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# STUDIES OF THE DIGESTION OF BRADYKININ, LYS-BRADYKININ, AND DES-ARG$^9$-BRADYKININ BY ANGIOTENSIN CONVERTING ENZYME*

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**Abstract**—We have studied the degradation of bradykinin, lysyl bradykinin and des-Arg$^9$-bradykinin by the angiotensin converting enzyme. Bradykinin was cleaved at two sites to produce the pentapeptide Arg-Pro-Pro-Gly-Phe plus dipeptides Ser-Pro and Phe-Arg. Lysyl bradykinin was cleaved similarly to release the same dipeptides plus the hexapeptide Lys-Arg-Pro-Pro-Gly-Phe. The tripeptidase activity of ACE was observed when des-Arg$^9$-bradykinin was digested. A single cleavage yielded the above pentapeptide plus Ser-Pro-Phe. Although des-Arg$^9$-bradykinin was the most rapidly digested, when mixtures of des-Arg$^9$-bradykinin and bradykinin or lysyl bradykinin were tested, virtually all of the bradykinin and most of the lysyl bradykinin was digested prior to the onset of digestion of des-Arg$^9$-bradykinin. This was shown to be due to inhibition of des-Arg$^9$-bradykinin cleavage by kinins and kinin-degradation products. The order in terms of potency was bradykinin > lysyl bradykinin > Ser-Pro $\gg$ Phe-Arg > Arg-Pro-Pro-Gly-Phe. The concentration of chloride ion was an important parameter which affected the rate of digestion of each substrate examined. des-Arg$^9$-bradykinin was not digested by ACE in the absence of sodium chloride and the rate of digestion increased as the chloride concentration was increased to 100–150 mM. On the other hand, increasing NaCl concentration was inhibitory for bradykinin digestion. The rate of Lys-bradykinin digestion was increased from 0 to 1 mM NaCl and decreased thereafter up to physiologic concentration. A half-maximal rate was seen at 100–150 mM NaCl compared to no salt. Of the divalent cations examined, cupric ion inhibited further digestion of des-Arg$^9$-bradykinin at physiologic concentrations. Our data indicate that the rate of degradation of kinins and the nature of the stable final cleavage products in plasma or serum (studied *in vitro*) are dependent upon the effects of chloride ion, metal ions, and the kinetic effects of multiple metabolites produced by at least two kininases.

We have shown recently that the degradation of kinins in human plasma and serum results in the formation of products that are not explicable by the enzymatic activities of carboxypeptidase N [1] or angiotensin converting enzyme [2, 3], the two known kininases present in plasma. Among these products are free phenylalanine and the tripeptide Ser-Pro-Phe [4, 5].

One of the kinins that can be produced is lysyl bradykinin (kallidin) formed by the cleavage of low molecular weight kininogen by tissue kallikreins [6]. Once formed, however, a plasma aminopeptidase removes the N-terminal lysine and converts it to bradykinin [7]. Bradykinin itself is the product of cleavage of high molecular weight kininogen by plasma kallikrein. Each of these appears to act upon receptors termed B-2 [8]. We have shown that the initial bradykinin degradation product in human plasma is des-Arg$^9$-bradykinin, and this has been shown recently to have activity upon blood vessels dependent upon a receptor designated B-1 [8]. The role of angiotensin converting enzyme (ACE), however, in the cleavage of lysyl bradykinin, bradykinin, and des-Arg$^9$-bradykinin has not been explored in detail. In this manuscript we have studied the interaction of ACE with each of these kinins, identified the resultant cleavage products, determined their relative rates of degradation alone and in mixtures, and defined kinetic effects of some of the later metabolites.

