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PLASMA ENZYMES THAT RELEASE KININS

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Abstract—The present work deals with a further characterization of the kinin liberation process in plasma. Purified plasma kallikrein, plasmin, and kininogenic substrates were prepared from human or equine plasma, and some of their properties were compared. Plasmin and kallikrein were shown to liberate kinins from horse plasma heated for 3 hr at 56°, a kallikreinogen-free substrate; "acid-treated" plasma or purified kininogen was used as substrate only by plasmin. Experiments with these last substrates allowed the conclusion that plasmin by itself has the capacity to release kinins. Hydrolytic actions of kallikrein and plasmin on *p*-toluene sulfonyl-L-arginine methyl ester (TAMe), benzoyl-L-arginine ethyl ester (BAEE), lysine ethyl ester (LEE), and benzoyl-L-arginine-amide (BAA) showed that plasmin hydrolyzes TAMe, BAEE, and LEE more effectively than does plasma kallikrein. BAA was not hydrolyzed by human or equine plasmin, but it was split by plasma kallikrein. Plasma kallikrein did not hydrolyze casein. A new method of plasma kallikrein purification was developed.

THE liberation of kinins by plasma enzymes has been attributed to the action of kallikrein as well as of plasmin on plasma globulins. The kinin-releasing activity of plasmin was first observed by Beraldo[1] and was also described by Rocha e Silva,[2] Schachter,[3] and Lewis,[4] but no evidence showing that this activity was an inherent property of the enzyme was presented. Actually, Schachter has admitted that dilution of serum only activates plasmin, which in turn would release kallikrein; Bhoola *et al.*[5] have further demonstrated that plasmin failed to release a smooth-muscle stimulant from plasma under conditions in which serum kallikrein was shown to be very effective. Nevertheless, a rapid release of kinin by human plasmin has been described by Eisen and Keele[6] and attributed by them[7] to a contamination with Hageman factor, a blood component which, besides its role in coagulation, has been related to the kallikreinogen-activating process by Margolis[8] and Webster and Ratnoff.[9]

In an attempt to characterize human plasmin and plasma kallikrein, Webster and Pierce[10] have verified the influence of various proteolytic inhibitors on these enzymes; although they behaved similarly with respect to their inhibition, there were differences in the ratio of such inhibition.

Recently Schachter[11] has reported that in his own experience serum kallikrein has been far more effective than plasmin in liberating kinins. He has not excluded, however, the existence of an indirect release of kinin by plasmin, a possibility suggested by Vogt[12] as a result of his observation that human plasmin liberates a kinin from normal dog plasma globulin, but not from globulin heated at 56° or from pure bovine kininogen. Indeed, these data supported the assumption that plasmin acted through an activating process of kallikreinogen.

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Most of the experiments mentioned previously were performed with crude enzymes and substrates obtained from various animal species. The present work was done with purified enzymes and substrates, from human or equine sources, in an attempt to clarify this subject.

MATERIAL AND METHODS

Preparation of enzymes

Human kallikrein. This was prepared as follows according to Lavras *et al.*[13] Human Horton's substrate[14] was submitted to ammonium sulfate fractionation, after neutralization with 1 M $NaHCO_3$; the fraction obtained between 0·35 and 0·05 saturation was further purified by chromatography on a DEAE-cellulose column,[15] a process leading to the separation of the enzyme from its substrate. The enzyme was eluted with the establishment of a gradient by introducing 0·05 M NaH_2PO_4–0·1 M NaCl into 0·005 M sodium phosphate buffer, pH 7·0. Its elution preceded that of the substrate.

