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KININS RELEASED FROM HORSE HEAT-ACID-DENATURATED PLASMA BY PLASMIN, PLASMA KALLIKREIN, TRYPSIN AND *BOTHROPS* KININOGENASE

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Abstract—Isolation and identification of kinins released from heat-acid-denaturated horse plasma by plasmin, plasma kallikrein, trypsin and *Bothrops* kininogenase was carried out. From heat-acid-denaturated plasma, plasmin released mainly methionyl-lysyl-bradykinin and small amounts of bradykinin and kallidin. Plasma kallikrein released only bradykinin from this substrate. Trypsin released mainly bradykinin and kallidin, and traces of methionyl-lysyl-bradykinin. *Bothrops* kininogenase released mainly bradykinin, but some kallidin was also found in the substrate-enzyme incubate.

MANY physiologists and pathologists employ the method of Diniz and Carvalho[1] for the determination of kininogen in plasma. The observation frequently reported in the literature that the substrate used in this method (plasma heated in acid medium) responds poorly to kallikrein seemed to be irrelevant after the finding that plasmin, shown to be the most potent endogenous kininogenase,[2] is as effective as trypsin in releasing kinin from this substrate (expressed in terms of bradykinin). However, in a further work[3] we have shown that the main kinin released by plasmin from kininogen 2 is methionyl-lysyl-bradykinin. It therefore seemed of some importance to identify the kinins released by plasmin from heat-acid denaturated plasma and to compare them with the kinins released by other kininogenases.

The experiments here described are concerned with the isolation and identification of the kinins released from heat-acid-denaturated horse plasma by plasmin, plasma kallikrein, trypsin and *Bothrops* venom kininogenase.

MATERIAL AND METHODS

Kinins. Bradykinin acetate, synthetic, was obtained from Sigma Chemical Company, St. Louis, U.S.A.; Methionyl-lysyl-bradykinin, synthetic, was a gift from Dr. E. Schroder, A.G., Berlin; Kallidin, synthetic, was kindly supplied by Sandoz Ltd., Basle. Loss of peptides in the diluted solutions was prevented by the addition of oxalic acid in the final concentration of 10^{-3} M.[4]

Trypsin, crystalline, Spofa, Czechoslovakia, was used. One mg trypsin hydrolyzed 106 μmoles of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) per min, when assayed by the method of Brown.[5]

Bothrops jararaca venom was obtained from Instituto Butantan, Brazil. The venom

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was dissolved in 0·9% NaCl, heated to 90°, immediately refrigerated, centrifuged, and the solution kept frozen while not in use.

Plasma kallikrein was obtained from horse plasma as described previously,[6] with the exception of rechromatography on DEAE-cellulose.

Plasmin was obtained from pre-plasmin, prepared from human plasma by the method of Robbins and Summaria[7] with minor modifications.[2] Activation and proteolytic activity were performed as described previously.[2]

Plasma. Fresh horse plasma, collected in a siliconized container (Silicone GKI, USSR) over potassium oxalate (3g/l. blood), was centrifuged at about 5000 rev./min. in polyethylene tubes and used immediately. It was denaturated by heat in acid medium as described by Diniz and Carvalho[1] under the following conditions. To 450 ml of 0·2% acetic acid heated to 50–60°, 50 ml of plasma were added, mixed, and the mixture was heated in a boiling water bath for 30 min. Fifteen ml 1 M NaOH were then added till pH 7·0–7·5 was reached, followed by 125 ml 0·2 M Tris-HCl buffer, pH 7·8, containing 60 mg of oxalic acid in order to impede the adsorption of the peptides on glass.[4]

Incubation with kininogenases. The whole of the mixture above was then incubated at 37° with one of the enzymes. The amount of *Bothrops* venom, trypsin, plasma kallikrein and plasmin used, expressed in mg protein, were respectively 4·5, 5, 8·5 and 12·5. The times of incubation were 30 min with *Bothrops* venom and trypsin; 60 min with plasma kallikrein and 150 min with plasmin, as recommended in our previous work.[2] Boiling ethanol (1300 ml) was then added to each incubate, the mixture kept 7–10 min in a boiling water-bath and then transferred to a refrigerator where it was kept for 18–20 hr and afterwards centrifuged. The alcohol was evaporated by distillation under reduced pressure at 44–50° and the aqueous solution was lyophilized.

