a product of Rohm and Haas, GMBH, Darmstadt, Germany. Synthetic bradykinin, pyroglutamylglycine, isoleucylprolylproline, and *N*-acetylglutamic acid were kindly supplied by Dr. S. Sakakibara of our Institute. Sephadex G-100, Sephadex G-25, Sephadex G-10, and CM-Sephadex C-50 were products of Pharmacia, Upsalla, Sweden.

Analytical Techniques. Gel filtrations were performed at about 4° in a cold room and column chromatography on CM-Sephadex C-50 was performed at room temperature. The amounts of peptides in each fraction from the columns were measured from their absorbancy at 280 mμ, and by color development with ninhydrin after alkaline hydrolysis. For alkaline hydrolysis, 0.2-ml aliquots of fractions of eluate from the column were heated with 1 ml of 2.5 N NaOH at 110° for 2.5 hr. The hydrolysates were mixed with 1 ml of 30% glacial acetic acid, and the ninhydrin reaction was performed by the method of Yemm and Cocking (1955). Amino acid analyses were carried out with a Japan Electron Optics Laboratory amino acid analyzer, JLC-5AH. Hydrazinolysis was performed by the method of Akabori *et al.* (1952), and the free amino acid liberated from the C terminal of the peptide was identified in the amino acid analyzer. Ornithine derived from arginine by hydrazinolysis was separated from lysine in the amino acid analyzer using a short column at pH 4.25. Direct Edman degradation for sequential analysis was performed essentially according to the method of Iwanaga *et al.* (1969), but almost all the peptides examined were soluble in ethylene chloride, so the ethylene chloride precipitation step was omitted and the water phase of the final step was subjected to the subsequent degradation. Subtractive Edman degradation was carried out by the method of Dopheide *et al.* (1967).

High-voltage paper electrophoresis was done at pH 3.5 (pyridine–acetic acid–water, 1:10:289, v/v) and pH 5.5 (pyridine–acetic acid–water, 100:35:4865, v/v). The solvents used for thin-layer chromatography and descending paper chromatography were 1-butanol–acetic acid–water (3:1:1, v/v) and 1-butanol–acetic acid–pyridine–water (15:3:10:12, v/v), respectively.

Preparation of Peptide Fragments. Hydrolysis of the bradykinin-potentiating peptides with *Streptomyces griseus* protease or collagenase was carried out in 0.01 M phosphate buffer, pH 8.0, and with trypsin in 0.1 M ammonium bicarbonate at 37°. The weight ratio of substrate to enzyme was 50:1 for tryptic hydrolysis and 10:1 for hydrolysis with protease from *S. griseus* or collagenase. Hydrolysates were applied to Toyo No. 51A filter paper and subjected to paper electrophoresis or paper chromatography. To locate the peptides on a guide strip cut from the paper, ninhydrin reagent, peptide reagent (*tert*-butyl hypochlorite–*o*-tolidine–KI) (Mazur *et al.*, 1962), Sakaguchi's reagent, and Ehrlich's reagent were used. The peptide zones on the paper, located by comparison to the guide strip, were eluted with 5% pyridine–acetic acid solution and lyophilized. The homogeneity of the peptide fragments was examined by paper electrophoresis at pH 3.5 and thin-layer chromatography (1-butanol–acetic acid–water, 3:1:1, v/v). The recovery of peptide fragments was calculated from amino acid analysis.

Biological Assays. Bradykinin activity was determined in a 16-ml organ bath by measuring contraction of guinea pig ileum in aerated tyrode solution and of estrus rat uterus in oxygenated de Jalon solution at 30°. Peptides were added just before addition of 0.05–0.5 ml of the standard bradykinin solution and the increase in size of contractions of the smooth muscles was estimated by comparison with that induced by a standard dose of synthetic bradykinin. As standards, a concentration of 1 μg of synthetic bradykinin per ml was used for assay with guinea pig ileum and 0.1 μg/ml for assay with rat uterus. The amounts of peptides required to double the effect of bradykinin on smooth muscles, which were independent of the amount of standard bradykinin added, were measured. Assuming the molar amount of potentiator B to potentiate twofold the action of bradykinin as 1.0, the relative potencies of other peptides were calculated from their molar ratios.

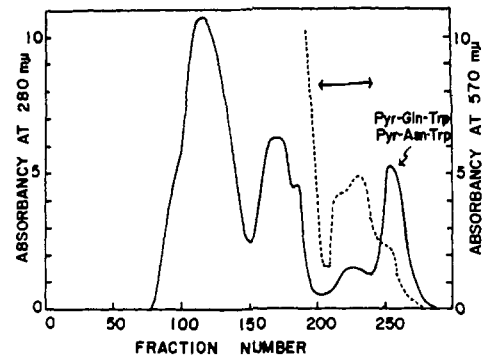


FIGURE 1: Gel filtration of the venom of *A. halys blomhoffii* on a column of Sephadex G-100. *A. halys blomhoffii* venom (10 g) was applied to a column (6 × 125 cm) of Sephadex G-100 equilibrated with 0.01 M phosphate buffer, pH 8.0, and fractions of 15 ml of eluate were collected at a flow rate of 50 ml/hr. The solid line shows the absorbancy at 280 mμ of each fraction. The dotted line shows the absorbancy at 570 mμ in the ninhydrin reaction after alkaline hydrolysis. The fraction with bradykinin-potentiating activity is indicated by the arrow.