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## MATERIALS AND METHODS

### *Materials*

Bradykinin (BK), des-Arg$^9$-bradykinin (DBK) and Lys-bradykinin (LBK) were obtained from Sigma (St. Louis, MO). Angiotensin converting enzyme (EC 3.4.15.1) was purified from rabbit lung and was provided by Dr. Richard L. Soffer, Cornell Medical Center, New York, NY. The specific activity was 0.1 unit/$\mu$g of protein (one unit released 1 $\mu$mole of hippuric acid from Hip-His-Leu per min at 37°). Phe-Arg, Ser-Pro and the pentapeptide Arg-Pro-Pro-Gly-Phe were purchased from Serva Biochemicals (Garden City, NY). HPLC-grade acetonitrile and all inorganic salts used were purchased from Fisher (Springfield, NJ); trifluoroacetic acid was from Pierce (Rockford, IL).

### *Enzyme digestion*

Incubation of BK, DBK and LBK with ACE was performed at 37° in a total volume of 100 $\mu$l containing 100 mM NaCl buffered at pH 7.4 with 3 mM sodium phosphate. Final concentrations of the substrates and the enzyme were 20 ng/$\mu$l and 0.4 ng/$\mu$l respectively. The latter value is the concentration of ACE in plasma [9]; the substrate concentration was chosen so as to yield clear peaks by high performance

liquid chromatography (HPLC). Phosphate ion is weakly inhibitory to ACE, but the concentration utilized approximates its level in serum [10]. At timed intervals, 30μl aliquots were quenched with 250 μl of trifluoroacetic acid solution [0.1% (v/v) in water], pH 2.1, and were directly loaded onto the HPLC column for analysis.

*High performance liquid chromatography*

HPLC analyses were performed with a Waters Associate system containing a set of 6000 A pumps, Data Module 730, System Controller 720, Automated Injector (Wisp) 710-B, and Extended Wavelength Module 440 absorbance detector. Peptide products were applied to a μ-Bondapack C-18 reverse phase column (3.9 mm i.d. × 30 cm) of 10 micron particle size (Waters), and the separations were carried out with a linear gradient of increasing concentrations of Solvent B [0.065% (v/v) trifluoroacetic acid in acetonitrile] from 10 to 50% in a span of 10 min. Solvent A was 0.1% (v/v) trifluoroacetic acid in deionized water, and the flow rate was maintained at 1.5 ml/min at ambient temperature. The products detected at 214 nm were identified by co-elution with peptide standards and were confirmed by amino acid analyses. Substrate degradation was calculated by comparing the integrated peak area to the peak area of a known amount of authentic standard.

*Amino acid analyses*

Amino acid analyses were performed by Dr. Marshal Elzinga, Brookhaven National Laboratories, Upton, NY, as follows. The peak obtained by HPLC was lyophilized and exposed to 6 N HCl overnight at 110°. The sample was dried under nitrogen, dissolved in 0.2 M sodium citrate buffer, pH 2.0, and loaded into the column. Separation and identification were performed utilizing the methods and apparatus described by Hewick *et al.* [11].

*Statistical analysis.* The means of different values were compared with Student's *t*-test.

## RESULTS

*Digestion of BK, LBK, and DBK by ACE*

Bradykinin was incubated with ACE and a time course of digestion was monitored by HPLC. In Fig. 1A (20 min time point), we could identify formation of the dipeptides Phe-Arg and Ser-Pro plus the heptapeptide Arg-Pro-Pro-Gly-Phe-Ser-Pro and the pentapeptide Arg-Pro-Pro-Gly-Phe. At this point, heptapeptide was more prominent than pentapeptide. At 40 min (Fig. 1B), bradykinin was further depleted and increased levels of all four degradation products were seen; the quantities of heptapeptide and pentapeptide were approximately equal. By 60 min, at which time over 90% of the bradykinin was degraded, heptapeptide was depleted and a prominent pentapeptide peak was clearly visible. With further incubation (not shown) all of the bradykinin and heptapeptide were converted to a mixture of Arg-Pro-Pro-Gly-Phe plus Ser-Pro and Phe-Arg. These products were stable to further degradation by ACE for over 24 hr.

