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AN IMPROVED METHOD FOR DETERMINATION OF THE TOTAL KININOGEN IN RABBIT AND HUMAN PLASMA

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Abstract—The method described by Diniz and Carvalho has been widely used for determination of the total kininogen (KGN) in plasma, but this method is also reported to produce bradykinin (BK) potentiators besides BK after incubation of the pretreated plasma with trypsin. The present experiments aim to establish a method to induce the full conversion of KGN into BK without potentiators and kininase. The pretreatment of plasma with heat (98°) and acetic acid (pH 3.5) used in Diniz's method was compared with five other pretreatments, namely non-treated, two ways of heating (at 98 or 60°) and two ways of acidification (at pH 3.5 or 2.0). The pretreated plasmas were incubated with trypsin in 0.2 M Tris buffer, pH 7.8. BK released was assayed on the isolated rat uterus. The full conversion of KGN into BK by trypsin was tested, comparing it with the amounts of BK released by highly purified hog pancreas kallikrein and highly purified snake venom kininogenase. Trypsin (200 μg) and 30 min of the incubation were sufficient to convert KGN into BK up to plateau level in non-treated and treated plasmas. The results indicated that the pretreatments of Diniz and 98° produced BK potentiators, and the non-treated and the pH 3.5-treated plasmas showed lesser amounts of BK formed, even in the presence of *o*-phenanthroline. The pretreatments of pH 2.0 and 60° produced no potentiators and caused the full conversion of KGN into BK, but the latter treatment did not inactivate kininase in rabbit plasma. Thus, it is concluded that the pretreatment of plasma with HCl (pH 2.0) was most suitable for the determination of total KGN in rabbit plasma. For human plasma, pretreatment by heating (60°) was also suitable, besides the pH 2.0 treatment.

Kininogen in plasma is a precursor of bradykinin, and the changes of the levels have been used as a good indicator for the involvement of the kallikrein–kinin system in a variety of pathological conditions, since free kinin in body fluids is short-lived and difficult to detect.

Kininogen is always determined as bradykinin after conversion into bradykinin by certain kininogenases, such as trypsin and glandular kallikreins. The method described by Diniz and Carvalho [1] has been used widely for the determination of the total kininogen in plasma, as trypsin is commercially available in a relatively pure form.

It has been pointed out, however, that the pretreatment of plasma with heat and acetic acid, which is used in this method, produces bradykinin potentiators besides bradykinin, after incubation of the pretreated plasma with trypsin [2–4]. Thus, kininogen levels in plasma determined by this method were higher than those determined by other methods [5–9], and the real values remained unknown because of the lack of a reliable determination method.

A method for determining the total kininogen in plasma should satisfy the following three points: (1) the full conversion of kininogen into bradykinin, (2) inactivation of kininase, and (3) no production of bradykinin potentiators.

The present work attempts an improvement of Diniz and Carvalho's method, and the results indicate the establishment of a method satisfy the three points mentioned above, using trypsin.

A part of the results was presented at the International Symposium on Inflammation and Anti-inflammatory therapy (Kinin '76) held at Rio de Janeiro, Brazil, November 1976 [10].

MATERIALS AND METHODS

Blood collection

Albino male rabbits (body weight 2.5 to 3.5 kg) were anaesthetized slightly with ether, and a polyethylene cannula (Hibiki No. 7) was inserted into the carotid artery. Blood was collected in 50-ml plastic tubes containing heparin (Sigma Co., St. Louis, MO.) (2 units (U.S.P. J-A.)/ml) by flowing out freely. Collection was stopped before spontaneous respiration ceased. Blood was immediately centrifuged at 25° at 1000 *g* for 15 min. Plasma was gently removed with a plastic pipette, and every 2 ml was distributed into small plastic tubes within 1 hr. Plasmas were kept at −20° until used. Since the kininogen levels in frozen plasma were not different from those in fresh plasma, the frozen plasmas were used for the establishment of the method. Plasma was pretreated within 20 min after thawing.

Human blood was collected from the cubital vein of healthy subjects (two males and one female) with a disposable plastic syringe and needle into 50-ml plastic tubes containing heparin (2 units/ml). Plasma was prepared in the same manner as was rabbit plasma.