Results

Isolation of Five Bradykinin-Potentiating Peptides from Venom. PREPARATION OF A BRADYKININ-POTENTIATING PEPTIDE FRACTION. Lyophilized *Agkistrodon halys blomhoffii* venom (10 g) was dissolved in 25 ml of 0.01 M phosphate buffer, pH 8.0, and the insoluble material was removed by centrifugation. The supernatant was applied to a column of Sephadex G-100 equilibrated with 0.01 M phosphate buffer, pH 8.0, and eluted with the same buffer. As shown in Figure 1, bradykinin-potentiating activity was found in low molecular weight fractions separating from the bulk of the venom protein. The last peak with high absorbancy at 280 mμ in Figure 1 was that of tripeptides, previously characterized as Pyr-Asn-Trp and Pyr-Gln-Trp (Kato *et al.*, 1966). The fractions (200–245) containing bradykinin-potentiating peptides were pooled, lyophilized, and applied to a column of Sephadex G-10 equilibrated with 5% acetic acid. Figure 2a shows that the bradykinin-potentiating peptides were eluted in the fractions of the void volume, and these fractions (29–38) were pooled, lyophilized, and applied to a column of Sephadex G-25. Contaminating proteins were removed in this way, and fractions 50–63 in Figure 2b were pooled and lyophilized. The yield of the mixture of bradykinin-potentiating peptides was 153.8 mg.

SEPARATION OF BRADYKININ-POTENTIATING PEPTIDES ON A COLUMN OF CM-SEPHADEX C-50. The above mixture of brady-

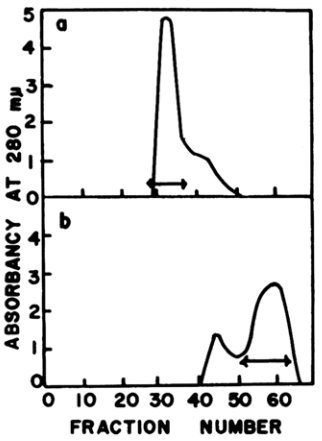


FIGURE 2: (a) Gel filtration of the eluate from the Sephadex G-100 column on a column of Sephadex G-10: column size, 3 × 88 cm; fraction size, 5 ml; flow rate; 50 ml/hr; eluent, water. (b) Gel filtration of the fractions from the Sephadex G-10 column on a column of Sephadex G-25: column size, 2 × 140 cm; fraction size, 5 ml; flow rate, 15 ml/hr; eluent, 5% acetic acid.

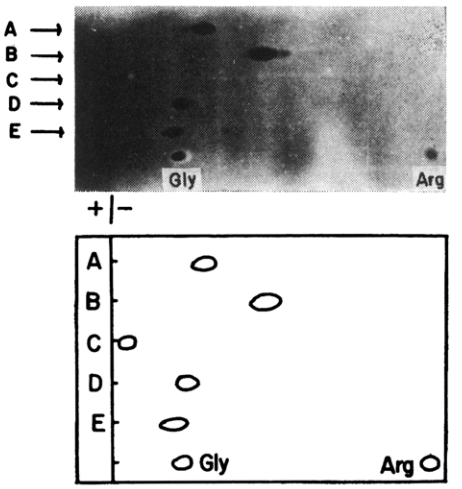


FIGURE 4: Paper electrophoresis of purified bradykinin-potentiating peptides. Paper electrophoresis was performed at pH 3.5 at 4000 V/60 cm for 1 hr. Peptides were located with peptide reagent. Sketch of the photograph is shown below.

kinin-potentiating peptides was purified further by chromatography on a column of CM-Sephadex C-50 equilibrated with 0.02 M pyridine–formic acid buffer, pH 3.1. As shown in Figure 3, four peaks with biological activity were separated on elution with the same buffer. Only one had absorbancy at 280 mμ, while all of the four peaks were ninhydrin positive after alkaline hydrolysis. A fifth biologically active peptide with no absorbancy at 280 mμ which was ninhydrin positive after alkaline hydrolysis was eluted with 2 M pyridine–acetic acid buffer, pH 5.0. These peptides with bradykinin-potentiating activity were designated as potentiators A, B, C, D, and E as shown in Figure 3, and the peptide fractions indicated by arrows in the figure were lyophilized. The yields of each peptide from 10 g of the venom were as follows: A (fractions 46–48), 9.6 mg; B (fractions 107–114), 68.8 mg; C (fractions 21–26), 36.8 mg; D (fractions 39–43), 6.4 mg; E (fractions 54–59), 22.4 mg. Thus, applying the separation system with CM-Sephadex C-50, four peptides were separated by elution with 0.02 M pyridine–formic acid buffer, pH 3.1, and one peptide with 2 M pyridine–acetic acid buffer, pH 5.0. Other ion-exchange resins, such as Dowex 50 and Amberlite IRC-50, were less effective for separating the peptides.

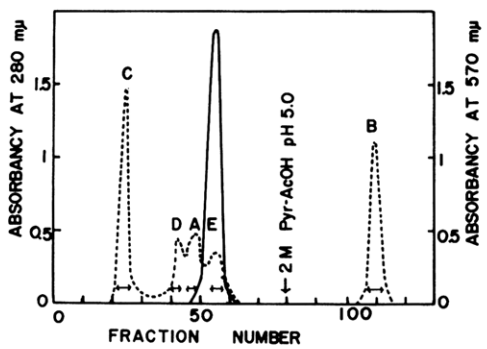


FIGURE 3: CM-Sephadex C-50 column chromatography of bradykinin-potentiating peptides. Peptides (75 mg) were applied to a column (1.5 × 115 cm) equilibrated with 0.02 M pyridine–formic acid buffer, pH 3.1, and 5-ml fractions of eluate were collected. After 80 fractions had been collected, the column was eluted stepwise with 2 M pyridine–acetic acid buffer, pH 5.0. The solid line shows the absorbancy at 280 mμ. The dotted line shows the absorbancy at 570 mμ in the ninhydrin reaction after alkaline hydrolysis.