Isolation of kinins. From the dry residue obtained after lyophilization, portions of 1 g were weighed out, dissolved in 5 ml distilled water and submitted to gel filtration on a G-75 Sephadex 2·5 × 25 cm column, equilibrated with water. Fractions of 4 ml were collected in an automatic fraction collector at a rate of 1 ml/hr, and their absorbance was measured in a spectrophotometer at 280 mμ. The biological activity of the fractions was tested on the guinea-pig ileum and the active fractions were pooled and lyophilized. The dry residue was dissolved in 5 ml distilled water and filtered through a G-25 Sephadex column (2·5 × 25 cm) equilibrated with water. Again the fractions were tested on the ileum for their biological activity and the active fractions were mixed and lyophilized. Samples of 0·1 g of the residue were dissolved in 1 ml of distilled water and 0·01 ml fractions of this solution were transferred to a sheet of Whatman chromatographic paper (N.1 or 3 MM) large enough to contain 10 to 20 original spots. After the electrophoresis-chromatographic procedure[9] two longitudinal full-length strips containing the first and last spots were cut off and stained to localize the ninhydrin-reacting spots. The remaining unstained chromatogram was cut horizontally in strips corresponding to each ninhydrin reacting spot and the strips of paper were eluted with the same volume of 0·2% acetic acid. The eluates were lyophilized, dissolved in 0·9% NaCl and their activity was tested on the ileum. The whole procedure was repeated with the remaining crude material as many times as necessary to permit a clear cut identification of the liberated peptides.

All the active eluates from the same incubation mixture were pooled and from each pool a sample was incubated with chymotrypsin to verify whether the activity found

depended on the presence of peptides. For this 0·2 ml of the eluate were added to 0·2 ml of 0·2 M Tris buffer, pH 7·8, and incubated with 25 μg of chymotrypsin for 30 min at 37°, the activity of the incubate being tested on the ileum at the end of 15 and 30 min. Samples of active material were also tested for their capacity to lower the blood pressure in the rat, increase capillary permeability in the rabbit and relax the rat duodenum.

After it was verified that the eluates contained kinins, the eluates were submitted to electrophoresis followed by chromatography, using kallidin, methionyl-lysyl-bradykinin and bradykinin as standards. If necessary separate spots were eluted and repeatedly submitted to electrophoresis followed by chromatography, or to one of the procedures only, always using the synthetic kinins as inner and outer standards.

RESULTS

Figure 1 shows the filtration pattern obtained when a lyophilized incubate of plasmin-substrate was passed through Sephadex G-75 and the extinction of the resulting fractions was measured at 280 mμ. It can be seen that three absorbance peaks can be distinguished but, the peak of pharmacological activity was localized at the

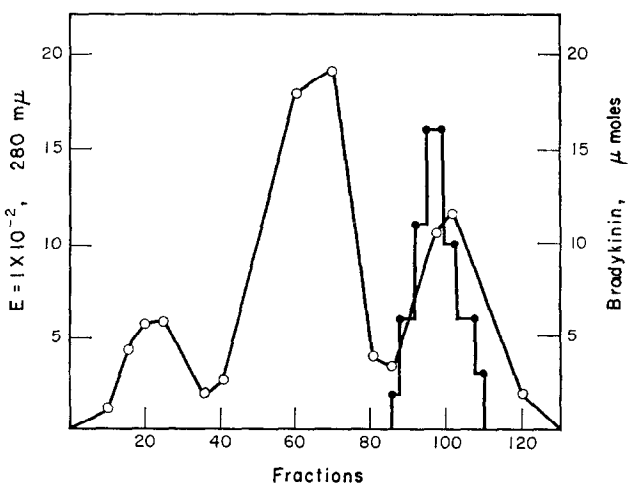


FIG. 1. Gel filtration pattern of lyophilized incubate of plasmin substrate on a 25 × 25 cm G.75 Sephadex column. The solvent was water. Extinction at 280 mμ (○—○); activity expressed in μmoles of bradykinin, as determined on the guinea-pig ileum (●—●).