Polypropylene tubes and vessels were used during

Table 1. Effect of temperature after collection of blood on the kininogen level (μg BK/ml of plasma) in plasma*

Plasma	No.	Temperature after collection kept for 3 hr		
		0°	Room temp. (22–5°)	37°
Rabbit	1	3.20	3.20	3.23
	2	2.70	2.90	2.72
	3	2.90	2.95	2.93
Human	1	4.02	4.09	4.08
	2	4.73	4.92	4.90
	3	5.23	5.02	5.22

* Comparisons at different temperatures were made with the same plasma. Three different experiments for rabbit and human plasma are shown.

this preparation and all contact with glass or negatively charged surfaces was carefully avoided during the whole procedure.

Temperature after collection of blood

The different temperatures used to keep blood after the collection were compared in rabbit and human plasma. Blood which was kept in the bath at 37°, at room temperature (22°), and in ice (0°) was centrifuged at 1000 *g* at fixed temperatures of 37, 25 and 0° respectively. Then the plasmas were pretreated at pH 2.0 as mentioned in Results, and the kininogen levels were determined. The time from collection to pretreatment was approximately 3 hr.

As shown in Table 1, the kininogen levels at the three temperatures were not significantly different from each other. Therefore, in the following experiments, blood was kept at room temperature and centrifuged at 25°.

Bioassay of kinin

Isolated rat uterus preparations were used for assay of kinin. Virgin rats weighing 120–200 g were injected i.p. and s.c. with 5 mg hexestrol (Hexron, Teikoku Zoki, Tokyo 12–24 hr before assay. One horn of the estrous rat uterus was suspended at 28° in a 10-ml organ bath filled with Munsick solution gassed with 95% O_2 + 5% CO_2. Methysergide (10^{-7} g/ml, Sandoz, Basel) was used on some occasions. The contractions of the uterus were recorded using an isotonic transducer (ME Commercials Ltd., Tokyo), connected to a pen recorder (Rikadenki Kogyo Ltd., Tokyo). Synthetic bradykinin (Protein Research Foundation, Minoh, Osaka) was used as the standard. The contact time for each sample was 90 sec, and the interval between tests was 5 min. The Munsick solution contained 114.0 mM NaCl, 6.2 mM KCl, 0.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 30.0 mM $NaHCO_3$ and 0.5 g glucose in 0.01 liter distilled water [11].

For recovery experiments of bradykinin throughout the procedure, 0.5 μg bradykinin (BK) was added in the presence of *o*-phenanthroline (0.4 mg) (1) to Tris buffer (1.75 ml), (2) to plasma (0.2 ml) + Tris buffer (1.55 ml) and (3) to trypsin (0.2 mg) + Tris buffer (1.65 ml). The mixture was incubated for 30 min at 37° and further procedure followed Diniz's method. The recoveries obtained were (1) 101.3 and 93.9 per cent, (2) 100.8 and 99.2 per cent and (3) 98.0 and 96.7 per cent.

Conversion of kallidin to bradykinin

The conversion of kallidin to bradykinin was tested by bioassay using isolated guinea pig ileum, which was suspended in 10 ml of organ bath filled with Tyrode solution gassed with 95% O_2 + 5% CO_2. Kallidin (Protein Research Foundation, Minoh, Osaka) (0.5 μg) in Tris buffer was incubated with 0.2 ml plasma in the presence of *o*-phenanthroline (0.4 mg). Since the response of the ileum to kallidin was approximately one-half that of bradykinin at the same dose, the conversion was tested by reaching the maximal response.

Kininase activity

Bradykinin (1.5 μg) was incubated with 0.2 ml of treated or non-treated plasma in 0.2 M Tris buffer (pH 7.8) in the presence or the absence of *o*-phenanthroline (0.4 mg). The total volume of the incubation mixtures was 3.0 ml. The amount of residual bradykinin was tested every 5 min on the rat uterus.