Each of the bradykinin-potentiating peptides separated by CM-Sephadex C-50 chromatography gave essentially a single spot on staining with peptide reagent after high-voltage paper electrophoresis at pH 3.5 (Figure 4). The peptides are of sufficiently high purity for amino acid sequence determination and the integral molar ratios of the constituent amino acids in Table I can be taken as an evidence for homogeneity. Potentiators B and E developed a purple color with ninhydrin while the others did not. Potentiators A, B, and D reacted with Sakaguchi's reagent, and only potentiator E reacted with Ehrlich's reagent.

Amino acid analyses of the five bradykinin-potentiating peptides showed that potentiators B and E, which were ninhydrin positive, contained lysine residues. Thus, the purple color developed by potentiators B and E with ninhydrin reagent is due probably to their lysine residues, and none of the bradykinin-potentiating peptides isolated have a free amino group in the N terminal. Table I shows that all these bradykinin-potentiating peptides contained 5 or 6 moles of proline residues and 1 mole of glutamic acid in a total of 11 amino acid residues. The high proline content seems to be a characteristic of the bradykinin-potentiating peptides of *Agkistrodon halys blomhoffii* venom. Potentiators A, B, C, and D had similar amino acid compositions, suggesting that they had homologous amino acid sequences. Potentiator E, the only one containing tryptophan, differed in biological activity from the other four peptides as mentioned in the next section.

BIOLOGICAL ACTIVITIES OF THE FIVE BRADYKININ-POTENTIATING PEPTIDES. The biological activities of the peptides were assayed on guinea pig ileum and rat uterus as described in the Experimental Section. Potentiator B was most effective on guinea pig ileum, 0.325 nmole/ml potentiating the action of bradykinin twofold (Figure 5). The last column of Table I shows a comparison of the bradykinin-potentiating activities of the five peptides on isolated guinea pig ileum. Potentiators A, D, and E had weaker activities on guinea pig ileum than B and C. Potentiator E showed about 50-fold more potentiating activity on rat uterus than on guinea pig ileum. Thus, 46.8 nmoles/ml of potentiator E was required to double the effect of bradykinin on guinea pig ileum while 0.93 nmole/ml was

TABLE I: Amino Acid Compositions of Five Bradykinin-Potentiating Peptides from *Agkistrodon halys blomhoffii* Venom.[a]

Potentiator	Amino Acid Composition (Mole per Mole of Peptide)											Moles of Amino Acid per Mole of Peptide	Relative[c] Biological Specific Activity (Molar Ratio)
	Lys	Arg	Glu	Pro	Gly	Ile	Leu	Asp	Ser	Val	Trp		
A		0.8 (1)	1.0 (1)	5.4 (6)	1.7 (2)	0.8 (1)						11	1/192
B	0.8 (1)	1.0 (1)	1.0 (1)	4.2 (5)	1.1 (1)	0.8 (1)	0.8 (1)					11	1
C			1.0 (1)	5.7 (6)	1.7 (2)	0.9 (1)	0.9 (1)					11	1/2
D		1.3 (1)	1.0 (1)	5.2 (6)	1.6 (1)	0.8 (1)	0.7 (1)					11	1/8
E	0.7 (1)		1.0 (1)	4.0 (5)				0.8 (1)	0.7 (1)	1.0 (1)	0.8[b] (1)	11	1/144

[a] Each peptide (0.1–0.5 mg) was hdrolyzed with constant-boiling HCl at 110° for 24 hr and subjected to amino acid analysis. The value for glutamic acid was taken as 1.0. Amino acids present at concentrations of 0.05 residue per molecule or less were not reported. [b] Tryptophan content was calculated by the method of Goodwin and Morton (1964). [c] The biological activity of each peptide was assayed on isolated guinea pig ileum and the relative biological specific activity was calculated as described in the Experimental Section.

required with rat uterus. The other four peptides had little bradykinin-potentiating activities on rat uterus.

According to Stewart *et al.*, 0.05–0.1 nmole/ml of the bradykinin-potentiating peptide from *Bothrops jararaca* venom was required to double the effect of bradykinin on guinea pig ileum.

All five bradykinin-potentiating peptides distinctly increased the contractile effect by bradykinin on smooth muscles, but they caused no contraction by themselves. Furthermore, the size of the contraction induced by a definite amount of bradykinin increased almost quantitatively with the amount of peptide added. Moreover, the sensitivity of isolated guinea pig ileum was enhanced by addition of a bradykinin-potentiating peptide to the organ bath, and this increased sensitivity persists even after washing the organ. The sensitizing activity of potentiator B was greatest, and the amount of potentiator B necessary to potentiate the action of bradykinin on guinea pig ileum twofold, clearly demonstrates its sensitizing activity. These results are shown in Figure 5. After contact with 5.2 nmoles of potentiator B the sensitivity of guinea pig ileum to bradykinin was greatly increased, and this increased sensitivity was not lost by washing [Figure 5 (1) (2)]. Potentiator C, on the other hand, had only a very slight sensitizing action [Figure 5 (4)].

Amino Acid Sequence of Potentiator B. Potentiator B was ninhydrin positive on filter paper, but no N-terminal amino acid could be detected even when 1.87 mg (1.6 μmoles) of sample was subjected to Edman's degradation. The C-terminal amino acid of potentiator B was found by hydrazinolysis to be proline and 0.34 mole of proline was recovered from 1 mole of potentiator B. The following enzymatic hydrolyses were used to obtain peptide fragments of potentiator B.