Horse kallikrein. Horse blood collected over potassium oxalate (3 g/liter blood) was immediately centrifuged at 0–5° to separate the plasma. After activation of the plasma kinin-releasing enzyme by acid treatment,[15] kininase was removed in conditions similar to those used by Hamberg;[16] after addition of 0·9% NaCl to plasma (0·25 ml/ml) the pH was adjusted to 2 with 2 M HCl and the mixture incubated in a water bath until it reached 80°, then rapidly heated to 90°. This material was dialyzed against 0·9% NaCl until the pH rose to 3·5; it was then neutralized to pH 7 with 2 M NaOH. The resultant "treated plasma" was lyophilized for storage or immediately submitted to two consecutive purifications by chromatography on diethylaminoethyl (DEAE)-cellulose as described by Henriques *et al.*[15] The gradient was established by adding 0·02 M phosphate (NaH_2PO_4–Na_2HPO_4) buffer, pH 6·5, into a chamber containing 0·005 M phosphate (NaH_2PO_4–Na_2HPO_4) buffer, pH 6·5. The elution pattern was followed by reading the u.v. absorption at 280 mμ. In the fractions corresponding to the peaks, protein determinations were carried out by the method of Lowry *et al.*[17]

Plasmin. Horse plasminogen was prepared by the method of Ronwin[18] and activated by urokinase according to Robbins and Summaria.[19] The crude plasmin obtained, as well as *Actase**, was purified by chromatography on DEAE-cellulose in the same conditions employed by Derechin *et al.*[20] for the purification of plasminogen. Elution and estimation of protein were followed by the same process used for kallikrein preparation.

Preparation of substrates

Untreated horse plasma. Plasma was obtained from horse blood collected over potassium oxalate (3 g/liter blood); after separation by centrifugation at 0–5° it was dialyzed against 0·9% NaCl and immediately used. The whole process was carried out in siliconized glassware.

Human and equine Werle's substrates (HW and EW). Kallikrein-and kallikreinogen-free substrates were obtained according to Werle *et al.*[21] by heating human or equine plasma at 56° for 3 hr and dialyzing against 0·9% NaCl; they were stored at −10°. Residual kininase activity was inhibited by incubation with 3×10^{-3} M EDTA[22] immediately before using.

Human and equine "acid-treated" substrates. Werle's substrates were treated according

* Johnson & Johnson; registered trade name for a preparation of human plasmin.

to the method used by Diniz and co-workers[23] for the estimation of bradykininogen.

Equine kininogen. The method of Henriques *et al.*[15] was used, starting from EW instead of from fresh horse plasma; a better purification of equine kininogen was obtained, since the yield increased by 100% and the purification was 1·33 times greater.

Kinin-releasing activity

The kinin liberated by the plasma enzymes from the substrates was assayed on the isolated guinea pig ileum.[15] The incubation was conducted either directly in the organ bath or, for incubations longer than 3 min, in test tubes; the reaction was then interrupted by 10-min boiling after lowering the pH to 5·5 with 1 M HCl. This mixture, neutralized with 1 M NaOH, was centrifuged, and aliquots of the supernatant were assayed. The relative potency of the samples was calculated from the responses to single and double doses[24] of both synthetic bradykinin and aliquots.

Dog blood pressure

Mongrel dogs anesthetized with morphine (10 mg/kg) plus Somnifene† (0·2 ml/kg) and injected with atropine sulfate (2 mg) and promethazine (50 mg) had their blood pressure recorded from the carotid artery. The blood pressure variation produced by the assay preparation was compared with that produced by pancreatic kallikrein (Padutin, 13·4 Frey units/mg).

Hydrolytic activity

This was determined on various substrates by different methods: on casein,[25] benzoyl-L-arginine amide (BAA),[26] lysine ethyl ester (LEE), *p*-toluene sulfonyl-L-arginine methyl ester (TAMe), and benzoyl-L-arginine ethyl ester (BAEE).[27]

RESULTS AND DISCUSSION

Purification of plasma kallikrein. The plasma substrate for the bradykinin-releasing enzyme from the venom of *Bothrops jararaca*, which precipitates with $(NH_4)_2SO_4$ (between 0·33 and 0·500 saturation is strongly contaminated with kininase and kinin-forming activity;[15] an attempt to purify this last activity of plasma has already been reported.[13] However, the method described in the present work for horse kallikrein gives betterpurification and yield. After adsorption of kininase-free "treated plasma" on a DEAE-cellulose column, the kinin-releasing activity was eluted between 0·005 M

TABLE 1. KININ-RELEASING ACTIVITY OF SAMPLES OBTAINED IN THE PURIFICATION OF HORSE PLASMA KALLIKREIN

Step	Procedure	Bradykinin released (μg/mg sample protein)*	Protein yield (%)
1	Separation of fresh plasma	0·014	100
2	Activation by acid treatment	0·084	19
3	Chromatography and rechromatography	1·54	3·8

* Kinin released by 30-min incubation at 37° with EW was assayed on isolated guinea pig ileum.

