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Kinin-Converting Activity in the Dog Pseudoglobulin Fraction from Heated Plasma and Kinin Liberation from Dog Kininogen by Guinea-pig Coagulating Gland Kallikrein (CGK)

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The pseudoglobulin fraction obtained from heated plasma (60°, 1 hr) of various animals (PFHP) is widely used as a substrate in kininogenase activity assay. In the present investigation, however, kinin-converting activity was found in dog PFHP; namely 100 μg of synthetic kallidin was completely converted to bradykinin within 1 hr on incubation with 300 mg of dog PFHP. The converting activity could be destroyed by further heating at 100° for 30 min.

Kinin-converting activity was present in various species of PFHP's, though the activity of dog PFHP was the most potent among the PFHPs checked in this investigation.

The kinin liberated from dog kininogen by guinea-pig coagulating gland kallikrein (CGK) was also identified by chromatographic analysis and by the DNS method. It has been reported that CGK liberates bradykinin from dog kininogen but it is now clear that CGK in fact liberates kallidin, like other glandular kallikreins, and that the kallidin is further converted to bradykinin by the contaminating kinin-converting enzyme in the substrate preparation.

Keywords——kinin-converting enzyme; guinea-pig coagulating gland kallikrein; kinin; kininogen; pseudoglobulin; aminoacyl-β-naphthylamidase activity; kinin analysis

The kinins liberated from kininogens by kininogenases vary according to the enzyme used. In general, plasma kallikrein liberates bradykinin and glandular kallikreins liberate kallidin rather than bradykinin.[2] Moriwaki and Schachter previously reported that although the guinea-pig coagulating gland kallikrein (CGK) is a glandular kallikrein, it did not liberate kallidin but formed bradykinin from dog kininogen, and this enzyme was not inhibited by the usual glandular kallikrein inhibitor, aprotinin.[3]

Recently, however, we found that the substrate, dog pseudoglobulin fraction obtained from heated plasma, used in the previous experiments[3] contained kallidin-converting activity to bradykinin. This finding suggested that CGK might liberate kallidin from dog kininogen, like other glandular kallikreins, and that this kallidin was then converted to bradykinin by the contaminating kinin-converting enzyme in the substrate.

This paper deals with the kinin-converting enzyme present in the pseudoglobulin fraction from heated plasma of various animals and with the nature of the kinin liberated from dog kininogen by CGK.

Materials and Methods

Materials——Synthetic bradykinin, kallidin, Leu-, Arg- and Lys-β-naphthylamide (Leu-, Arg- and Lys-β-NA) were purchased from the Protein Research Foundation (Osaka). β-Naphthylamine and Tween 20 were obtained from Kanto Kagaku Kogyo Co. (Tokyo). SP-Sephadex C-50 was from Pharmacia Fine Chemicals (Uppsala, Sweden), silica gel H from E. Merck (Darmstadt, Germany), Wakogel Q-23 from Wako Junyaku Co. (Tokyo), 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) from Tokyo Kasei Kogyo

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2) M. Schachter, *Physiol. Rev.*, **49**, 509 (1969).
3) C. Moriwaki and M. Schachter, *J. Physiol.*, **219**, 341 (1971).

Co. (Tokyo) and Fast Garnet GBC from K and K Laboratories (New York, U.S.A.) were also used in this investigation. DNS-bradykinin (DNS-BK) and DNS-kallidin (DNS-KD) were prepared in our laboratory.[4]

Preparation of CGK——CGK was partially purified from guinea-pig coagulating gland using a part of the method of Moriwaki *et al.*[5] The preparation had a kinin-liberating activity of 1.9 μg BK eq./min/E_{280} when assayed by the Magnus method using isolated guinea-pig ileum.

Preparation of the Substrates——The pseudoglobulin fractions from heated plasma (PFHPs) of various animals were prepared according to the procedure of Moriwaki and Schachter.[3] The collected fresh plasma was kept at 60° for 1 hr, then dialyzed against 0.9% NaCl for 20 hr at 4°. Insoluble material was separated by centrifugation (8000 rpm, 20 min). Next, an equal volume of saturated ammonium sulfate solution was added and the resulting precipitate was collected by centrifugation (8000 rpm, 20 min). The precipitate was dissolved in water to one-third of the original plasma volume and dialyzed against distilled water for 20 hr at 4°. This solution was lyophilized and used in the following experiments as a substrate for kinin liberation.