It is known that kininase I removes the C-terminal arginine from BK to produce the octapeptide termed des-Arg$^9$-bradykinin (DBK). We therefore next examined the ability of ACE to cleave DBK. As can be seen in Fig. 2A–C, with increasing incubation with time, DBK was cleaved into two products. Amino acid analysis of each peak demonstrated the formation of the pentapeptide Arg-Pro-Pro-Gly-Phe and, preceding it, the tripeptide Ser-Pro-Phe. With prolonged incubation up to 24 hr, no further degradation products were observed. Since lysyl bradykinin (LBK) is the other major kinin produced by kallikreins, we next studied the ability of ACE to degrade LBK. The result (not shown) resembled that seen with bradykinin in that sequential dipeptidase activity was seen liberating Phe-Arg and then Ser-Pro. The larger remaining fragments were an octapeptide and hexapeptide, each of which had an amino terminal lysine as confirmed by amino acid analysis. These are more polar and therefore eluted

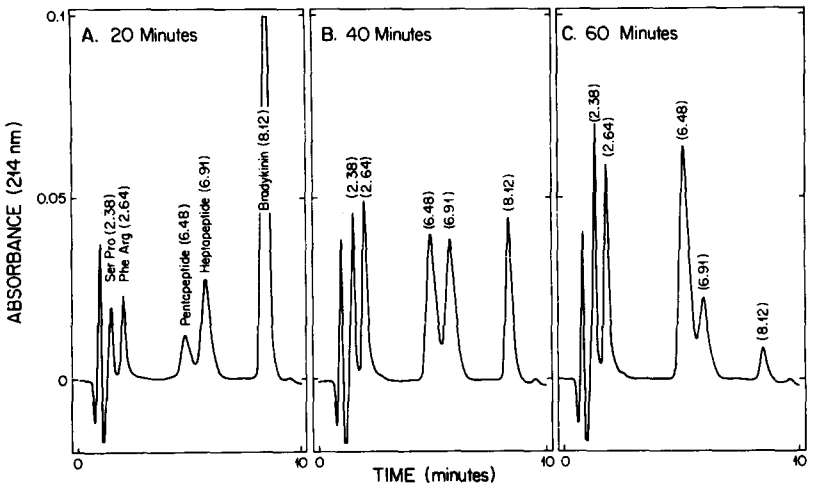


Fig. 1. Elution pattern of bradykinin degradation by ACE on a reverse phase HPLC column as described in Materials and Methods. Assessment of time points at 20, 40, and 60 min are shown for parts A, B, and C respectively. In part A the identity of each peak is indicated and its retention time, in minutes, is given in parentheses; only the latter is indicated in B and C.

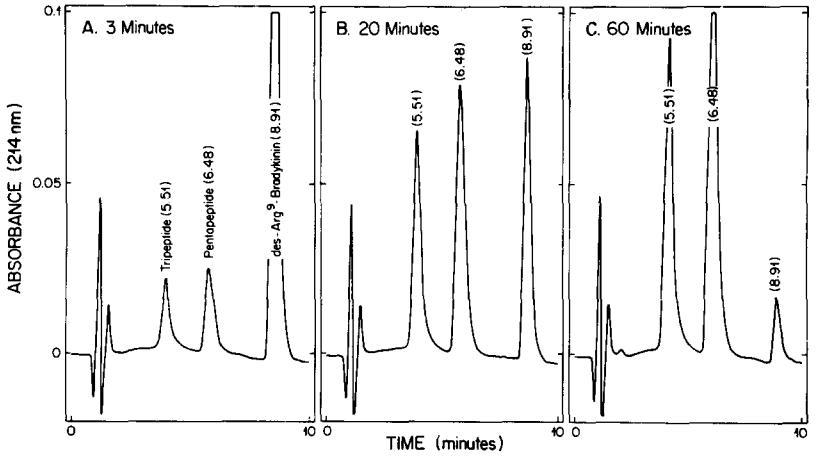


Fig. 2. Elution pattern of the degradation of des-Arg$^9$-bradykinin by ACE showing time points obtained after 3 (A) 20 (B) and 60 (C) min. The major peaks are identified in section A and retention times, in minutes, are shown in parentheses. The experimental conditions for degradation and elution are described in Materials and Methods.