TRYPTIC HYDROLYSIS. Potentiator B (3.57 mg) was incubated with 0.07 mg of trypsin in 0.2 ml of buffer at 37° for 1 hr. After tryptic digestion two fragments, B-T-1 and B-T-2, were separated by high-voltage paper electrophoresis at pH 3.5. B-T-2 stained strongly and B-T-1 weakly with ninhydrin. No other spots giving coloration were found. The peptides were extracted as described in the Experimental Section, and each was subjected to amino acid analysis. The amino acid compositions of intact potentiator B, and B-T-1 and B-T-2 are shown in Table II. The recovery of each peptides was as follows: B-T-1, 60%, B-T-2, 60%. Isoleucine was liberated from B-T-1 by aminopeptidase M, and proline was detected after hydrazinolysis. Quantitatively 0.9 mole of isoleucine and 0.5 mole of proline were obtained per mole of B-T-1. B-T-1 contained 1 mole of isoleucine and 2 moles of proline (Table II), so its amino acid sequence was deduced to be

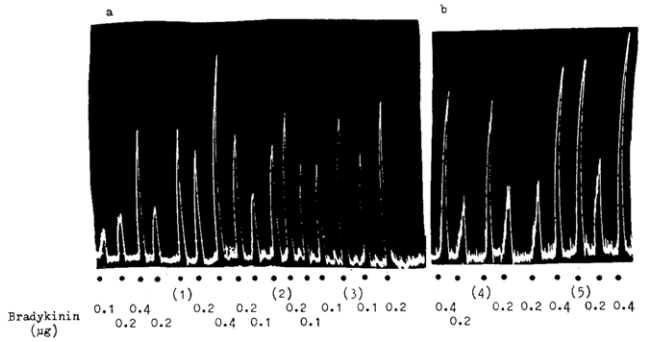


FIGURE 5: Bradykinin-potentiating activity of natural and synthetic potentiators B and C on guinea pig ileum. Bradykinin activity was assayed by contraction of isolated guinea pig ileum in aerated tyrode solution at 30°. To a 16-ml organ bath, 0.1–0.4 μg of synthetic bradykinin was added at 4-min intervals as indicated. Contraction was measured for 1 min, and then the organ was washed with tyrode solution. For measurement of bradykinin-potentiating activity, bradykinin-potentiating peptides were added just before addition of bradykinin: (a) (1) natural potentiator B (5.2 nmoles) plus bradykinin (0.2 μg); (2) natural potentiator B (5.2 nmoles) plus bradykinin (0.1 μg); (3) synthetic potentiator B (6 nmoles) plus bradykinin (0.1 μg); (b) (4) natural potentiator C (13 nmoles) plus bradykinin (0.2 μg); (5) synthetic potentiator C (10 nmoles) plus bradykinin (0.2 μg).

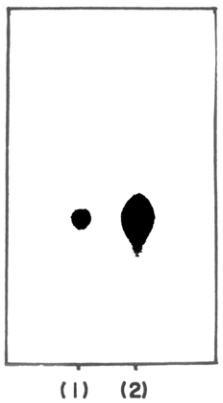


FIGURE 6: Identification of B-T-1 with synthetic isoleucylprolylproline by thin-layer chromatography: solvent, 1-butanol–acetic acid–H_2O (3:1:1, v/v). Peptides were located with peptide reagent: (1) B-T-1; (2) Ile-Pro-Pro.

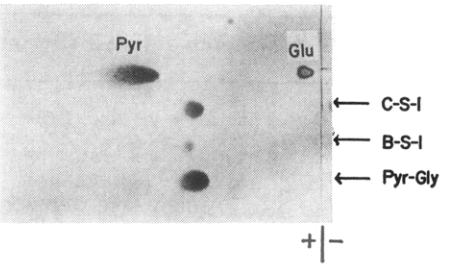


FIGURE 7: Identification of C-S-1 and B-S-1 with synthetic pyroglutamylglycine by paper electrophoresis. Paper electrophoresis was performed at pH 3.5 at 4000 V/60 cm for 30 min. Peptides were located with peptide reagent.

Ile-Pro-Pro. This sequence was confirmed by comparison to a synthetic peptide on thin-layer chromatography (Figure 6). The C-terminal residue of B-T-2 was found to be lysine by hydrazinolysis, and 0.43 mole of lysine was recovered per mole of B-T-2. No N-terminal amino acid was detected by Edman's degradation using 1.8 μmoles of B-T-2. Carboxypeptidase B liberated lysine from B-T-2, and the remaining peptide, separated from lysine by paper electrophoresis at pH 3.5, was ninhydrin negative. Thus, it was deduced that B-T-2 has lysine as its C terminal, and has no free amino group in the N terminal. The amino acid composition of lysine-less-B-T-2 is shown in Table II. Hydrazinolysis showed that proline was the C-terminal amino acid of this lysine-less-B-T-2. Therefore, the C-terminal part of B-T-2 was determined to be Pro-Lys. Thus, sequential analyses of the two peptide fragments obtained by tryptic digestion showed that trypsin hydrolyzed only the Lys–Ile bond in potentiator B, and the amino acid sequence of the five amino acid residues at the C terminal of potentiator B should be Pro-Lys-Ile-Pro-Pro.