Chromatographic Analysis of Kinins Liberated by CGK——The incubation mixture of the substrate and CGK was centrifuged at 5000 rpm for 30 min at 4° and the supernatant was applied to a siliconized silica gel column (0.9×10 cm).[6] After washing the column with distilled water until proteins could no longer be detected in the effluent, adsorbed kinins were eluted with about 1 ml of a mixture of AcOH: pyridine (3: 1). The eluate was dried under a stream of N_2 gas and dissolved in 2 ml of 0.1 M ammonium formate buffer, pH 7.0. It was then applied to an SP-Sephadex C-50 column (0.9×27 cm) equilibrated with 0.1 M ammonium formate buffer, pH 7.0, and linear gradient elution was carried out with 0.1—0.5 M of the same buffer (total 200 ml). The biological activity of the eluate was assayed on rat uterus. A standard curve was prepared using synthetic bradykinin (2 ng to 10 ng).

Assay for Kinin-Converting Activity Using Isolated Rat Uterus——The sensitivity of the rat uterus to bradykinin is 1—3 times than to kallidin.[7] Thus, kinin conversion from kallidin to bradykinin can be qualitatively confirmed by the increase of the contractile response of the uterus. For the measurement of kinin-converting activity, 0.1 ml of the dog or bovine PFHP solution (25 mg/ml, in 0.02 M phosphate buffer, pH 7.4) was incubated with 0.1 ml of kallidin solution (500 ng/ml, in saline) at 30° for various periods, then 10 μl of this mixture was poured into a 10 ml bath in which an isolated rat uterus was suspended in aerated De Jalon's solution at 30°, and the contractile response of the uterus was recorded isotonically. A standard curve was prepared with synthetic kallidin.

Aminoacyl-β-naphthylamidase Activity Assay——Aminoacyl-β-naphthylamidase activities in the PFHPs and plasma were assayed by a slight modification of the method of Guimarães *et al.*[8] The sample solution (0.5 ml) was added to a mixture of 1.0 ml of substrate solution (amino-β-NA: 1 mM) and 1.5 ml of 0.01 M Tris-HCl buffer, pH 7.0, and the mixture was incubated for 1 hr at 37°. The reaction was terminated by adding 0.5 ml of Fast Garnet GBC solution (0.1% in 0.2 M acetate buffer, pH 4.2, containing 10% v/v of Tween 20), and the developed red color was read at 520 nm. When insoluble material was detected in the reaction mixture, the solution was centrifuged to eliminate it (3000 rpm for 10 min at room temperature). A standard curve was prepared with β-naphthylamine in 40% EtOH. The activities were expressed in p moles of amino-β-naphthylamide hydrolyzed/min/mg of protein at 37°, pH 7.0.

Results

Separation of Bradykinin and Kallidin by SP-Sephadex C-50 Chromatography

A mixture of bradykinin and kallidin was applied an SP-Sephadex C-50 column and linear gradient elution was carried out. Two distinct peaks were obtained (Fig. 1-A). Fig. 1-B shows the elution pattern of the biological activity of kinin when kallidin alone was applied to the column. Kallidin was eluted with 0.38 M ammonium formate buffer, pH 7.0, so that the earlier activity peak in Fig. 1-A appearing at 0.25 M of the same buffer is presumably bradykinin and the later one kallidin. Thus, bradykinin and kallidin could be separated into 2 distinct fractions by SP-Sephadex C-50 chromatography.