Agents

Trypsin was a product of National Biochemical Corporation (twice crystallized, salt free, bovine pancreas). The doses used in this experiment did not show any significant kininase activity. Highly purified hog pancreas kallikrein was a gift of Dr. C. Kuzbach (Bayer AG, Germany). The activity was 1290 kallikrein units (K.U.)/mg. Highly purified snake venom kininogenase was a gift of Dr. H. Kato and Dr. S. Iwanaga (Protein Research Institute, Osaka University, Osaka). This kininogenase was purified from venom of the snake, *Agkistrodon halys blomhoffii*, and showed the esterase activity of 10 TAME units/mg of protein (one unit equals the activity when 1 μmole TAME was esterified for 1 min, when incubated with 10 μmoles TAME at 36.5°, at pH 8.5 [12]). This kininogenase was reported to release bradykinin from both high molecular weight (HMW) and low molecular weight (LMW) kininogen of ox [12, 13]. The following substances were also used: *o*-phenanthroline (Nihon Rikagaku Yakuhin Co. Ltd., Tokyo), 1 N HCl

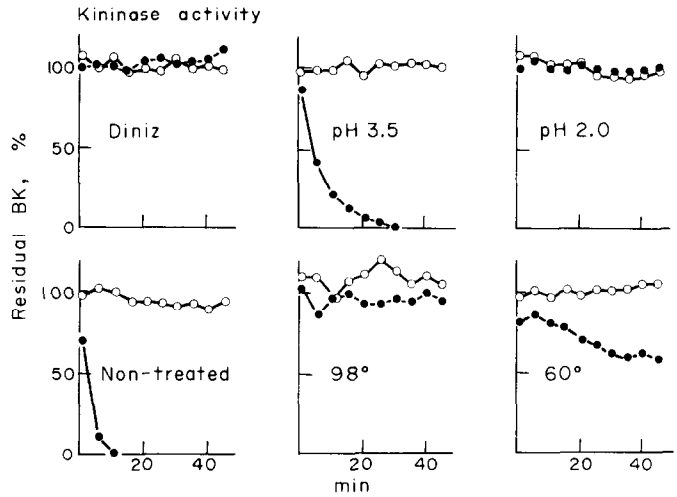

Fig. 1. Kininase activity of rabbit plasmas pretreated differently. Ordinate shows the amounts of residual bradykinin after incubation with 0.2 ml of rabbit plasma, expressed as per cent of bradykinin added at zero time. Abscissa indicates the time of incubation (min). Bradykinin (BK) (1.5 μg) was incubated with the pretreated plasma (0.2 ml) in the presence (○——○) or the absence (●——●) of *o*-phenanthroline (0.4 mg).

and 1 N NaOH (Wako Chemicals, Tokyo), and Tris(hydroxymethyl)aminoethane (Sigma Co.).

Statistical tests

The Student's *t*-test was used to evaluate the significance of differences.

RESULTS

Rabbit plasma

Plasma pretreatments. In order to find a method for the full conversion of kininogens to bradykinin by trypsin without bradykinin potentiators and kininases, rabbit plasma was pretreated in different ways.

As shown in Table 2, in Diniz's method, 0.2 ml plasma was added to 1.8 ml of 0.2% acetic acid (pH 3.5) and boiled (98°) for 30 min. The pretreated plasma was then neutralized with 0.05 ml of 1 N NaOH (monitored with Toyo Roshi universal paper) and incubated with 200 μg trypsin in 0.2 M Tris buffer at pH 7.8 for 30 min. The incubation was terminated using warm ethanol and heating for 10 min at 70°. After evaporation of the supernatant, the dried residue was dissolved in 0.9% saline (the sample solution) and the bradykinin released was assayed using the rat uterus.