earlier than the analogous fragments derived from the degradation of bradykinin. The comparative rate of cleavage of equimolar amounts of each of the three ACE substrates is shown in Fig. 3 and was determined by plotting the percent substrate depletion versus time. DBK was the most rapidly cleaved ($P < 0.05$ at each determination for 0–50 min compared to the rate of BK digestion). Although the difference between LBK and BK was not statistically significant, the value for LBK degradation at every time point and in each of three experiments was greater than the degradation of BK.

*Degradation of substrate mixtures by ACE*

The results shown in Fig. 3 would suggest that ACE digests DBK more rapidly than LBK or BK. However, a different result was obtained when equimolar mixtures of any two of these substrates were interacted with ACE. In Fig. 4 (parts A–E), a mixture of DBK and BK was degraded by ACE. It can be seen that there was no digestion of DBK until all of the BK was degraded. Once BK had been depleted, we first observed a decrease in the DBK

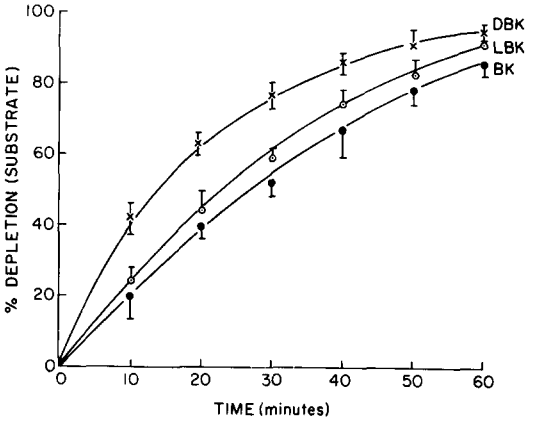


Fig. 3. Time curves of the degradation of BK, DBK, and LBK as determined by the rate of substrate depletion. The percent depletion at each time point was monitored by HPLC and the result plotted against time in minutes. The mean and standard error of three determinations at each time point is shown.

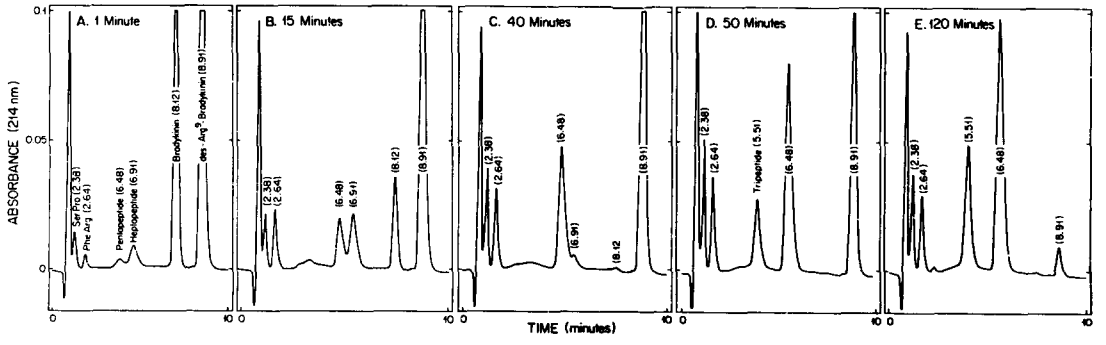


Fig. 4. Time course of digestion of a mixture of BK and DBK by ACE as assessed by HPLC analysis. Five time points are shown in parts A to E between 1 and 120 min of digestion. The major peaks are identified in part A and the retention times are given in parentheses. The digestion was performed under the experimental conditions described in Materials and Methods except that the concentrations of BK and DBK were 10 ng/$\mu$l each.