4) K. Kizuki, C. Moriwaki, Y. Hojima, and H. Moriya, *Chem. Pharm. Bull.*, **24**, 1742 (1976).
5) C. Moriwaki, N. Watanuki, Y. Fujimoto, and H. Moriya, *Chem. Pharm. Bull.*, **22**, 628 (1974).
6) H. Yoshida, K. Matsumoto, T. Nakajima, and Z. Tamura, *Chem. Pharm. Bull.*, **19**, 1691 (1971).
7) E. Schröder, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös and A.F. Wilde, Springer-Verlag, Berlin-Heiderberg-New York, 1970, pp. 324—350.
8) J.A. Guimarães, D.R. Borges, E.S. Prado, and J.L. Prado, *Biochem. Pharmacol.*, **22**, 3157 (1973).

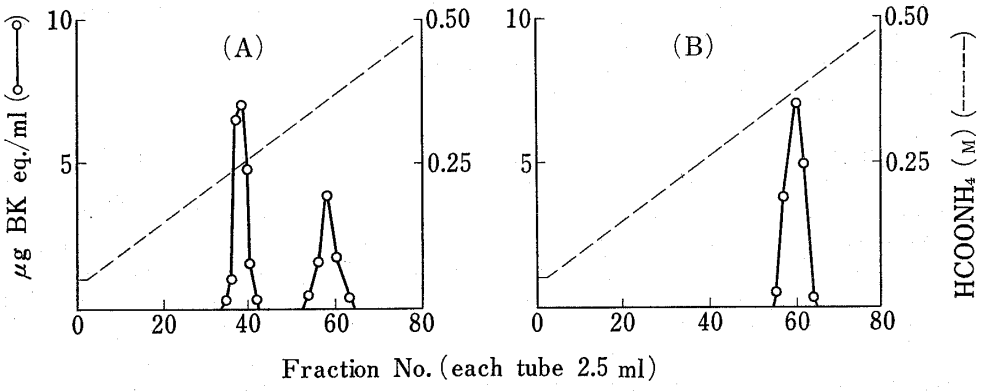

Fig. 1. Elution Profiles of Bradykinin and Kallidin from a SP-Sephadex C-50 Column

Synthetic bradykinin and kallidin were separately dissolved in 0.1 M ammonium formate buffer, pH 7.0, to give a concentration of 100 μg/ml, and the mixture of 1 ml of them (A) and 1 ml of kallidin solution (B) were directly applied to an SP-Sephadex C-50 column. Linear gradient elution was carried out as described in "Materials and Methods".

Kinin-Converting Activity in Dog PFHP

Dog PFHP (300 mg) and synthetic kallidin (100 μg) were incubated for 1 hr at 37°, pH 7.4, and the kinins in this incubation mixture were analyzed by SP-Sephadex chromatography (Fig. 2). As shown in Fig. 2-A, only one biologically active peak was eluted from the column. The position of this peak coincided with that of bradykinin, and no biological activity was found around the fractions in which kallidin would be detected; synthetic kallidin was completely converted to bradykinin by dog PFHP. On the other hand, when PFHP which had been further heated at 100° for 30 min was incubated with kallidin, it failed to convert kallidin to bradykinin (Fig. 2-B). The converting activity in the PFHP was also inhibited by 2×10^{-3} M *o*-phenanthroline, but not completely; minor biological activity was detected around the bradykinin fraction (Fig. 2-C). These results indicate that synthetic kallidin was completely converted to bradykinin by an enzyme in dog PFHP; the kinin-converting enzyme in the dog PFHP was still active after heat treatment at 60° for 1 hr but it was almost completely inactivated by further heating at 100° for 30 min.

Table I shows the effects of heating (60°) on the enzymic activities and the kininogen content in dog plasma. Kinin-converting activity still remained even after 4 hr at 60°, whereas the kininase activity had completely disappeared after 1 hr. The kininogen content gradually decreased with increase of incubation time.

Figure 3 shows the increment of the uterus contractile response of kallidin on incubation with PFHPs, probably due to the conversion of kallidin to bradykinin. The biological activity of kallidin (2.5 ng) was increased about 3-fold by incubation with dog PFHP for 5 min, but no further increase of the response was observed upon an additional 5 min incubation. Thus, it can be concluded that 2.5 ng of kallidin was completely converted to bradykinin by 125 μg of dog PFHP within 5 min at 30°. The bovine PFHP also increased the contractile activity of kallidin on the uterus gradually, but the response was less than that upon 5 min incubation of dog PFHP and kallidin even after 20 min (Fig. 3-I and -E), so the converting activity of bovine PFHP was much weaker than that of dog PFHP.