last absorbance peak. When the active fractions were pooled together, lyophilized and filtered through Sephadex G-25 again three absorbance peaks were found but, the peak of biological activity was now shifted to just before the last absorbance peak (Fig. 2). The filtration patterns on G-75 found with the other incubates were similar to that just described for the plasmin incubate but different values of absorbance were observed. After filtration of the active G-75 fractions on Sephadex G-25 three extinction peaks were found with venom kininogenase, while two absorbance peaks were observed with trypsin and kallikrein.

Figure 3 shows the first electrophoresis-chromatographic separation of the pooled

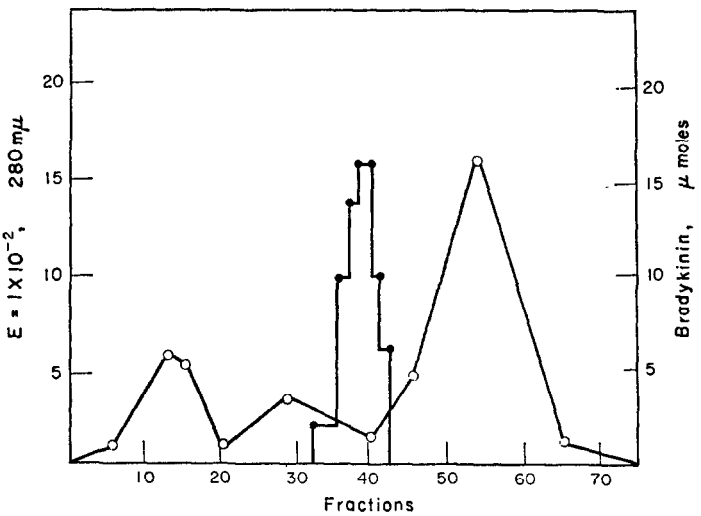


FIG. 2. Gel filtration pattern on a 2·5 × 25 cm G-25 Sepadex column of pooled and lyophilized active fractions obtained by gel-filtration on a G-75 Sephadex column (see Fig. 1). The solvent was water. Extinction at 280 mμ (○——○); activity expressed in μmoles of bradykinin, as determined on the guinea-pig ileum (●——●).

biologically active fractions obtained after the second Sephadex purification of the incubates of the substrate with kallikrein, trypsin, *Bothrops* venom and plasmin, respectively. It was observed that in the trypsin-incubate three spots were biologically active (1, 2 and 6 in Fig. 4). With kallikrein only spot 4 was found to be biologically active. With plasmin three spots were biologically active (1, 2 and 6), as can be seen in Fig. 5, and with *Bothrops* venom two spots were biologically active (2 and 6), as shown in Fig. 6. Spot 5, found in the chromatograms of all incubates, stained yellow with ninhydrin, and if rechromatographed showed the same R_f as proline, giving blue colour with isatin, but showed no activity on the ileum. All active eluates became inactive after incubation with chymotrypsin; Fig. 7 shows the action of chymotrypsin on one eluate obtained from trypsin-treated substrate.

Although, as shown in Fig. 3, the first electrophoresis-chromatography analysis permits only a rough resolution of the ninhydrin-reacting material present in the incubates, in fact it reflects the actual composition of the active peptides in the incubate analysed. A second electrophoresis-chromatography analysis of the pooled active peptides obtained in this first analysis is shown in Fig. 8. Thus from the trypsin-substrate incubate three ninhydrin reacting spots were obtained roughly corresponding to methionyl-lysyl-bradykinin, kallidin and bradykinin. From the venom-substrate incubate two ninhydrin-reacting spots are shown roughly corresponding to kallidin and bradykinin. From the kallikrein incubate only one ninhydrin-staining spot was obtained, corresponding to bradykinin. All these ninhydrin-reacting substances were found to be biologically active on the guinea-pig ileum. A similar chromatographic performance for the plasmin incubate revealed three ninhydrin-reacting spots corresponding to methionyl-lysyl-bradykinin, bradykinin and kallidin.