† Commercial name registered by Roche Laboratories for a mixture of diethyl- and alyl-isopropil-barbituric acid diethylamides.

and 0·02 M phosphate (NaH_2PO_4–Na_2HPO_4) buffer, pH 6·5, and the bulk of protein remained adsorbed. Rechromatography of the most active fractions gave a 3·8% final protein yield, representing a 110-fold purification (Table 1).

This purified protein, besides its rapid kinin-releasing activity, produced hypotension in the dog, which characterized it as plasma kallikrein.[28, 29]

Purification of plasmin. The purification of crude horse plasmin by chromatography on a DEAE-cellulose column was followed by the determination of its caseinolytic

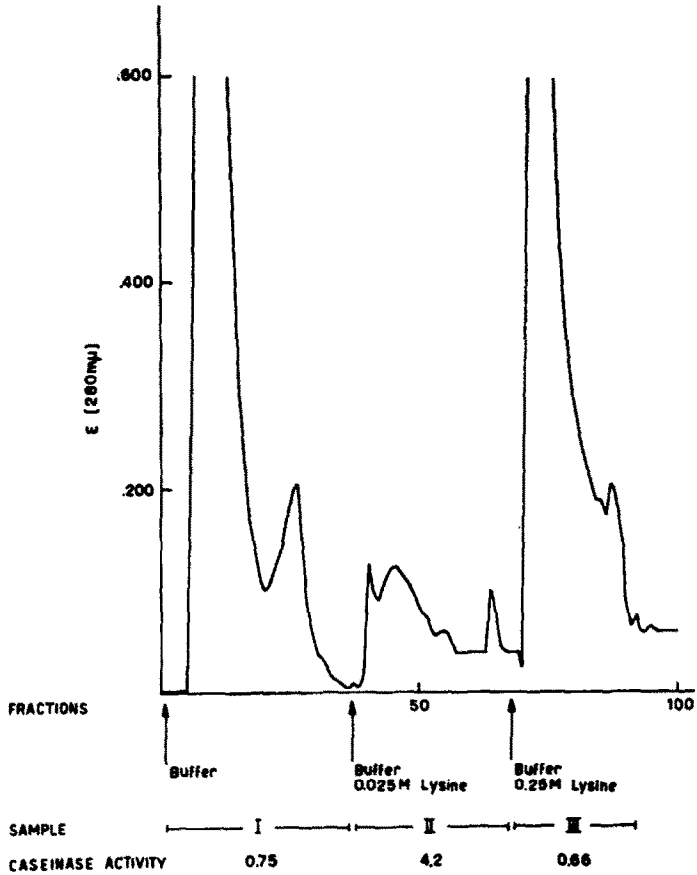

FIG. 1. Chromatography on DEAE-cellulose column of equine plasmin (caseinase activity 1·05) in 0·02 M ammonium acetate buffer, pH 9. Caseinase specific activity is expressed as the increase (multiplied by 10) in optical density at 280 mμ, of perchloric acid filtrate, per milligram protein in the sample.

activity. By elution with 0·02 M ammonium acetate buffer, pH 9, containing 0·025 M lysine, a protein peak, four times more caseinolytic than the starting material, was obtained (Fig. 1). This purified plasmin released kinin.

Material from peak I was 1·5 times more potent as a kinin-releaser than crude plasmin. Since its caseinolytic activity was lower than the initial material, it could be considered as a kinin-releasing enzyme contamination in the crude plasmin.

On the other hand, submitting to the same chromatographic process the commercial human plasmin *Actase*, having initial caseinase activity of the same degree as the purified equine plasmin, a further purification was obtained. The peak of protein eluted with 0·02 M ammonium acetate buffer, pH 9, containing 0·25 M lysine, was almost twice as caseinolytic as the Actase preparation (Fig. 2) and showed an increase in its kinin-releasing activity (Fig. 3). The purification of both activities indicates that plasmin itself has the capacity to liberate kinin from equine acid-treated substrate.