Aminoacyl-β-naphthylamidase Activities of Various PFHP's and Plasma

As mentioned above, dog and bovine PFHPs contained kinin-converting activity, but the rate of conversion from kallidin to bradykinin could not be measured accurately due to the lack of specific assay procedures for these two peptides. Meanwhile, the kinin-converting enzyme in the human plasma was found to split amino-β-NAs, and the activities measured with Leu-, Arg- and Lys-β-NAs were almost parallel with the kinin-converting activity.[8]

The aminoacyl-β-NAase activities of PFHP's and those of the plasma from various animals are listed in Table II. The hydrolysis of Leu-β-NA by the plasma of various species was greatly decreased by heat treatment. This indicates that most of the kinin-converting enzyme in the plasma is inactivated by treatment at 60° for 1 hr, but not completely. The hydrolysis rate of Leu-β-NA by dog PFHP was 48 pmol/min/mg, which was the highest among PFHP's and about 10 times that of bovine PFHP. This observation was in good accord with the fact that the kinin-converting activity of dog PFHP was much stronger

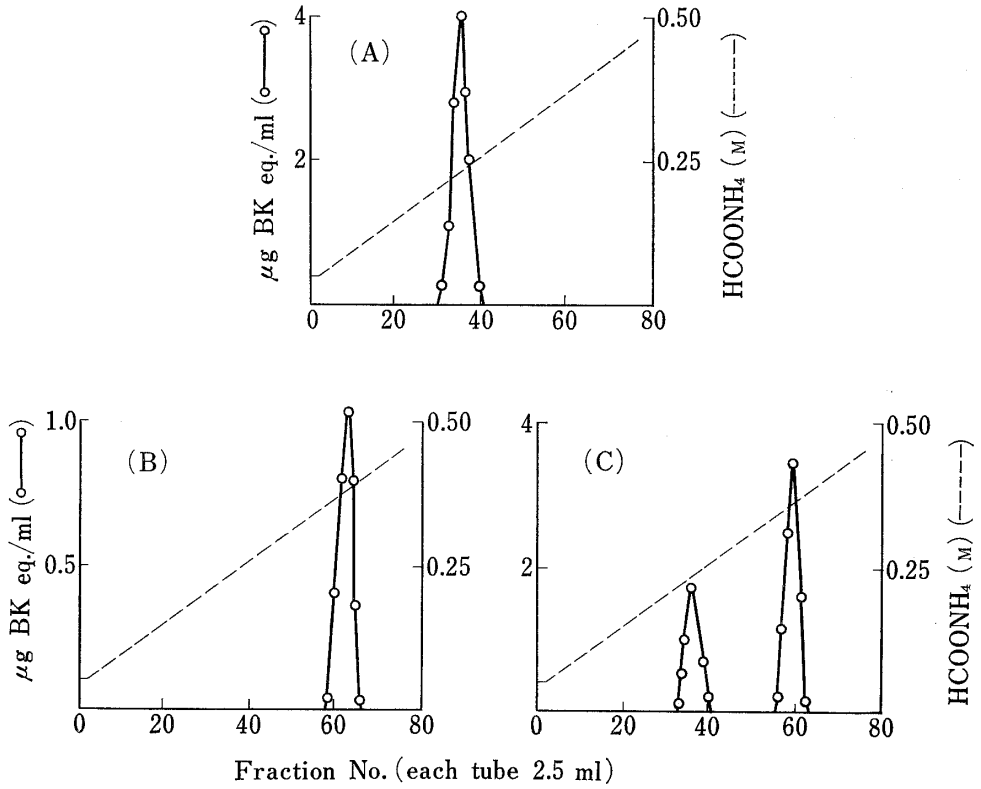

Fig. 2. Conversion of Kallidin to Bradykinin by Dog PFHP

Kallidin (100 μg) was incubated with 300 mg of dog PFHP (A) or further heated PFHP at 100° for 30 min (B) without *o*-phenanthroline. (C): Kallidin (100 μg) was incubated with 300 mg of the PFHP in the presence of 2×10^{-3} M *o*-phenanthroline. The kinin analysis was carried out as described in "Materials and Methods".