Table 2. Pretreatment of plasma

		Heated		Acidified	
Diniz	Non-treated	98°C	60°C	pH 3.5	pH 2.0
0.2 ml Plasma					
1.8 ml Acet. acid pH 3.5	1.8 ml H_2O	1.8 ml H_2O	1.8 ml H_2O	1.8 ml Acet. acid pH 3.5	1.8 ml 0.03 N HCl pH 2.0
98°C, 30′ Neutralized		98°C, 30′	60°C, 30′	37°C, 15′ Neutralized	37°C, 15′ Neutralized
⇓ Incubation { 0.2 M Tris buffer (pH 7.8) Trypsin					
⇓ ← 5 ml of absolute alcohol					
Evaporation					
⇓					
Bioassay					

This pretreatment was compared with five other pretreatments shown in Table 2: non-treatment, boiling (at 98°) or heating at 60° in distilled water for 30 min, and acidification by 0.2% acetic acid at pH 3.5 or by 0.03 N hydrochloride at pH 2.0, at 37° for 15 min. Acidified plasmas were neutralized with 1 N NaOH. These plasmas, pretreated in different ways, were incubated with trypsin, and the bradykinin released was assayed in the same manner as in Diniz's method. In the following discussions, these pretreatments are called non-treated, Diniz, 98°, 60°, pH 3.5, and pH 2.0 respectively.

Kininase activities. As non-treated plasma obviously showed potent kininase activity, the effects of the pretreatments on kininase activity were studied. The pretreatments of Diniz, pH 2.0 and 98° abolished the kininase activity in plasma, whereas the kininase activity still remained after the pretreatment at pH 3.5 and 60° in rabbit plasma (Fig. 1). Addition of 0.2 ml *o*-phenanthroline (2 mg/ml) to 0.2 ml plasma inhibited the kininase activity, as shown by open circles. In the following experiments, this amount of the kininase inhibitor was added to all samples irrespective of the pretreatments.

Full conversion of kininogen into bradykinin. In order to know whether trypsin induced the full conversion of kininogen into bradykinin, two other kininogenases, highly purified hog pancreas kallikrein and highly purified snake venom kininogenase, were used. The increased doses of both enzymes formed more bradykinin from rabbit kininogen, and the formation reached a plateau at 10 K.U. and 20

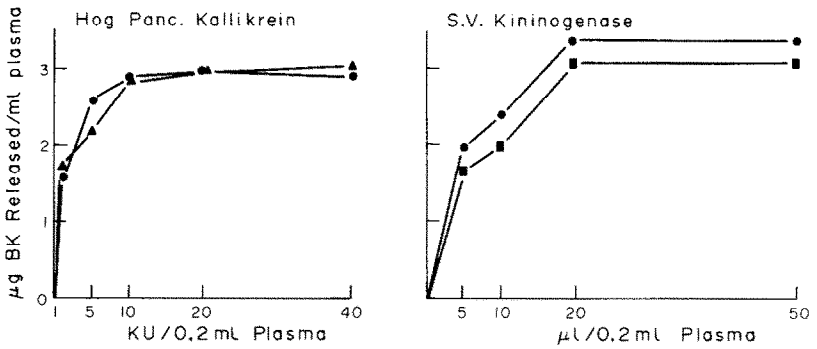

Fig. 2. Kinin formation from non-treated rabbit plasma by the highly purified hog pancreas kallikrein (HPK) and the highly purified snake venom kininogenase (SVK). Ordinate indicates the bradykinin (BK) amounts (μg) released/ml of plasma. Abscissa shows kallikrein units (K.U.) of HPK or μl of SVK solution added to 0.2 ml plasma in the incubation mixtures.

μl/0.2 ml of plasma for hog pancreas kallikrein and the snake venom kininogenase respectively (Fig. 2). Thus, 20 K.U./0.2 ml of plasma for hog pancreas kallikrein and 20 μl/0.2 ml of plasma for snake venom kininogenase were used as the amount of enzyme which converted the total kininogen into bradykinin.

An incubation time of 30 min was sufficient for full conversion by both enzymes.

Hog pancreas kallikrein is known to release kallidin instead of bradykinin, so the conversion to bradykinin by aminopeptidases in rabbit plasma was tested in isolated guinea pig ileum. Incubation of kallidin with 0.2 ml of rabbit plasma caused rapid conversion, which was completed within 15 min. Thus, 30 min of incubation time in the present experiments was sufficient.