HYDROLYSIS WITH A PROTEASE FROM *Streptomyces griseus*. Potentiator B (10 mg) was hydrolyzed in 0.5 ml of buffer with 1 mg of protease from *S. griseus* for 24 hr. The hydrolysate was subjected to paper electrophoresis at pH 3.5, and four spots, B-S-1 to B-S-4, were detected with ninhydrin reagent and peptide reagent. Each peptide was extracted as described in the Experimental Section, and its amino acid composition was examined (Table III). The recovery of each peptide was as follows: B-S-1, 23%; B-S-2, 22%; B-S-3, 24%; B-S-4, 20%. Results of amino acid analysis and paper electrophoresis at pH 3.5 showed that B-S-2 was identical with B-T-1 (Ile-Pro-Pro) and B-S-3 with B-T-2. B-S-1, which had the amino acid composition Glu(1) and Gly(1), was ninhydrin negative on filter paper, but was detectable with peptide reagent. B-S-1 was identified as Pyr-Gly by comparison to the synthetic compound (Figure 7). In this paper electrophoresis, pyroglutamic acid migrated faster than *N*-acetylglutamic acid, and they were clearly distinguishable from each other. The amino acid sequence of B-S-4 was analyzed using 2.4 μmoles of the sample by subtractive Edman degradation and was shown to be Leu-Pro-Pro-Arg- (Table IV). Since the C-terminal part of B-T-2 (= B-S-3) was Pro-Lys and potentiator B contained 1 lysine residue, the amino acid sequence of B-S-4 was deduced by reference to the amino acid composition of B-T-4 (Table III), to be Leu-Pro-Pro-Arg-Pro-Lys.

HYDROLYSIS WITH COLLAGENASE (CLOSTRIDIOPEPTIDASE A). Potentiator B (5.2 μmoles) was incubated with collagenase for 24 hr at 37°. Three spots, designated as B-C-1, B-C-2, and B-C-3 according to their mobilities from the origin, were detected with peptide reagent on paper electrophoresis of the hydrolysate at pH 3.5. The recovery of each peptides was as follows: B-C-1, 40%; B-C-2, 23%; B-C-3, 25%. As shown in Table V, B-C-2, from its amino acid composition, was deduced to be intact potentiator B. The amino-terminal part of B-C-3 was determined to be Pro-Lys-Ile by direct Edman degradation. Therefore, since B-C-3 had the amino acid composition Pro(3), Lys(1) (Table V), the amino acid se-

TABLE II: Amino Acid Compositions and Terminal Amino Acids of Potentiator B and Its Tryptic Peptides.

Amino Acid	Potentiator B	B-T-1	B-T-2	Lys-less-B-T-2
Lys	(1)		0.8 (1)	
Arg	(1)		0.8 (1)	0.8 (1)
Glu	(1)		1.0 (1)	1.0 (1)
Pro	(5)	1.8 (2)	2.6 (3)	2.7 (3)
Gly	(1)		0.9 (1)	1.1 (1)
Ile	(1)	1.0 (1)		
Leu	(1)		0.9 (1)	0.9 (1)
N terminal	Undetectable	Ile 0.9	Undetectable	
C terminal	Pro 0.34	Pro 0.5	Lys 0.43	Pro 0.8

TABLE III: Amino Acid Composition of Peptide Fragments Obtained from a Hydrolysate of Potentiator B with *S. griseus* Protease.

Amino Acid	B-S-1	B-S-2	B-S-3	B-S-4
Lys			0.9 (1)	1.1 (1)
Arg			1.1 (1)	1.1 (1)
Glu	1.0 (1)		1.0 (1)	
Pro		2.6 (2)	2.7 (3)	3.3 (3)
Gly	0.95 (1)		1.0 (1)	
Ile		1.0 (1)		
Leu			0.9 (1)	1.0 (1)

TABLE IV: Subtractive Edman Degradation of B-S-4.[a]

Cycle	Leu	Pro	Arg	Lys	Yield (%)
0	0.9	3.3	1.1	1.0	
1		3.8	1.2	1.0	62
2		*2.0*	1.2	1.0	100
3		*1.3*	1.0	1.0	97
4		1.1	*0.3*	1.0	57

[a] The value for lysine was taken as 1.0. The yield was calculated by amino acid analysis after each cycle.

TABLE V: Amino Acid Compositions of Peptide Fragments Obtained from a Hydrolysate of Potentiator B with Collagenase.

Amino Acid	B-C-1	B-C-2	B-C-3	Arg-less-B-C-1
Glu	1.0 (1)	1.0 (1)		1.0 (1)
Pro	1.5 (2)	3.9 (5)	2.7 (3)	2.0 (2)
Gly	1.0 (1)	1.1 (1)		0.96 (1)
Ile		0.9 (1)	1.0 (1)	
Leu	0.8 (1)	0.8 (1)		0.94 (1)
Lys		0.6 (1)	0.8 (1)	
Arg	0.8 (1)	0.7 (1)		

TABLE VI: Amino Acid Composition of Peptide Fragments from a Hydrolysate of Potentiator C with *S. griseus* Protease.

Amino Acid	Potentiator C	C-S-1	C-S-2	First Step[a] of C-S-2
Glu	(1)	1.0 (1)		
Pro	(6)		5.4 (6)	6.2 (6)
Gly	(2)	1.0 (1)	1.1 (1)	1.4 (1)
Ile	(1)		1.0 (1)	1.0 (1)
Leu	(1)		1.0 (1)	0.3 (0)

[a] The water phase after the first step of Edman degradation of C-S-2 was subjected to amino acid analysis.

quence of the peptide was deduced to be Pro-Lys-Ile-Pro-Pro. B-C-1 was ninhydrin negative on filter paper so B-C-1 may be located at the N-terminal part of potentiator B. B-C-1 (0.7 μmole) was incubated with 180 μg of carboxypeptidase B in 0.1 ml of 0.01 M phosphate buffer, pH 8.0, at 37° for 3 hr. Arginine was detected as the sole free amino acid in the hydrolysate by paper electrophoresis at pH 3.5. The resulting arginine-less-B-C-1, isolated from the hydrolysate by paper electrophoresis at pH 3.5, had the amino acid composition, Glu(1), Pro(2), Gly(1), and Leu(1). By reference to the amino acid sequences of B-S-1, the dipeptide obtained by digestion with *Streptomyces* protease, and of B-S-4, described in the previous section, the amino acid sequence of B-C-1 was deduced to be Pyr-Gly-Leu-Pro-Pro-Arg. The results obtained with B-C-1 and B-C-3 and the amino acid compositions of the peptides obtained by hydrolysis with collagenase (Table V), showed that an arginyl–prolyl bond in potentiator B was hydrolyzed with the commercial collagenase used.