TABLE I. Effect of Incubation Time at 60° on the Enzymic Activities and Kininogen Content in Dog Plasma

Incubation time at 60° (hr)	Enzymic Activities		Kininogen content (ng BK eq./mg)
	Kinin-converting	Kininase	
1	++	N.D.	20.0
2	++	N.D.	12.5
3	+	N.D.	11.0
4	+	N.D.	8.8

Various dog PFHPs, heated as indicated, were prepared as described in "Materials and Methods". The enzymic activities and kininogen contents were measured. Kininase activity assay was carried out by the Magnus method as described in our previous paper.[4] Kininogen content in PFHP was determined by adding trypsin to PFHP solution (1 mg/ml) in the presence of 8-hydroxyquinoline, which is a plasma kininase inhibitor (1 mg/ml). The liberated kinin was assayed by the Magnus method. Kininogen content was expressed as ng of bradykinin equivalent/mg of PFHP. Kinin-converting activity was confirmed by analytical column chromatography as described previously. ++: kallidin (100 μg) was completely converted to bradykinin by 300 mg of dog PFHP in 1 hr; +: a considerable amount of kallidin remained but bradykinin was detected; N.D.: not detected.

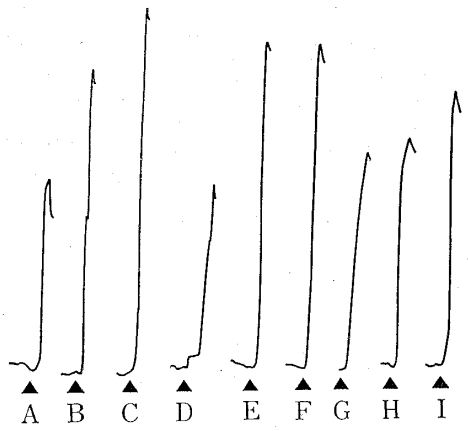

Fig. 3. Conversion of Kallidin to Bradykinin by Dog and Bovine PFHPs

A, B and C: Kallidin 2.5, 5.0 and 10.0 ng, respectively.
D, E and F: Dog PFHP was incubated with kallidin for 0, 5 and 10 min, respectively, at 30° and aliquots of these mixtures (2.5 ng kallidin eq.) were assayed.
G, H and I: Bovine PFHP was incubated with kallidin for 0, 10 and 20 min, respectively, and aliquots of these mixtures were assayed. (For details, see "Materials and Methods").

Table II. Aminoacyl-β-naphthylamidase Activities of Various PFHP's and Plasma

Sample	Specific activity (pmol/min/mg protein)		
	Leu-β-NA	Arg-β-NA	Lys-β-NA
PFHPs			
Dog	48	10	3
Cow	5	6	3
Sheep	28	44	13
Rat	3	N.D.	N.D.
Man	7	2	N.D.
Guinea-pig	28	19	5
Plasma[b]			
Dog	424[a]	—	—
Cow	34[a]	—	—
Sheep	285[a]	—	—
Rat	396[a]	—	—
Man[8]	275	174	94
Guinea-pig[9]	7000	—	1200

a) pmol/min/E_{280}.
b) stored at −20°.
N.D.: not detected.

than that of bovine PFHP (Fig. 3). It is noteworthy that bovine plasma contained weak Leu-β-NA hydrolyzing activity as compared with other plasmas. The aminoacyl-β-NAases in various species of PFHP, except sheep PFHP, split Leu-β-NA most rapidly and Lys-β-NA most slowly. This observation agrees well with results on the kinin-converting enzyme in plasma and liver reported by Guimarães *et al.*[8] and Borges *et al.*[9]

Identification of the Kinin Released from Dog Kininogen by CGK

Only one biologically active peak which coincided with bradykinin was obtained in SP-Sephadex C-50 chromatography of the incubation mixture of CGK and dog PFHP, whereas two peaks were detected with the further heated preparation of PFHP (Fig. 4).