Conditions for incubation with trypsin. The increased doses of trypsin were incubated with 0.2 ml of each plasma pretreated differently. A typical experiment is shown in Fig. 3. More than 200 μg trypsin produced the same amount of bradykinin after all pretreatments and the non-treatment. Therefore, 200 μg trypsin was used for the experiments, as in Diniz's method.

The incubation time with 200 μg trypsin was concluded to be 30 min in plasma for all treatments, because the bradykinin formation became maximal or reached a plateau at 30 min of incubation, as shown in Fig. 4. The pH for incubation was adjusted to pH 7.8 with 0.2 M Tris buffer, as in Diniz's method.

Bradykinin potentiators and the amount of bradykinin released from kininogen. The amounts of bradykinin (BK) equivalent/ml of plasma, respectively, and was constant even if the volume of the animals, as shown in Fig. 5. When the amounts of bradykinin released by the hog pancreas kallikrein or the snake venom kininogenase were calculated as the amount/1 ml of the original plasma, the calculated amount was 2.9 ± 0.1 and 3.0 ± 0.1 μg of bradykinin (BK) equivalent/ml of plasma, respectively, and was constant even if the volume of the sample solutions added to the organ bath was increased from 0.05 to 0.2 ml. On the other hand, the bradykinin levels/1 ml of plasma in the sample solution of Diniz were increased with the increased volumes of the sample solution added to the organ bath. The value after addition of 0.2 ml of the sample solution was significantly higher than that of

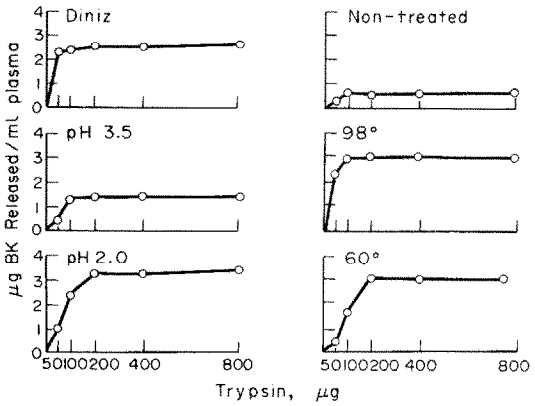

Fig. 3. Kinin formation from differently treated plasma by trypsin. Ordinate shows the amounts (μg) of bradykinin (BK) released/ml of plasma. Abscissa indicates the amounts of trypsin added to the incubation mixture. Experiments were performed with the same plasma.

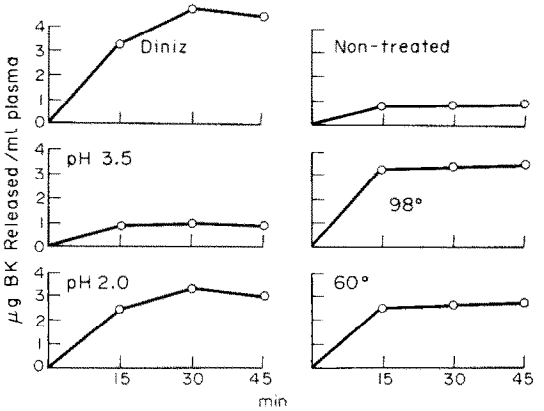

Fig. 4. Time course of kinin formation from differently pretreated rabbit plasma by trypsin. Ordinate shows the amounts (μg) of bradykinin (BK) released/ml of plasma. Abscissa indicates the time of incubation.