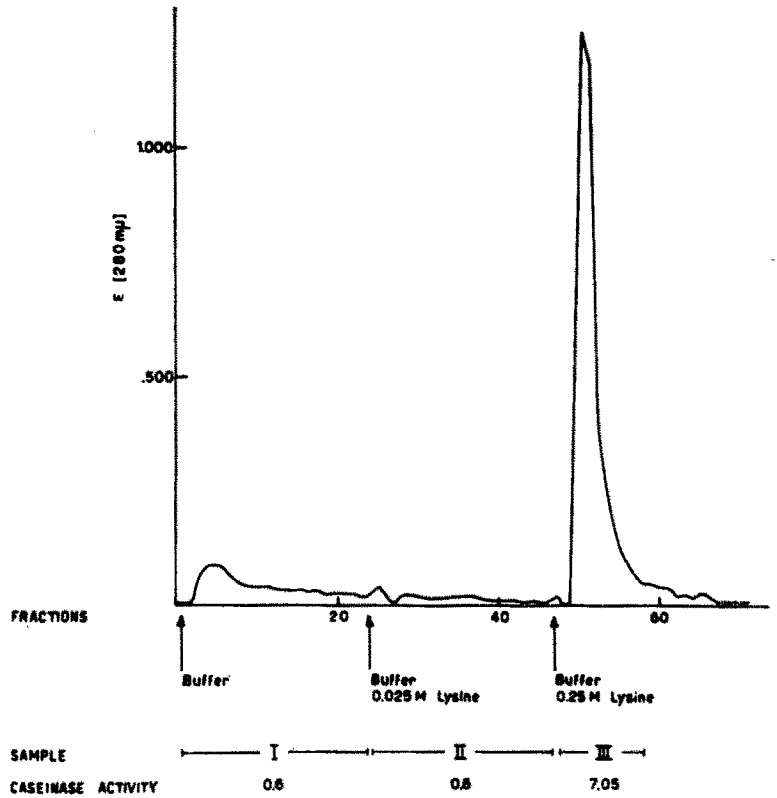

FIG. 2. Chromatography on DEAE-cellulose column of human plasmin (Actase) (caseinase activity 3·99). Same conditions as in Fig. 1.

Comparative properties of purified plasma kallikrein and plasmin. Before starting the comparative experiments on kinin-releasing activities of human and equine plasmin and kallikrein, the enzyme specificity for substrates of human or horse origin was investigated. In contrast to human and equine plasmin and human kallikrein which can liberate kinin from HW or EW, equine kallikrein attacks only EW. This was therefore the common substrate for kallikrein and plasmin, whatever their origin.

According to Werle *et al.*[21, 30] kallikreinogen is destroyed by heating plasma at 56° for 3 or even 1 hr. This was confirmed for EW by the fact that after its acidification

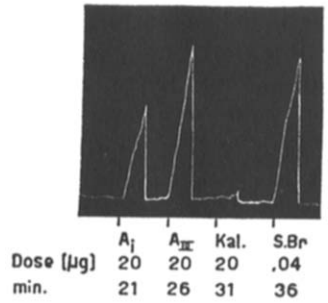

FIG. 3. Kinin-releasing activity of human plasmin (Actase) before (A_I) and after (A_{III}) chromatography on DEAE-cellulose column, assayed on isolated guinea pig ileum. Human plasmin (A_I or A_{III}) and horse kallikrein (Kal) were incubated with equine acid-treated substrates for 30 min at 37°, the reaction was interrupted, and aliquots of the incubation mixtures, corresponding to 20 µg enzyme protein were tested. Blanks for enzymes and substrates showed no kinin liberation (SBr, synthetic bradykinin).

by the method of Horton,[14] no kinin-releasing activity was present. In parallel experiments conducted with untreated plasma as controls, Horton's activation method permitted detection of kallikreinogen (Fig. 4). The absence of kallikreinogen in EW was also confirmed by the dilution method[31] by heating EW diluted with Tyrode's solution for 30 min at 37°. Moreover, no kinin liberation by this process was detected.