Judging from the substrate specificity of collagenase (Yagisawa *et al.*, 1965) it seems unlikely that an arginyl–prolyl bond was actually cleaved by collagenase activity. However, commercial collagenase contains clostridiopeptidase B (Mitchell and Harington, 1968) and purified clostridiopeptidase B hydrolysed the arginyl–prolyl bond in methionyllysylbradykinin (Mitchell, 1968). So the arginyl–prolyl bond in potentiator B may have been cleaved by clostridiopeptidase B contaminating the commercial preparation of collagenase.

FULL STRUCTURE FOR POTENTIATOR B. From the results described above, the peptide fragments so far analyzed can be arranged as shown in Table VIII, and the amino acid sequence of potentiator B is deduced to be: Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro.

Amino Acid Sequence of Potentiator C. When potentiator C (1.7 μmoles) was incubated with a protease from *S. griseus* in 0.2 ml of buffer for 24 hr at 37°, unhydrolyzed potentiator C and two peptide fragments, C-S-1 and C-S-2, were detected by applying the incubation mixture on paper chromatography (1-butanol–pyridine–acetic acid–water, 15:10:3:12, v/v). C-S-1 and C-S-2, were preparatively separated by the paper chromatography, from the *S. griseus* protease hydrolysate of 9 μmoles of potentiator C. The amino acid compositions of the two peptide fragments are shown in Table VI. The recovery of each peptides was 51% for C-S-1 and 60% for C-S-2. C-S-1 which had the amino acid composition Glu(1) and Gly(1), was ninhydrin negative and identified as Pyr-Gly by electrophoresis at pH 3.5 (Figure 7). The amino acid composition of C-S-2 was determined by Edman degradation using 0.56 μmole of sample. The first step was analyzed by both direct and subtractive Edman degradation. The last column of Table VI shows the amino acid composition of the water phase after the first step of Edman degradation of C-S-2. The amount of leucine was markedly reduced in the water phase. Table VII shows the PTH-amino acids of 8 steps identified by direct Edman degradation of C-S-2. Although PTH-leucine and PTH-isoleucine were not distinguished in this analytical system, the PTH-amino acid of the 7th step was deduced to be PTH-isoleucine, because the N-terminal amino acid of C-S-2 was determined to be leucine by subtractive Edman degradation. The PTH-amino acid of the 8th step was PTH-proline and free proline was found in the water phase by amino acid analysis. The C-terminal amino acid of potentiator C was also found to be proline by hydrazinolysis (0.3 mole per mole), so the amino acid sequence of C-S-2 was deduced to be Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro. Thus, together with the findings that C-S-1 was Pyr-Gly and the amino acid composition of potentiator C was Glu(1), Leu(1), Pro(6), Gly(1), and Ile(1), the whole amino acid sequence of potentiator C was concluded to be as follows: Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro.[2]

Confirmation of the Structures of Potentiators B and C by Comparison to Synthetic Peptides. Tables VIII and IX

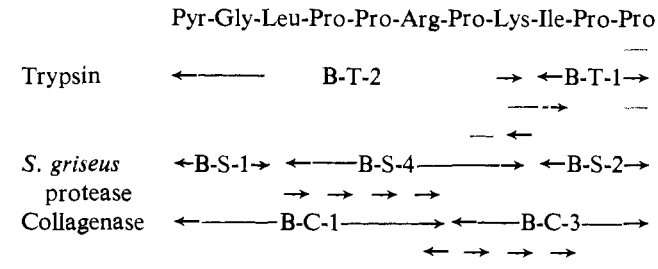
[2] Sequence analysis of potentiator C by mass spectrometry is now in progress. Previously the existence of N-terminal pyroglutamic acid was confirmed; the order of nine amino acid residues from the N terminal was identical with the expected structure (K. Okada of Kanazawa University, to be published).

TABLE VII: Direct Edman Degradation of C-S-2.[a]

Cycle	PTH-Amino Acid (Found)	Repetitive Yield (%)
1	Leu[b]	78
2	Pro	100
3	Pro	100
4	Gly	91
5	Pro	75
6	Pro	91
7	Ile[b]	81
8	Pro	56
8[c]	Pro	

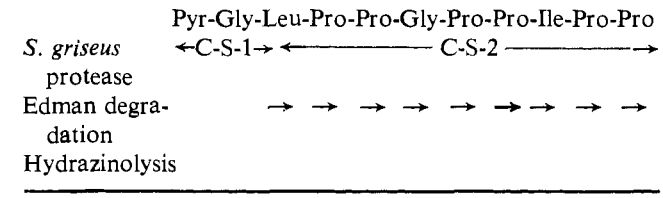
[a] The repetitive yield was calculated from the absorbancy at 269 mμ at each step. [b] The N-terminal amino acid of C-S-2 was determined to be leucine by subtractive Edman degradation, as shown in the last column of Table VI, so that, the PTH-amino acid from the 7th cycle was deduced to be isoleucine. [c] The water phase of the 8th step was subjected to amino acid analysis without acid hydrolysis, and free proline was found.