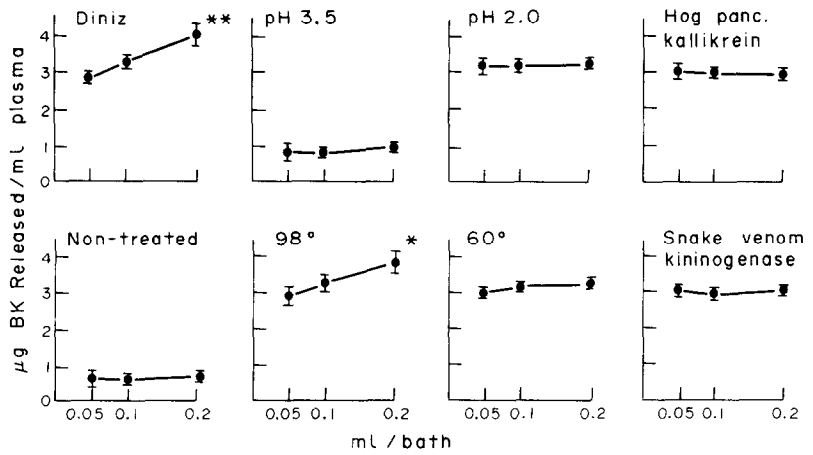

Fig. 5. Presence of bradykinin potentiators and the amounts of bradykinin released from differently pretreated rabbit plasma. Ordinate shows the amounts (μg) of bradykinin (BK) expressed as the amounts in terms of ml of the original plasma. Abscissa indicates the volumes of the sample solutions added to the organ bath. The comparisons were made with the same plasma. The values indicate the mean of nine plasmas from three animals with standard errors. The presence of potentiators was indicated by the fact that the apparent amount of bradykinin/ml of plasma with 0.2 ml of the sample solution from Diniz's plasma was significantly higher than that with 0.05 ml of the solution (**$P < 0.01$). The bradykinin amount with 0.2 ml of the sample solution from 98°-treated plasma was also significantly higher than that with 0.05 ml (*$P < 0.05$).

0.05 ml ($P < 0.01$). This indicated clearly the co-existence of bradykinin potentiators in the sample solution. The 98°-treated plasma showed the same upward tendency ($P < 0.05$). Thus, it was obvious that only boiling in Diniz's method caused the formation of potentiators.

The non-treated plasma and the pH 3.5-, pH 2.0- and 60°-treated plasma did not contain the bradykinin potentiators.

The amounts of bradykinin released from the pH 2.0-treated plasma (3.2 ± 0.1 μg BK/ml of plasma) and 60°-treated plasma (3.1 ± 0.1 μg BK/ml of plasma) were not different from those released by the hog pancreas kallikrein and by the snake venom kininogenase, whereas the amounts of bradykinin from the non-treated plasma (0.7 ± 0.1 μg BK/ml of plasma) and the pH 3.5-treated plasma (0.9 ± 0.1 μg BK/ml of plasma) were lower even in the presence of a sufficient amount of *o*-phenanthroline, a kininase inhibitor. From the results, the pretreatments of pH 2.0 and 60° were suitable for the present purpose. The latter pretreatment, however, did not

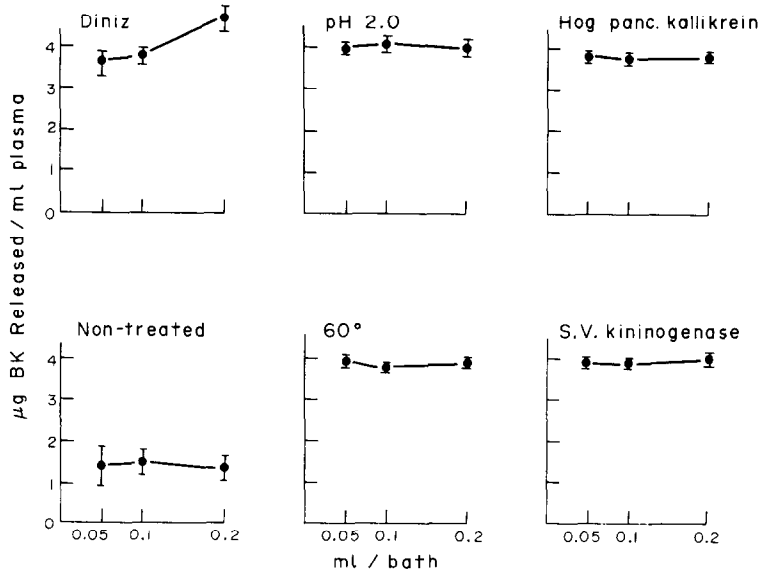

Fig. 6. Presence of bradykinin potentiators and the amounts of bradykinin released from differently pretreated human plasma. Ordinate shows the amounts (μg) of bradykinin (BK) expressed as the amounts in terms of 1 ml of the original plasma. Abscissa indicates the volumes of the sample solution added to the organ bath. The comparisons were made with the same plasma. The values indicate the mean of three plasmas from three subjects with standard errors. The sample solution of human plasma treated by Diniz also contained bradykinin potentiators.