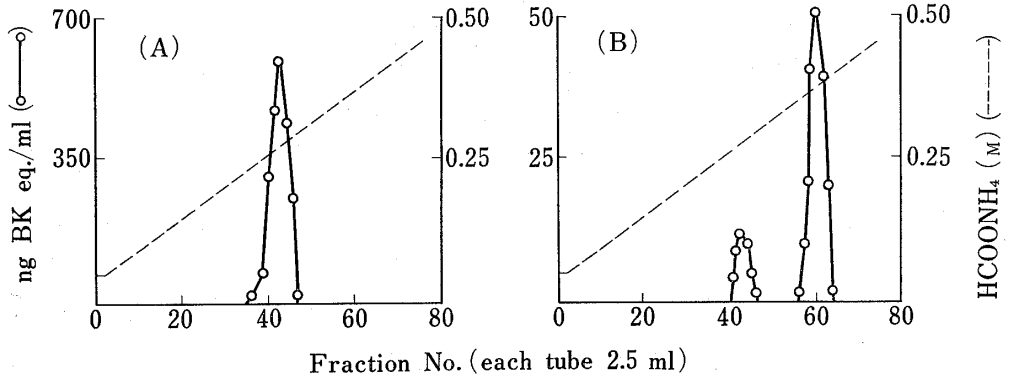

Fig. 4. SP-Sephadex C-50 Column Chromatographic Analysis of the Kinin Liberated from Dog Kininogen by CGK

CGK solution (0.6 μg BK eq./min/ml) (5 ml) was incubated with 20 ml of dog PFHP (A) or further heated (100°, 30 min) PFHP solution (B) for 1 hr at 37° in the presence of 8-hydroxyquinoline (0.8 mg/ml). The liberated kinins were analyzed according to the procedures described in "Materials and Methods". PFHP, 50 mg/ml, dissolved in 0.01 M phosphate buffer, pH 7.4, was used.

9) D.R. Borges, J.L. Prado, and J.A. Guimarães, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **281**, 403 (1974).

The main peak in Fig. 4-B was kallidin and the minor one was bradykinin. However, the net amount of bradykinin must be extremely small, since the sensitivity of rat uterus to bradykinin is 1—3 times that to kallidin.[7]

Figure 5 shows the results of analysis of kinins liberated from dog kininogen by CGK on silica gel H thin layer plates. The dog PFHP (3 g) and the further heated PFHP (3 g) were separately incubated with CGK (1.8 μg BK eq./min) for 1 hr at 37°, pH 7.4, and the liberated kinins were separated by the same procedures as in Fig. 4. The active fractions which correspond to the peak in Fig. 4-A and the main peak in Fig. 4-B were pooled separately and lyophilized. Each sample was dansylated according to the method of Gray and Hartly[10] with slight modifications,[4] and chromatographed on a silica gel H plate using 2PrOH: MeOAc: 28% NH_4OH (9: 7: 4). Only one yellow fluorescent band was detected other than DNS–OH and DNS–NH_2 in each sample, and the fluorescent bands from Fig. 4-A and -B coincided with those of authentic DNS-bradykinin and -kallidin, respectively. DNS–OH and DNS–NH_2 are by-products always generated in dansylation.

These results led us to the conclusion that dog PFHP contains a heat-stable kinin-converting enzyme, and that CGK acts on dog kininogen directly to release kallidin, which is then further converted to bradykinin by the above enzyme.