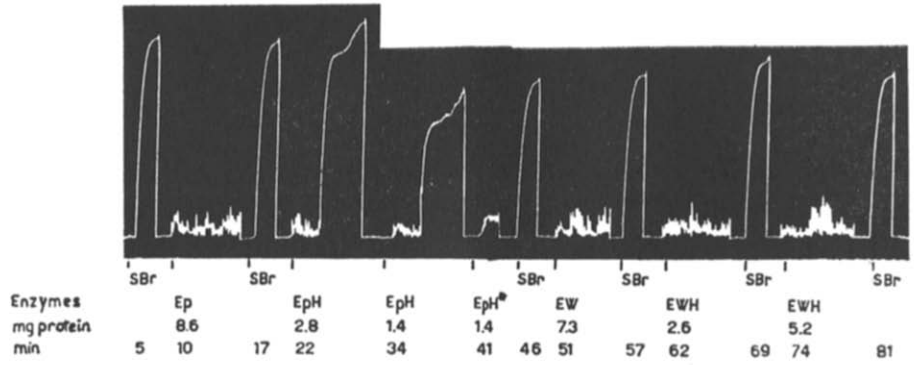

FIG. 4. Demonstration of lack of kallikreinogen in EW by Horton's activation process. Kinin-releasing activity, with EW (22 mg protein) as substrate, assayed in the isolated guinea pig ileum. EW after acidification (EWH) revealed no kinin-releasing activity while untreated plasma (Ep) activated by the same method (EpH), used as control, showed kallikrein activity as a result of the presence of kallikreinogen in Ep. SBr, 0·02 µg synthetic bradykinin. EpH* was assayed without substrate as control.

As may be seen in Fig. 5, Actase and horse plasma kallikrein were able to liberate a kinin from EW. Equine acid-treated substrate, known as a poor substrate for plasma kallikrein,[32] proved to be better for plasmin. Purified plasma kallikrein, in the same doses as plasmin, was actually unable to liberate a kinin from equine acid-treated substrate (Fig. 3). This suggests that a denaturation occurred during

the vigorous treatment necessary to obtain this substrate. The specific enzyme (plasma kallikrein) no longer reacted with it, but the less specific enzyme plasmin was still capable of releasing the polypeptide.

In the purification procedure of equine kininogen, the activities of plasmin and plasma kallikrein were assayed in every step of the preparation. Untreated plasma, EW, EW treated as recommended by Horton,[14] and the fraction precipitated between

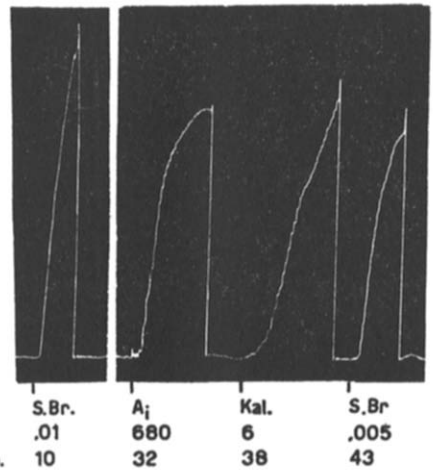

FIG. 5. Kinin-releasing activity of human plasmin (Actase,A_1) and horse plasma kallikrein (Kal) on EW; 3-min incubation of each plasma enzyme and substrate directly in the guinea pig ileum bath. Enzymes or substrate had no effect on the gut by themselves.

0·35 and 0·50 saturation with ammonium sulfate were found to be substrates for both enzymes. However, after chromatography of this last material, the equine kininogen, fractions eluted with phosphate buffer (NaH_2PO_4, 0·1 M–NaCl, 0·2 M), were found to be better substrates for trypsin than for plasmin, but they were not substrates for plasma kallikrein (Fig. 6). They were followed by other fractions from which plasma kallikrein as well as plasmin released the polypeptide, which is taken to indicate that, even if some denaturation occurred, it did not affect the kinin release.

In Fig. 7, equine kininogen was shown to be a suitable substrate for horse urinary kallikrein, having specific activity of 169,[33] suggesting further that if any denaturation occurred during the purification procedure, it did not interfere with the kinin release obtained with horse urinary kallikrein, a more specific enzyme than trypsin and plasmin. Thus, we conclude that in horse plasma there are two substrates for kinin-releasing enzymes, one of which is not attacked by plasma kallikrein.