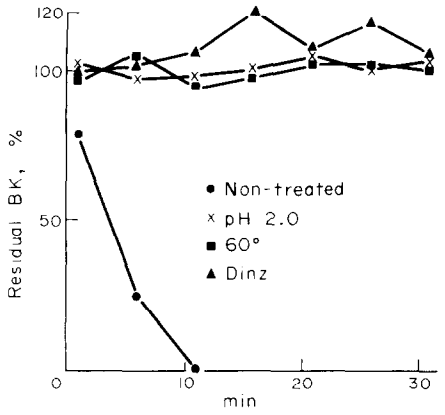

Fig. 7. Kininase activity of human plasma pretreated differently. Ordinate shows the amounts of residual bradykinin (BK) incubated with 0.2 ml of human plasma, expressed as per cent of bradykinin added at zero time. Abscissa indicates the time of incubation (min). The pretreatment of human plasma at 60° blocked the kininase activity. This differed from rabbit plasma.

inactivate kininase in rabbit plasma as shown in Fig. 1. Therefore, it could be concluded that the pretreatment of plasma at pH 2.0 was the best.

Human plasma

Human plasmas were pretreated in a manner similar to rabbit plasma. The non-treatment and the pretreatments of pH 2.0 and 60° were compared with Diniz's method.

Trypsin (200 μg) and 30 min of incubation time were also sufficient to convert the total kininogen into bradykinin in human plasma, as in rabbit plasma. In human plasma, 5 K.U./0.2 ml of plasma of the hog pancreas kallikrein and 1 μl/0.2 ml of plasma of the snake venom kininogenase released the maximal amount of bradykinin from human plasma. Thus, 20 K.U. and 20 μl for 0.2 ml plasma were used for the hog pancreas kallikrein and the snake venom kininogenase, respectively, as in rabbit plasma.

As shown in Fig. 6, the pretreatment by Diniz's method also produced bradykinin potentiators. The pretreatments of pH 2.0 and 60° did not produce the potentiators, and the incubation of the pretreated plasmas with trypsin resulted in sufficient conversion of the kininogen into bradykinin (4.0 ± 0.1 for pH 2.0 and 3.9 ± 0.1 μg BK/ml of plasma for 60°), in comparison with the levels of bradykinin released by the standard kininogenases (3.8 ± 0.1 for the hog pancreas kallikrein and 4.0 ± 0.1 μg BK/ml of plasma for the snake venom kininogenase).

Both pretreatments of pH 2.0 and 60° were valid in human plasma, because kininase in human plasma was inactivated by the pretreatment of 60°, as shown in Fig. 7.

DISCUSSION

The discovery of bradykinin was partly based on the fact that this peptide was released from plasma by the proteolytic action of trypsin [14]. Since then, the amount of kininogen, a precursor of bradykinin, usually has been determined by this kininogenase [1], as the determination of kininogen molecules by immunochemical assay has not succeeded yet. Moreover, the commercial availability of trypsin in a relatively pure form accelerated the use of Diniz's method. The inconsistency of kininogen level assessed by this method as compared with other methods was at least partly due to the co-existence of bradykinin potentiators.

The incubation of untreated plasma with a sufficient amount of trypsin did not result in the full conversion of kininogen into bradykinin, as shown in this experiment (Fig. 3). Thus, the denaturation of plasma, which induced the inactivation of kininase, the destruction of trypsin inhibitors, and probably molecular changes of kininogen, is definitely required to determine the kininogen level.

In this experiment, improvement was attempted by replacement of the pretreatment of plasma in boiling acetic acid (Diniz's method) with heating at 98 or 60°, or with acidification at pH 3.5 or 2.0.

The treatment of plasma at 60°C to destroy plasma kallikrein and Hageman factor was introduced by Margolis [15] and Eisen [16] and recommended by Webster for preparing substrate for kininogenases [17].