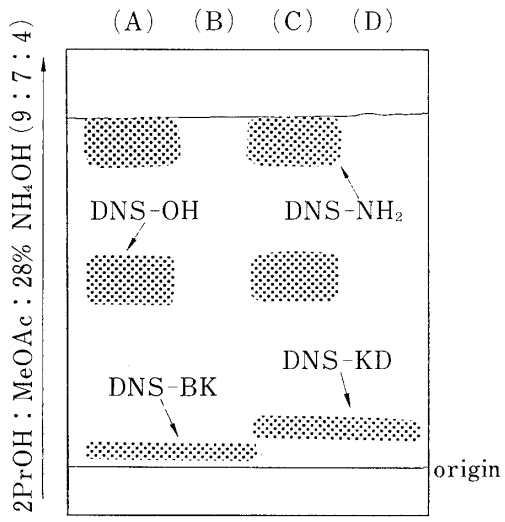

Fig. 5. Thin Layer Chromatogram of Dansylated Samples of the Active Fractions from SP-Sephadex C-50 Chromatography

Sixty ml of dog PFHP or further heated PFHP solution (50 mg/ml) was incubated with 15 ml of the CGK solution used in Fig. 4 for 1 hr at 37° in the presence of 8-hydroxyquinoline. The liberated kinins in these mixtures were separated by SP-Sephadex C-50 chromatography and lyophilized. After that, samples corresponding to the active peak in Fig. 4-A (A) and the main peak in Fig. 4-B (C) were dansylated and chromatographed on silica gel H plates. (B) and (D): authentic DNS-bradykinin and -kallidin, respectively.

Discussion

For kininogenase activity assay, the pseudoglobulin fraction from heated or acid-treated plasma is often used as the crude kininogen. The treatment of the plasma is essential to destroy kininogenases and their precursors, kininases and proteinase inhibitors which may interfere in the kininogenase assay. Indeed, most of these substances can be destroyed by the above treatment. However, kinin-converting arylaminopeptidases in plasma, which convert kallidin and Met-Lys-bradykinin to bradykinin before the kinins are inactivated by kininases,[8] were found to be relatively stable to heat treatment. Such enzymic activity was not completely inactivated by heat treatment at 60° for 1 hr, and the PFHPs retained some activity to hydrolyze amino-β-NAs. The dog PFHP especially contained a considerable amount of activity as compared with the other PFHPs (Table II and Fig. 3). As already reported by Borges *et al.*, kinin-converting arylaminopeptidase in plasma is different from leucine aminopeptidase (hog kidney) as regards substrate specificity.[9]

In 1971, Moriwaki and Schachter reported that dog PFHP failed to convert kallidin to bradykinin, and that CGK directly liberated bradykinin from the kininogen. In their experiments, 5 mg of PFHP was incubated with 100 μg of kallidin (37° for 30 min, pH 7.0) for the detection of the kinin-converting activity, while they incubated 10 g of PFHP with

10) *a*) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 59p (1963); *b*) *Idem*, *ibid.*, **89**, 379 (1963).

0.1 g of CGK for the analysis of the liberated kinins. In the present investigation, 300 mg of PFHP was used for the detection of the kinin-converting activity, and this amount of dog PFHP was sufficient to convert 100 μg of kallidin completely to bradykinin, as shown in Fig. 2-A (37°, for 1 hr, pH 7.4). Therefore, in their experiments, the existence of the kinin-converting activity might have been overlooked due to the small amount of PFHP used relative to kallidin. In contrast, 10 g of the substrate used in the detection of the liberated kinin may have been too much. They obtained 520 μg of bradykinin in the incubation mixture of CGK and the substrate, but judging from our present results the same amount of kallidin would be completely converted to bradykinin under their experimental conditions. Thus, it seems likely that CGK liberates kallidin from dog kininogen, like other glandular kallikreins.

Contamination by kinin-converting enzyme in the substrate is a serious problem in the kininogenase assay of enzymes. In general, the kininogenase activity of an enzyme is expressed as the equivalent amount of bradykinin which can be liberated from a substrate in 1 min, so that if kallidin-releasing enzymes, such as pancreatic or urinary kallikreins, were assayed by the Magnus method, the experimental values would differ from the kininogenase activity according to the amount of contaminating kinin-converting enzyme present, since the sensitivities of guinea-pig ileum or rat uterus to kallidin and Met-Lys-bradykinin are less than that to bradykinin. This, of course, will not affect bradykinin-releasing enzymes, such as plasma kallikrein and trypsin. Nevertheless, from a practical point of view it is desirable to employ a substrate which is free from kinin-converting activity, since the kinin liberated by a kininogenase is not always identified.