Since (1) plasmin releases kinin from horse plasma treated according to Werle's procedure, a kallikreinogen-free substrate, and (2) plasmin forms kinins from purified horse kininogen whereas horse plasma kallikrein does not, we may conclude that plasmin itself has the ability to release kinin, as we suggested previously from the results obtained with plasmin of different degrees of purification. However, the fact that, a smaller concentration of human or horse plasmin is necessary to liberate a kinin from untreated equine plasma than from EW (Fig. 8) indicates the existence

of a concomitant indirect mechanism of action through the activation of kallikreinogen, as postulated by Vogt.[12]

These data contradict Vogt's conclusion that plasmin is able to form plasma kinin only by this indirect mechanism.

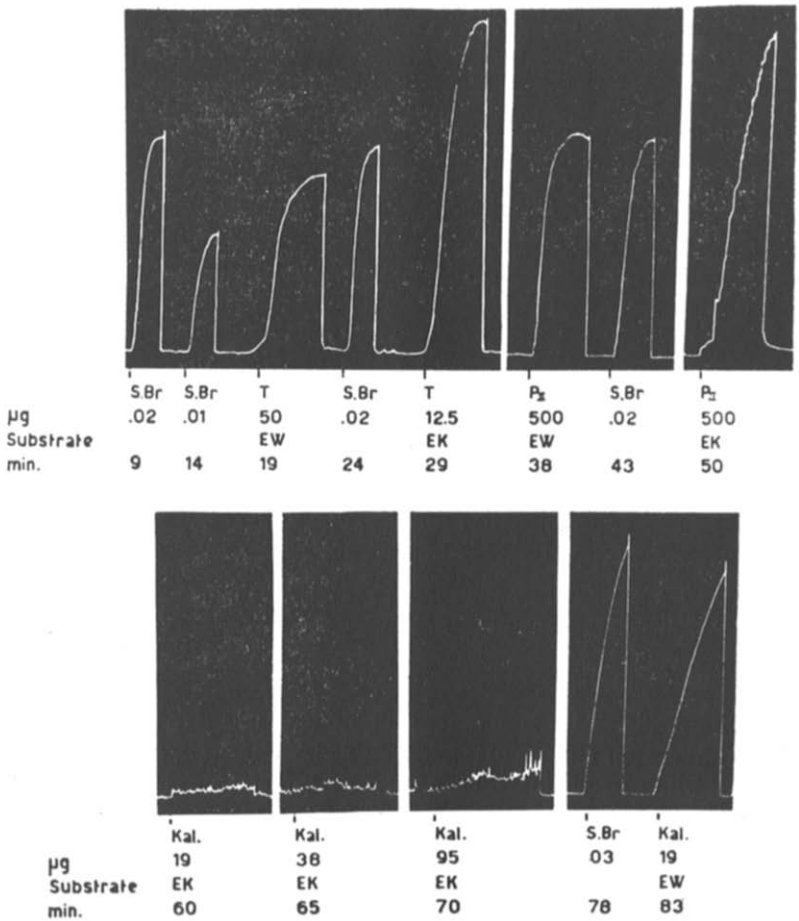

FIG. 6. Liberation of kinin by trypsin (T), purified equine plasmin (P$_{II}$), or horse kallikrein (Kal) from EW (22 mg protein) or EK (126 µg protein), directly in the ileum bath. It is noteworthy that kallikrein did not act on EK, even in a dose 5 times higher than that effective on EW.

TABLE 2. ESTEROLYTIC ACTIVITIES* OF HUMAN PLASMIN AND HORSE PLASMA KALLIKREIN

Enzyme	Substrate		
	LEE	TAMe	BAEE
Human plasmin	0·55	0·69	0·75
Horse plasma kallikrein	0·07	0·19	0·28

* Esterolytic activity expressed in micromoles of hydrolyzed substrate per minute and per milligram of enzyme protein.