This preliminary identification was confirmed after analysing separately the eluate

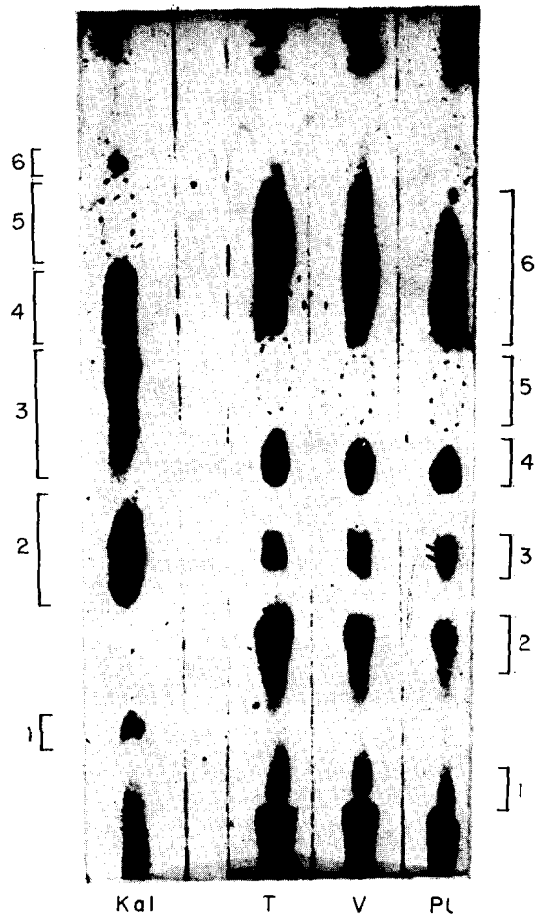
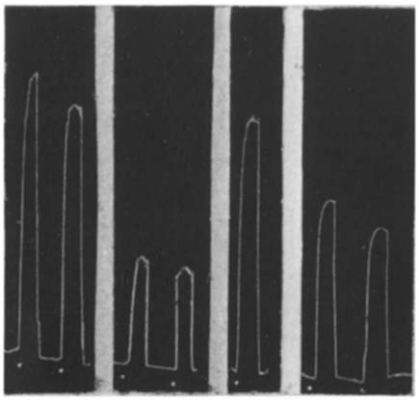
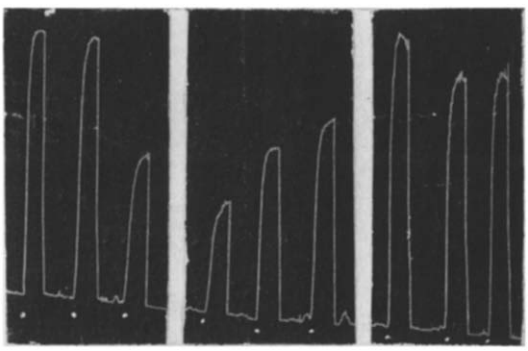


FIG. 3. Descending chromatography (after electrophoresis) of the enzyme-substrate incubates purified by filtration on Sephadex G-75 and G-25. Paper, Whatman No. 1; solvent, butanol:acetic acid:water (63:10:27). Enzymes: kallikrein (Kal), Trypsin (T), Bothrops venom kininogenase (V) and plasmin Pl).



	Br	6	Br	1	Br	2	Br	
$1 \times 10^{-3} \mu g$	20		10		20		15	
ml		0·05		0·10		0·03		
min	70	73	82	85	97	106	109	

FIG. 4. Activity on the guinea-pig ileum of the eluates of spots 1,2 and 6 from a chromatogram of trypsin-substrate incubate similar to that shown in Fig. 3. Spot 1 (1); spot 2 (2); spot 6 (6); bradykinin (Br).