TABLE VIII: Summary of the Sequential Analysis of Potentiator B.[a]



[a] (—) Hydrazinolysis, (←) carboxypeptidase B, (⇢) aminopeptidase M, (→) Edman degradation.

TABLE IX: Summary of Sequential Analysis of Potentiator C.



summarize the results of sequential analyses on potentiators B and C. Chemically synthetized peptides[3] with the amino acid sequences proposed by the authors were kindly supplied by Dr. S. Sakakibara and Mr. T. Kimura of the Peptide Center of our Institute. The natural potentiators B and C had the same mobilities and R_F values as the corresponding synthetic peptides on paper electrophoresis and paper chromatography, as shown in Figure 8. Moreover, hydrolysates of both the natural and synthetic peptides with a protease from *S. griseus* gave the same patterns on paper electrophoresis and paper chromatography.

As shown in Figure 5, the bradykinin-potentiating activities of the synthetic and natural peptides, potentiators B and C, were almost identical. Twofold potentiation of the action of bradykinin on guinea pig ileum was obtained with 0.33 nmole/ml of natural potentiator B and with 0.38 nmole/ml of synthetic material. Moreover, twofold potentiation of the action of bradykinin on guinea pig ileum was achieved with 0.81 nmole/ml and 0.62 nmole/ml of natural and synthetic potentiator C, respectively.

Furthermore, the identity of natural bradykinin-potentiating peptides with synthetic peptides was confirmed by the measurements of another biological activity, inhibition on lung angiotensin-converting enzyme. The inhibition on angiotensin-converting enzyme from dog lung with the bradykinin-potentiating peptides from *Bothrops jararaca* venom was found by Ferreira *et al.* (1970b). The inhibitory activity of potentiator B and C to the dog lung angiotensin-converting enzyme was kindly measured by Dr. Y. S. Bakhle (Department of Pharmacology, Royal College of Surgeons, England).[3] The inhibitions were measured at 4 different concentrations for potentiator B and at 3 different concentrations for potentiator C. The concentrations to inhibit 50% of enzyme activity were 130–150 pmoles/ml for synthetic and natural potentiators B and 3000 pmoles/ml for synthetic and natural potentiators C. Thus the synthetic and natural potentiators, B and C, were identical in their strong inhibition on the enzyme.

Discussion

As described earlier in this paper, one of the bradykinin-potentiating factors found in the venom of *Bothrops jararaca* was recently determined to be a pentapeptide, Pyr-Lys-Trp-Ala-Pro, by Ferreira *et al.* (1970a). Bradykinin-potentiating peptides have been found in the venoms of *Agkistrodon* and *Bothrops* species together with the bradykinin-releasing enzyme, so the existence of such peptides in some species may be of physiological importance. The bradykinin-potentiating peptide found in the venom of *Agkistrodon halys blomhoffii* by the authors was separated into five biologically active entities; potentiators A, B, C, D, and E. The amino acid sequences of two of them, B and C, presented in this paper were confirmed by chemical synthesis.

Potentiators B and C have 5 and 6 moles of prolyl residue, respectively, among their 11 amino acid residues. It was difficult to estimate the proline content of the potentiators accurately because it was so high. Thus on amino acid analysis the quantitative value for proline was not accurate, and initially it was tentatively taken as 4 moles (Kato and Suzuki, 1969). The lack of a free N-terminal amino group and the existence of C-terminal proline renders the peptides resistant to aminopeptidase or carboxypeptidase, and so various normal methods of sequential analysis cannot be used. Furthermore, potentiator C is highly resistant to proteolytic enzymes, such as chymotrypsin, pepsin, thermolysin, and nagarse, only

[3] The results on the chemical synthesis of potentiators B and C, and the activities of the synthetic and the natural peptides on the angiotensin-converting enzyme were presented orally at the Second American Peptide Symposium, held in Cleveland, Ohio, under the chairmanship of Dr. F. W. Bumpus, Aug 17–19, 1970. The Proceedings will be published by Gordon & Breach Science Publishers, New York, N. Y. (T. Kimura, H. Kato, S. Sakakibara, and T. Suzuki).

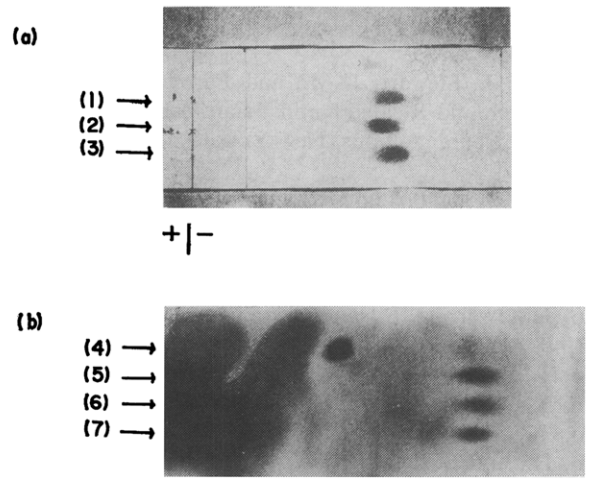


FIGURE 8: Identification of potentiators B and C with the corresponding synthetic peptides by paper electrophoresis and paper chromatography. (a) Paper electrophoresis of synthetic and natural potentiator B. Paper electrophoresis was performed at pH 3.5 at 3000 V/60 cm for 2 hr. Peptides were located with ninhydrin reagent: (1) natural potentiator B; (2) synthetic potentiator B; (3) natural potentiator B plus synthetic potentiator B. (b) Paper chromatography of synthetic and natural potentiator C: solvent, 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v). Peptides were located with peptide reagent: (4) Pyr-Gly; (5) natural potentiator C; (6) natural potentiator C plus synthetic potentiator C; (7) synthetic potentiator C.