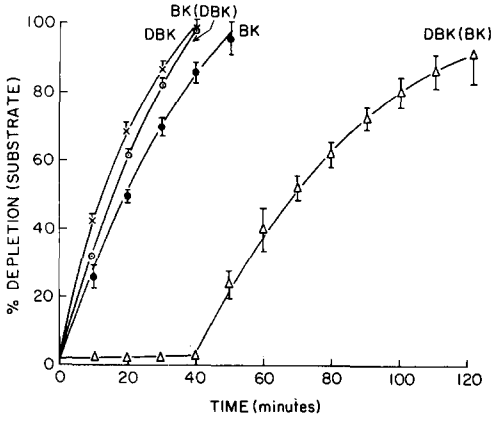


Fig. 5. Time courses of the ability of ACE to degrade BK and DBK when tested alone and when mixed together. The progress curves are identified for each substance whose degradation was being measured by HPLC and in parentheses the substance mixed with it. The percent depletion of each at the time points indicated is plotted versus time in minutes. The concentration of the substrates was 20 ng/μl when tested alone, whereas it was 10 ng/μl in a mixed experiment. All other conditions were the same as described in Materials and Methods. The mean and standard error of four determinations at each time interval is shown.

peak, and formation of the tripeptide Ser-Pro-Phe, a characteristic cleavage product of DBK. When the same experiment was performed with LBK substituted for BK, the result was similar, i.e. LBK was degraded prior to DBK. However, in contrast to degradation of a mixture of BK and DBK, some DBK was degraded before total depletion of LBK. Finally, when LBK and BK were mixed and digested by ACE, (data not shown), there appeared to be simultaneous degradation of each to form the expected dipeptides Ser-Pro and Phe-Arg, and peptides of 5, 6, 7, and 8 amino acids. In summary, BK, and to a slightly lesser degree, LBK, appeared to impede degradation of DBK. When all three were mixed, the predicted result (not shown) was found in that DBK degradation was only seen after BK (and about 90% of the LBK) was depleted.

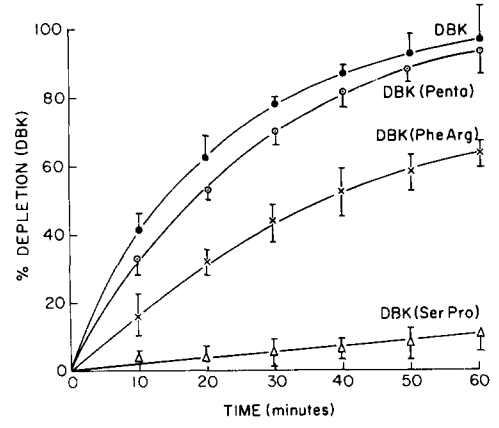


Fig. 6. Time course of the ability of ACE to digest DBK in the presence of the bradykinin degradation products shown in parentheses. The percent depletion as monitored by HPLC is plotted versus time in minutes. The concentrations of DBK, pentapeptide, Phe-Arg and Ser-Pro were 20 ng/μl, 12.6 ng/μl, 7 ng/μl and 4.4 ng/μl respectively (equimolar). The rest of the conditions were the same as in Materials and Methods. The mean and standard error of four separate experiments is shown for each time point.