	Br	Br	Br	1	2	Br	6	Br	Br
$1 \times 10^{-3} \mu g$	20	20	10			15		20	20
ml				0·10	0·03		0·06		
min	67	70	73	76	79	82	90	93	96

FIG. 5. Biological activity on the guinea-pig ileum of the eluates of spots 1, 2 and 6 eluted from a chromatogram of plasmin-substrate incubate similar to that shown in Fig. 3. Spot 1 (1); spot 2 (2); spot 6 (6); bradykinin (Br).

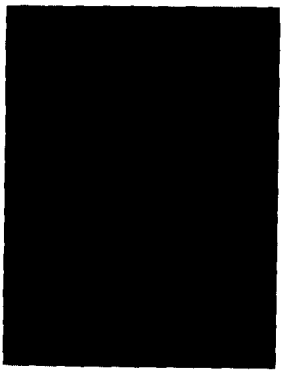


FIG. 6. Biological activity on the guinea-pig ileum of the eluates of spots 2 and 6 from a chromatogram of *Bothrops* venom-substrate incubate similar to that shown in Fig. 3. Spot 2 (2); spot 6 (6); bradykinin (Br).

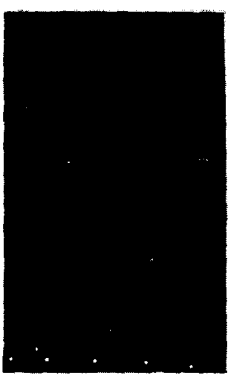


FIG. 7. Action of chymotrypsin on an eluate of spot 6 (Fig. 3) of an unstained chromatogram (following electrophoresis) of trypsin-substrate incubate. Bradykinin (Br); incubate of eluate without chymotrypsin (A); incubate of eluate with chymotrypsin, after 15 min (B); the same, after 30 min incubation (C).

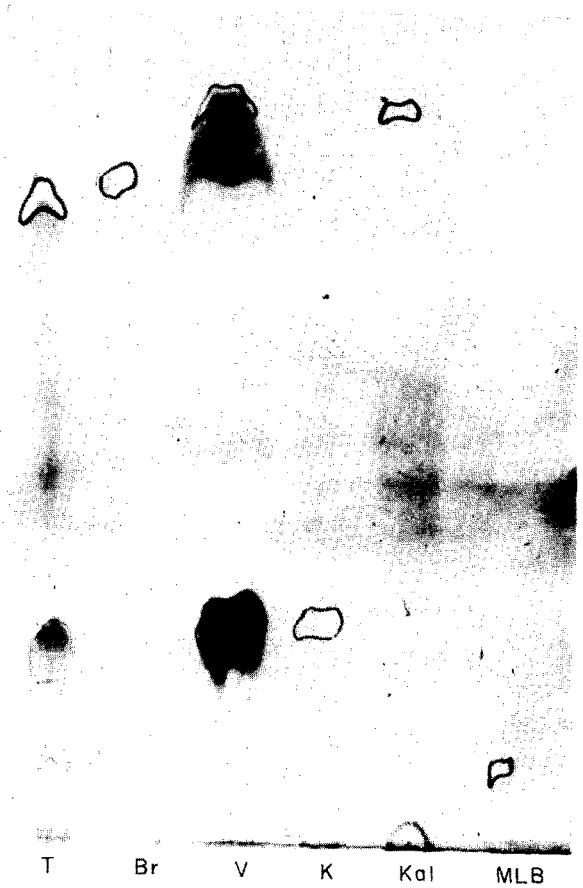


FIG. 8. Rechromatography of active eluates obtained after chromatography following electrophoresis of trypsin- (T), *Bothrops* venom- (V) and kallikrein- (Kal) substrate incubates. Bradykinin (Br); kallidin (K); methionyl-lysyl-bradykinin (MLB). Staining with ninhydrin.