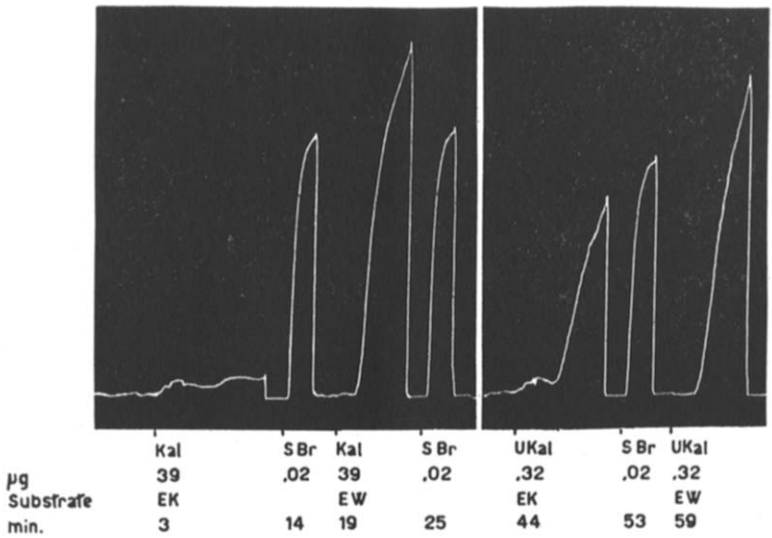

FIG. 7. Liberation of kinin by horse plasma kallikrein (Kal) and urinary kallikrein (UKal) from EK (100 µg protein) and from EW (22 mg protein) directly in the ileum bath. UKal acted on EK substrate not attacked by Kal.

In addition to the kinin-forming activity of the plasma enzymes, their esterolytic activity on LEE, TAMe and BAEE were also compared; plasmin showed a higher hydrolytic activity (Table 2) on the three substrates.

These enzymes showed a different action on BAA, since this synthetic substrate

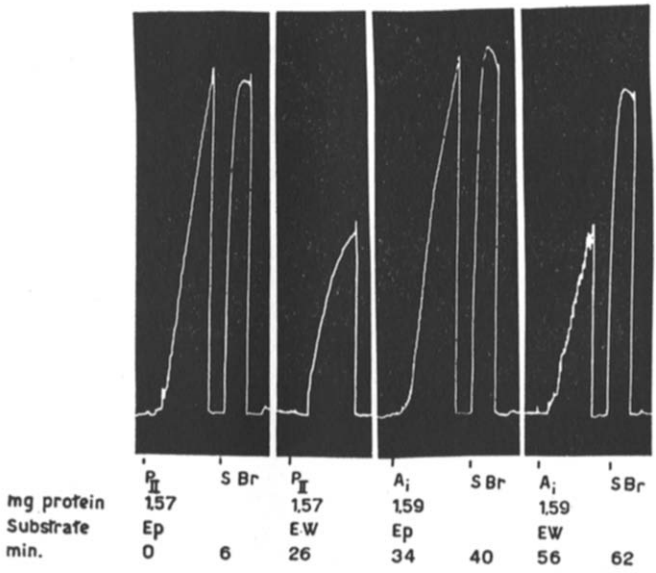

FIG. 8. Kinin-releasing activity of human (A_1) and horse (P_{II}) plasmin on equine untreated plasma Ep (26 mg protein) and on EW (22 mg protein). The amount of kinin released by A_1 or P_{II} from Ep is greater than that liberated from EW. Assay directly in the ileum bath. SBr, 0·05 µg synthetic bradykinin.

was not hydrolyzed by human or horse plasmin in doses up to 2 mg, whereas hydrolysis was effected by plasma kallikrein in an 8-fold lower concentration. On casein, however, this last enzyme, even when used in doses as high as 2 mg, showed no hydrolytic effect; these experiments were controlled by carrying out parallel casein hydrolysis by plasmin and trypsin.

Acknowledgements—Synthetic bradykinin was obtained through the courtesy of Dr. E. D. Nicolaides from Parke Davis & Co.; many thanks are due to Dr. Francisco Antonacio, of Colsan, for the supply human plasma. Padutin and horse urinary kallikrein were kindly supplied by Drs. J. L. Prado and Eline S. Prado from the Laboratórios de Farmacologia e Bioquímica, Escola Paulista de Medicina; we are also grateful to them for helpful criticism during the writing of this paper.

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