When the mixture of BK and DBK was digested by ACE, not only did the presence of BK completely inhibit DBK digestion, but when DBK was subsequently digested, its rate of degradation was diminished. The data regarding the digestion of BK and DBK alone and when combined are plotted in Fig. 5. Most striking was the prominent lag prior to the onset of DBK digestion when it was mixed with bradykinin. However, it can be seen that, once DBK digestion began, it was retarded significantly relative to its rate of digestion when tested alone. For example, during the first 20 min of DBK digestion alone, 80% was depleted. Once DBK digestion began in the presence of BK (the 40 min time point), only 35% was degraded during the first 20 min. We next considered the possibility that the presence of bradykinin degradation products might have inhibited the ability of ACE to digest DBK. Figure 6 illustrates the rate of DBK in the presence of an equimolar mixture of Arg-Pro-Pro-Gly-Phe as well as either Ser-Pro or Phe-Arg. It can be seen that Ser-Pro caused a prominent diminution in the rate of DBK digestion ($P < 0.01$), whereas Phe-Arg had a lesser effect ($P < 0.05$) and pentapeptide had little or no effect ($P > 0.05$). The mixture of all three gave marked inhibition which was additive. No comparable inhibition of BK or LBK digestion upon addition of each of these peptides was evident.

*Effect of pH*

The effect of pH upon degradation of BK, LBK, and DBK by ACE is shown in Fig. 7. The pH optimum for BK or LBK was 7.5–8, whereas that for DBK was clearly at 8.5. While each was inhibited under more acidic conditions, the effect on the degradation of DBK was pronounced in that a prominent decrease in rate of digestion was seen as the pH was changed from 7.0 to 6.5. Although the rate of

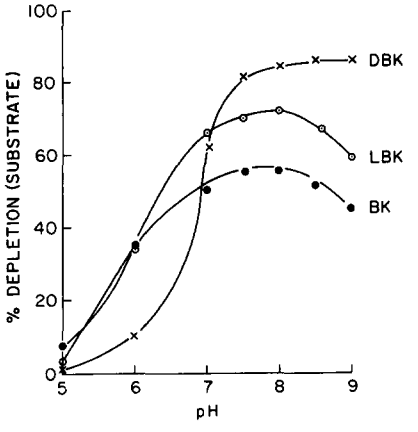


Fig. 7. Percent depletion (measured by HPLC) of BK, LBK, and DBK after digestion by ACE for 30 (DBK) and 40 (LBK and BK) min in 3 mM sodium phosphate buffer (pH 5–9) made with differing proportions of $Na_2HPO_4$ plus $NaH_2PO_4$.

digestion of BK and DBK decreased as the pH approached 9, no effect upon DBK digestion was seen.

*Effect of ions*

We next examined the effect of NaCl concentration upon the rate of degradation of BK, LBK, and DBK by ACE. As the NaCl concentration was increased from 0 to 250 mM (Fig. 8), the rate of BK digestion was inhibited, while the rate of degradation of LBK was increased from 0 to 1 mM and then decreased thereafter. The rate of DBK digestion was found to be very dependent upon NaCl concentration. In fact, when NaCl was absent, no degradation took place. As the NaCl concentration was increased, the rate of digestion also increased and appeared optimal at 100–150 mM which approximates physiologic conditions (note: in all previous experiments the NaCl concentration was 100 mM). We examined the effect of different anions and cations upon digestion of BK, DBK and LBK by ACE tested at a 1 mM (LBK) or a 10 mM (BK and DBK) concentration (Table 1). Substituting potassium or ammonium ions for sodium had no effect upon the degradation rates of any of the ACE substrates. We then changed the anion, keeping the sodium constant. Under these conditions, DBK, LBK, and BK were digested at the rate corresponding to 0 NaCl concentrations seen in Fig. 8, i.e. DBK was not digested at all, BK was not inhibited, and the mild enhancement of LBK digestion at 1 mM was not seen. This result suggests that the inhibitory and enhancing effects seen upon ACE digestion of its substrates is a function of chloride ion.

We next studied the effects of different cations upon the digestion of BK, LBK, and DBK by ACE. No appreciable alteration in the rate of digestion of any of the substrates was seen when calcium or zinc was varied from 0.1 to 1.0 mM. A striking effect upon the rate of DBK digestion was seen with cupric ion. As the copper concentration was increased to 20 $\mu$M (physiologic range 11–22 $\mu$M) [12], 60% inhibition was observed. No significant effect upon BK or LBK digestion was seen unless the concentration of copper was >100 $\mu$M.