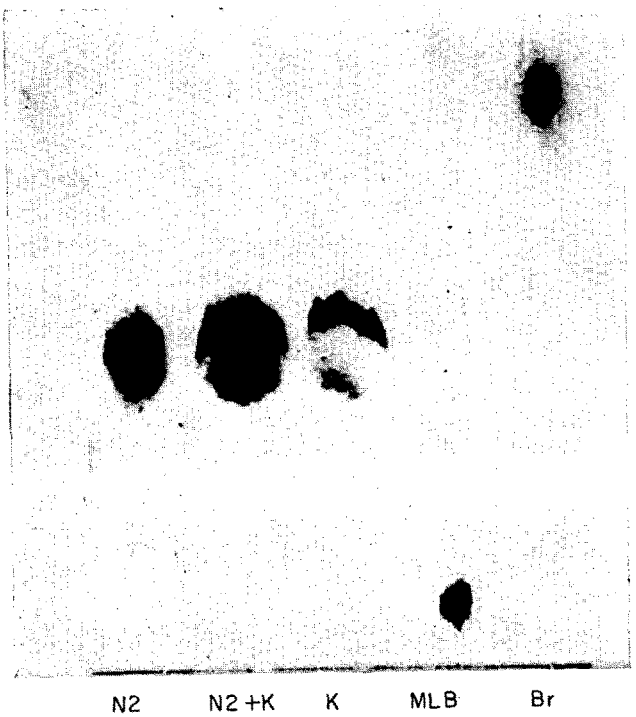


FIG. 9. Rechromatography following electrophoresis of a spot corresponding to kallidin (N_2) isolated from an incubate of *Bothrops* venom + substrate. Kallidin (K); methionyl-lysylbradykinin (MLB); bradykinin (Br). Staining with ninhydrin.

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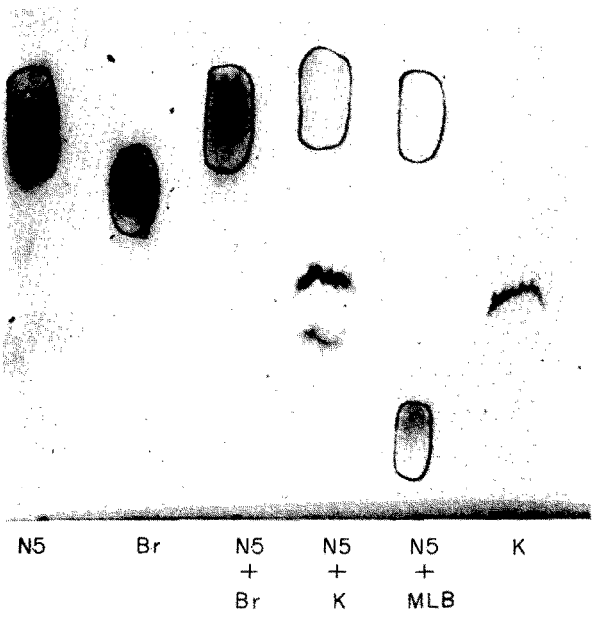

FIG. 10. Chromatography following electrophoresis of the eluate of a spot corresponding to bradykinin (N_5) obtained from purified venom-substrate incubate. Bradykinin (Br); kallidin (K); methionyl-lysyl-bradykinin (MLB). Staining with ninhydrin.