A. halys blomhoffii

Potentiator B	Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro
Potentiator C	Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro
Potentiator E[a]	Pyr-Lys-Trp-Asp-Pro-Pro-Pro-Val-Ser-Pro-Pro

Bothrops jararaca

BPF 5a[b]	Pyr-Lys-Trp-Ala-Pro
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

FIGURE 9: Amino acid sequences of bradykinin-potentiating peptides from the venoms of *A. halys blomhoffii* and *Bothrops jararaca*. (a) Reported by Kato and Suzuki (1970c). (b) Reported by Ferreira *et al.* (1970a). They designated this pentapeptide as BPF5a because it is the first bradykinin-potentiating factor with five amino acid residues isolated from the venom of *Bothrops jararaca*.

the glycyl–leucyl bond being partially cleaved by a protease from *S. griseus*. Thus, in sequential studies on potentiator C, chemical synthesis of peptides was very useful, for example in the following case. During sequential studies on potentiator C, a peptide with the amino acid sequence Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Ile-Pro-Pro was synthesized. But, the bradykinin-potentiating activity of this peptide with one less prolyl residue than potentiator C was much weaker than that of natural potentiator C. Thus, the sequence of potentiator C was deduced by chemical synthesis in parallel with results of Edman degradation and was concluded to have the structure shown in Figure 9. This experience was like that of Elliott in sequential analysis of bradykinin. A compound with one less proline than bradykinin was tentatively suggested by Elliott but when this peptide was synthetized by Boissonnas, it had no bradykinin activity. Then, from bioassay of chemically analogous synthetic peptides, it appeared that bradykinin might have the structure, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, and this was confirmed by sequential analysis of natural bradykinin (Boissonnas *et al.*, 1963).

Potentiators A and D have similar amino acid compositions to potentiators B and C, and their amino-terminal parts were confirmed to be Pyr-Gly, although their whole amino acid sequences have not been determined yet. The amino acid sequence of the fifth bradykinin-potentiating peptide, potentiator E, was recently determined (Kato and Suzuki, 1970c) and is shown in Figure 9.

As shown in Figure 9, a high proline content and N-terminal pyroglutamic acid seem to be common features of bradykinin-potentiating peptides in the venom of *Agkistrodon halys blomhoffii*. Partial similarities of the peptides with bradykinin in their amino acid sequences are found in the sequences, -Pro-Pro-, -Pro-Pro-Gly-, and -Arg-Pro-. The bradykinin-potentiating peptides and bradykinin have a high proline content, so they may also have some resemblances in tertiary structure, and it is hoped that bradykinin-potentiating peptides may be useful in studies on the mode of induction of contraction of smooth muscle by plasma kinin. Potentiator E had weak bradykinin-potentiating activity on guinea pig ileum but potent activity on rat uterus. The pentapeptide reported by Ferreira *et al.* (1970a) had strong activity on guinea pig ileum although its N-terminal part was very similar to that of potentiator E. Further studies are required to see whether these biological characteristics depend on the integrity of the peptide chain or on the presence of certain amino acids at definite positions.

Proteolytic enzymes, such as trypsin and chymotrypsin, increase the sensitivity of guinea pig ileum or rat uterus to bradykinin, as reported by Edery (1964, 1965). Bradykinin-potentiating peptides from snake venoms also increase the sensitivity of isolated smooth muscle, and this increased sensitivity is not removed by washing. Thus, the bradykinin-potentiating peptides reported here not only increase the size of contraction induced by bradykinin, but also enhance the sensitivity of the organ to bradykinin. The sensitivity of the smooth muscle to bradykinin increased steadily on repeated contact with bradykinin-potentiating peptides.

Many workers have suggested that bradykinin-potentiating substances inhibit kininases in the plasma and tissues. Sulfhydryl compounds which have bradykinin-potentiating activity, inhibited plasma kininases (Ferreira and Rocha e Silva, 1962). A bradykinin-potentiating peptide, isolated from human plasma after digestion with trypsin, was found to have arginine or lysine as its C-terminal amino acid, and it competitively inhibited the activity of carboxypeptidase B with bradykinin-destroying activity (Hamberg *et al.*, 1969). Yang *et al.* (1970) reported that one of the kininases in human plasma seemed to be identical with the angiotensin-converting enzyme. Ferreira *et al.* (1970b) reported that kininases and the angiotensin-converting enzyme from dog lung were inhibited by bradykinin-potentiating peptides from the venom of *Bothrops jararaca*. Recently, it has been found that potentiators B and C strongly inhibited the activity of the angiotensin-converting enzyme in dog lung (personal communication from Dr. Y. S. Bakhle), and this is an interesting character of bradykinin-potentiating peptides. All the bradykinin-potentiating factors so far isolated have been demonstrated to inhibit the enzymatic destruction of bradykinin. This suggests that the increase in the effect of bradykinin is due to inhibition of the inactivation of the peptide in isolated tissues. However, bradykinin is destroyed by the guinea pig ileum too slowly to explain the rapid action of a compound with bradykinin-potentiating activity, such as cysteine (Picarelli *et al.*, 1962). From the results obtained so far, a possible explanation for the mode of action of bradykinin-potentiating peptides would be that they inhibit kininase activity. However, when further

results are obtained it may not be possible to explain the mechanism of their potentiating effect as due to their inhibitory actions on bradykinin-destroying enzymes.

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