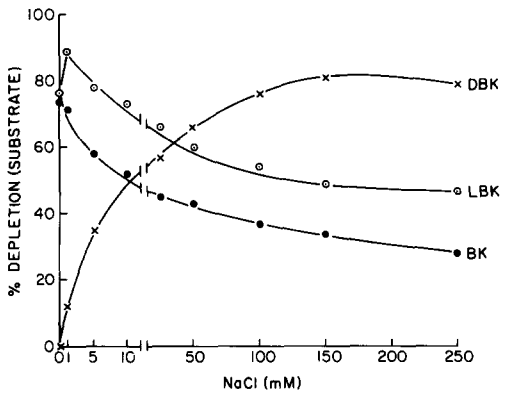


Fig. 8. Effect of NaCl concentration upon the degradation of BK, DBK, and LBK at 20, 30 and 30 min time points, respectively, as monitored by HPLC. The percent substrate depletion is plotted versus the salt concentration in millimolar. Experiments were performed in a total volume of 100 $\mu$l containing different concentrations of NaCl, indicated in the figure, buffered with 3 mM sodium phosphate, pH 7.4.

Table 1. Effect of monovalent anions and cations on the degradation of BK, DBK and LBK* by ACE

| Salt† | Percent depletion‡ | | |
|---|---|---|---|
| | BK | DBK | LBK |
| None | 74 | 0 | 76 |
| NaCl | 50 | 48 | 86 |
| KCl | 50 | 48 | 86 |
| $NH_4Cl$ | 52 | 50 | 85 |
| NaAc | 75 | 0 | 77 |
| $NaHCO_3$ | 74 | 0 | 75 |
| $NaNO_3$ | 73 | 0 | 76 |

* Time of digestion: 20 min for BK and 30 min for DBK and LBK.

† Tested at 10 mM for BK or DBK (increased concentration of some salts alters pH) and 1 mM for LBK (peak effect).

‡ Percent depletion was determined by HPLC analysis at the indicated time. Assays were performed in a total volume of 100 $\mu$l containing different salts buffered at pH 7.4 with 15 mM sodium phosphate. Final concentrations were as follows: enzyme, 0.4 ng/$\mu$l; substrates, 20 ng/$\mu$l; and salts, 10 mM for BK or DBK and 1 mM for LBK.

## DISCUSSION

In this manuscript, we have assessed, in detail, the degradation of BK, DBK and LBK by angiotensin converting enzyme. When digestion of BK by ACE was examined, two sequential cleavages were clearly seen as has been observed previously [5, 13] and, but for addition of the amino terminal lysine, LBK behaved similarly. However, it is known that the first major cleavage of BK in plasma (as studied *in vitro*) is by a carboxypeptidase to remove the C-terminal arginine [4, 14, 15] and leave DBK. DBK can then be further digested by ACE and a tripeptide product, Ser-Pro-Phe, is obtained in addition to the pentapeptide Arg-Pro-Pro-Gly-Phe. This tripeptidase activity of ACE has been reported previously [16] for DBK and other substrates, and our data confirm this observation. Previous studies have not examined mixtures of these substrates, or the effect of each degradation product upon cleavage of BK, DBK and LBK. Further, systematic study of the effects of pH and ionic strength, and anion and cation requirements on ACE digestion of each substrate has not been reported.