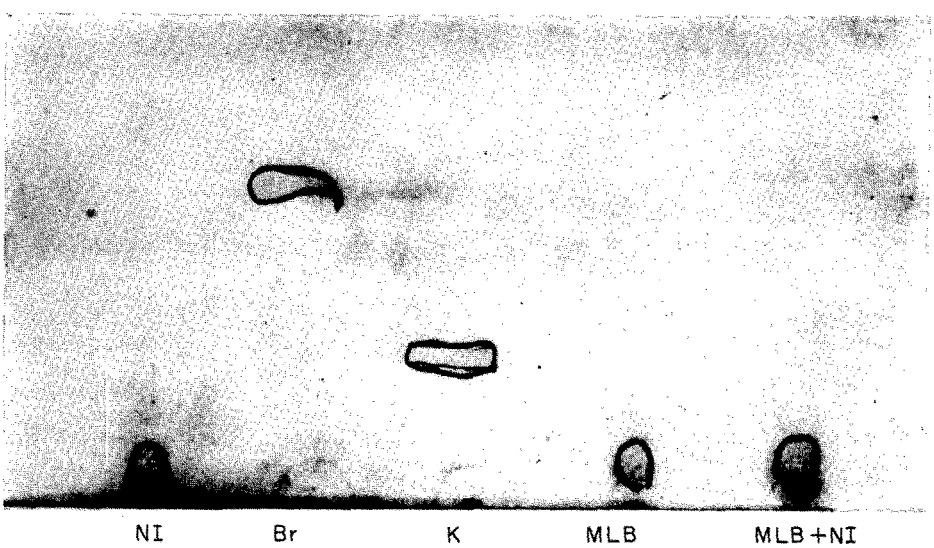

FIG. 11. Rechromatography after electrophoresis of an eluate (1) of the spot corresponding to methionyl-lysyl-bradykinin obtained after repeated electrophoresis-chromatography of a purified plasmin-substrate incubate. Bradykinin (Br); kallidin (K); methionyl-lysyl-bradykinin (MLB). Staining with ninhydrin.

of each spot by electrophoresis-chromatography or by only one of these two procedures. Illustrative examples of these identifications are shown in Figs. 9, 10 and 11. Figure 9 shows the rechromatography after electrophoresis of an eluate corresponding to kallidin isolated from the incubate of denaturated plasma with *Bothrops* venom. It can be seen that it has the same R_f as synthetic kallidin when chromatographed separately or added to a sample of kallidin. Figure 10 shows the electrophoresis-chromatography behaviour of the eluate of a spot corresponding to bradykinin (incubate with venom enzyme), alone and added to synthetic bradykinin or kallidin. Figure 11 shows that the electrophoresis-chromatography behaviour of the eluate corresponding to methionyl-lysyl-bradykinin (from the incubate with plasmin) is identical to that of synthetic methionyl-lysyl-bradykinin.

From the incubate of heat-acid-denaturated plasma with plasmin mainly methionyl-lysyl-bradykinin and small amounts of bradykinin and kallidin were isolated. In trypsin incubate mainly bradykinin and kallidin and traces of methionyl-lysyl-bradykinin were found. From venom-enzyme incubate mainly bradykinin and some kallidin were isolated. In the incubate of kallikrein only bradykinin was found.

DISCUSSION

Bradykinin, the hypotensive peptide first isolated by Andrade and Rocha e Silva[9] from incubate of trypsin with plasma globulins, was later found by Elliott, Lewis and Horton[10] to be a nonapeptide, and was the first member of the plasma kinins to be well characterized. Later Webster and Pierce[11] have shown that, from acid-treated human plasma, trypsin releases not only bradykinin but also kallidin, which constituted 2·5% of the total activity found in their trypsin incubates. Although in the experiments described here we did not estimate the percentage of each active peptide present in the trypsin incubates, the kallidin and bradykinin spots found in our chromatograms were of similar size and colour intensity, indicating that the proportion of kallidin was higher than that reported by Webster and Pierce.[11] Apart from bradykinin and kallidin, traces of methionyl-lysyl-bradykinin were also observed in our trypsin incubates. As it has been shown that trypsin converts not only kallidin[11] but also methionyl-lysyl-bradykinin[12] to bradykinin, the finding of larger or smaller amounts of these peptides possibly depends on the time of incubation of plasma with this enzymes and also on the presence of amidase in plasma, which has been shown to convert kallidin to bradykinin.[13] These factors might explain the findings of Zuber and Jaques[14] and Hamberg, Bumpus and Page[15] who did not find kallidin in incubates of trypsin with bovine plasma. Since we used heat-acid-denaturated plasma for these experiments, our incubates can be considered free of aminopeptidase.

As concerns plasma kallikrein, our findings coincide with those of Webster and Pierce[11] for acid-treated plasma. Activity was only found in the eluates of electrophoresis chromatogram spots with the R_f of bradykinin. Kallidin or methionyl-lysyl-bradykinin detectable by our electrophoresis-chromatography procedure were not found.

Plasmin released from heat-acid-denaturated plasma mainly methionyl-lysyl-bradykinin and easily detectable amounts of kallidin and bradykinin. In a previous paper[3] we reported the release of methionyl-lysyl-bradykinin by plasmin from purified kininogen 2. These findings might explain the puzzling fact that after the isolation of methionyl-lysyl-bradykinin from bovine plasma by Elliott and Lewis[16] later workers,

who did not use plasmin in their experiments, consistently failed to find this peptide in the incubates of purified kininogen or crude substrates with various kininogenases. It is possible that in the experiments of Elliott and Lewis,[16] plasmin was activated during the long period of treatment with acid and prolonged dialysis followed by neutralization.[17]

As we have shown recently,[2] plasmin releases practically the same amount of kinin (expressed as bradykinin) as trypsin from fresh or denaturated plasma, while kallikrein releases from the same substrate 1/10 of the amount of "kinin" released by any of those two enzymes. Therefore as methionyl-lysyl-bradykinin is the main product released by plasmin from heat-acid-denaturated plasma, and while trypsin releases mainly bradykinin and kallidin from this substrate, and as synthetic bradykinin was found to be two to five times as active as pure methionyl-lysyl-bradykinin on isolated smooth-muscle preparations,[16] it would seem that the amount of methionyl-lysyl-bradykinin released by plasmin from this substrate is much higher than the amount of bradykinin that trypsin is able to release from it. This observation, at least to some extent, puts in doubt whether the use of the method of Diniz and Carvalho[1] gives the right interpretation of the reserves of kininogen present in plasma. It might well be that the parallelism observed previously for the amount of "kinin" released by trypsin and plasmin from crude horse plasma substrates[2] is also found in various physiological and pathological conditions.

REFERENCES

1. C. R. DINIZ and I. F. CARVALHO, *Ann. N.Y. Acad. Sci.* **104**, 77 (1963).
2. P. BUDNITSKAYA, E. GAPANHUK and O. B. HENRIQUES, In press.
3. O. B. HENRIQUES, E. GAPANHUK, N. KAURITCHEVA and P. BUDNITSKAYA, *Biochem. Pharmac.*, **18** 1788, (1969).
4. L. M. GREENBAUM, K. YAMAFUJI and T. HOSODA, *Biochem. Pharmac.* **14**, 411 (1965).
5. M. E. BROWN, *J. Lab. Clin. Med.* **55**, 616 (1960).
6. O. B. HENRIQUES, A. A. C. LAVRAS, M. FICHMAN and Z. P. PICARELLI, *Biochem. Pharmac.* **15**, 31 (1966).
7. K. C. ROBBINS and L. SUMMARIA, *J. biol. Chem.* **238**, 952 (1963).
8. E. GAPANHUK and O. B. HENRIQUES, *J. Chromatog.* **32**, 782 (1968).
9. S. O. ANDRADE and M. ROCHA E SILVA, *Biochem. J.* **64**, 701 (1956).
10. D. F. ELLIOTT, G. P. LEWIS and E. W. HORTON, *Biochem. J.* **76**, 16P (1960).
11. M. E. WEBSTER and J. V. PIERCE, *Ann. N.Y. Acad. Sci.* **104**, 91 (1963).
12. E HABERMANN and G. BLENNEMANN, *Arch. exp. Path. Pharmak.* **249**, 357 (1964).
13. E. G. ERDOS, A. G. RENFREW, E. M. SLOANE and J. R. WOHLER, *Ann. N.Y. Acad. Sci.* **104**, 222 (1963).
14. H. ZUBER and R. JAQUES, *Helv. Chim. Acta.* **43**, 1128 (1960).
15. U. HAMBERG, F. M. BUMPUS and I. H. PAGE, *Biochim. biophys. Acta* **52**, 533 (1961).
16. D. F. ELLIOTT and G. P. LEWIS, *Biochem. J.* **95**, 437 (1965).
17. L. R. CHRISTENSEN and C. M. MACLEOD, *J. Gen. Physiol.* **28**, 559 (1945).