When we examined the rates of cleavage of BK, DBK, and LBK by ACE, it was somewhat surprising to see that DBK was cleaved most rapidly. On the other hand, this was clearly not the case when DBK was mixed with BK or LBK and their rate of degradation determined. In the presence of BK, complete inhibition of DBK degradation was seen (Fig. 5), and DBK digestion commenced only after BK was degraded completely. Further, the rate of DBK digestion was then much diminished compared to its rate of cleavage when examined alone. When the cleavage products of bradykinin digestion were tested as potential inhibitors of DBK digestion, Ser-

Pro was most inhibitory, while Phe-Arg and pentapeptide had lesser effects. When digestion of a mixture of DBK and LBK was examined, the effect of the presence of LBK was similar to BK but slightly less in degree. The order of inhibition we observed would therefore be bradykinin > Ser-Pro ≫ Phe-Arg > Arg-Pro-Pro-Gly-Phe. We did not observe inhibition between LBK and BK when digested together with ACE. The aforementioned metabolites do, however, have an effect upon the rate of degradation of BK and LBK, but the differences are not as profound as is seen with DBK.

Other workers have examined the effect of kinin metabolites upon the activity of ACE but utilized differing ACE substrates and did not systematically assess each of the relevant kinin cleavage products. For example, Dorer *et al.* [17] also report no inhibition of ACE by pentapeptide using Hippuryl-Gly-Gly as substrate, while Kariya *et al.* [18] demonstrated inhibition of ACE by bradykinin and other related peptides (Arg-Pro, Arg-Pro-Pro, and Phe-Ser-Pro) using angiotensin I or synthetic peptides as substrates. In the latter study, the peptides tested for inhibition are not known to be formed by plasma kininases. Finally, Phe-Arg has been shown to inhibit ACE digestion of hippuryl dipeptides [19] while angiotensin I and II, and angiotensinogen-related peptides inhibit digestion of BK by ACE [20].

The effect of chloride ion upon the degradation of bradykinin has been controversial. Some workers [17, 18] report no effect of chloride ion upon degradation of BK by ACE. Others [21, 22] have found a modest enhancement of bradykinin degradation by ACE between 1 and 100 mM (peak at 10 mM); above 100 mM the rate approximated that seen with no NaCl. In our system (3 mM sodium phosphate, pH 7.4) we consistently observed a diminution in the rate of degradation by as little as 1 mM NaCl, and the inhibition increased to 100 mM and then leveled off at higher salt concentrations. Other studies used 5 mM HEPES, [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] buffer or potassium phosphate buffer, pH 7.5; otherwise the experiments appeared identical. Thus, we are uncertain as to the explanation for the differences reported. Digestion of LBK by ACE was augmented up to 1 mM NaCl, returned to baseline at 5 mM NaCl (equal to no NaCl), and was then diminished up to 100 mM NaCl. Digestion of DBK, in contrast, was markedly augmented by NaCl starting at 1 mM, and these data are in agreement with previously published observations [17]. Chloride ion appeared to be responsible for each of these effects independent of the associated cation (Table 1).

If ACE were a major plasma kininase, our results indicate that bradykinin would be preferentially degraded prior to cleavage of any des-Arg$^9$-bradykinin that had been generated via carboxypeptidase N. However, our prior *in vitro* results [4, 5] in serum indicate that ACE does not digest bradykinin prior to its conversion to des-Arg$^9$-bradykinin. Thus, the latter product is, in fact, the major substrate for ACE. The chloride concentration of plasma or serum is optimal for this reaction to proceed (Fig. 8). The products of this reaction, Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe, appear unstable in serum; free Phe is released, and one of the final products, Ser-Pro, is a major ACE inhibitor. Thus, as the final kinin degradation products accumulate, there is feedback inhibition of this enzyme. Further studies will explore the kinetics and mechanism of ACE inhibition by kinin metabolites as well as the striking inhibition of des-Arg$^9$-bradykinin cleavage in the presence of other kinins. In contrast to drugs such as captopril, inhibition of activity restricted to some substrates may be observed. For example, elevated levels of copper or Ser-Pro will inhibit ACE activity upon DBK, but not upon BK. Such observations may have importance for the physiologic or pathologic effects of kinins. For *in vivo* studies, one must consider not only the rate of degradation and the products formed by plasma kinin degradation but also the effect of tissue degradation of kinins and clearances of the end products.

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