It was reported earlier by Werle [18] that acidification of plasma reduced the ability to inactivate kallikrein, and Gaddum [19], Horton [20] and Eisen [16] reported that kallikrein inhibitors and kininase in plasma were destroyed after treatment of plasma at pH 2.0. Recently α_2-macroglobulin has been reported to be destroyed by acidification [21].

The present experiments confirmed that the pretreatment of plasma at pH 2.0 destroyed kininase activity in plasma.

Eisen [16], Horton [20] and Jasani *et al.* [22] reported that the treatment of plasma at pH 2.0 released kinin after neutralization. A main kinin product from acid-treated plasma was reported to be methionyl-lysyl-bradykinin [23] and this might be due to the activation of pepsinogen in plasma [24]. This kinin release, however, does not seem to be critical for the method in the present study, because (1) the kinin release from acid-treated plasma was generally slow, compared with other activation, (2) methionyl-lysyl-bradykinin is easily converted to bradykinin by trypsin [23, 25, 26], and (3) if it does occur, this is part of the conversion of kininogen into bradykinin, and the total amount of bradykinin should not be changed if it was not destroyed by kininases. The conversion by trypsin was confirmed by us and was completed within 15–20 min with 4 μg methionyl-lysyl-bradykinin under the present experimental conditions. The non-treated rabbit and human plasmas also converted this kinin into bradykinin within 15–20 min.

Kininogen in undenatured plasma did not seem to be converted fully into bradykinin by trypsin (up to 800 μg) in the present experiment. This is in confirmation of another paper [27], but the reason is not known in the present study.

The present experiments also reveal that the pretreatment of plasma by boiling merely caused the release of bradykinin potentiators by trypsin in Diniz's method, and that acidification of plasma at pH 3.5 by acetic acid did not cause the full conversion of kininogen into bradykinin by trypsin.

In human plasma, pretreatment at 60° resulted in

the destruction of kininase. Thus, this pretreatment can be used in human plasma. This is in confirmation of a previous paper [7]. The same pretreatment at 60°, however, could not be used for rabbit plasma or other laboratory animals, because kininase was not destroyed. The claim that pretreatment of plasma at 60° could be used in the presence of plasma kininase inhibitors cannot be accepted, since high concentrations of kininase inhibitors such as *o*-phenanthroline are required, and their effect is not always reliable.

From the present results, it can be concluded that acidification of plasma at pH 2.0 by HCl is most suitable as pretreatment, and it results in the full conversion of kininogen into bradykinin without kininase and bradykinin potentiators, after incubation of the pretreated plasma with trypsin.

The amount of bradykinin released by trypsin in rabbit plasma (3.2 ± 0.1 μg of BK equivalent/ml of plasma) was not different from that released by the two highly purified kininogenases. This value is about one third as low as the mean value of 8.8 μg BK/ml of plasma (1 unit = 0.44 μg of synthetic bradykinin [28]), reported by Diniz and Carvalho [1] and agrees very well with Aarsen's data [2] of 2.9 μg BK/ml of plasma, which was obtained by exclusion of bradykinin potentiators using Amberlite CG-50.

The kininogen levels in plasma from three healthy human subjects obtained by the present method was 4.0 ± 0.1 μg BK/ml of plasma. This value is also less than half that reported by Diniz *et al.* (9.3 μg BK/ml of plasma) and is approximately the same or slightly lower than levels reported by Jacobsen (2.8 to 4.5 μg BK/ml of plasma [5]), Brocklehurst and Zeitlin (6.1 ± 1.4 μg BK/ml of plasma) and Mouri (5.63 ± 0.76 μg BK/ml of plasma [7]), using different methods.

The temperature used to store blood from collection to pretreatment is known to influence kininogen levels, since the kallikrein–kinin system in plasma may be activated at low temperatures; this is called cold activation [29]. Diniz and Carvalho [1] also reported that plasma kept at 0° for 1 day showed a low level of kininogen. Comparison of kininogen levels in blood kept at different temperatures reveals that the temperatures did not cause significant influence on the kininogen levels, at least for 3 hr (Table 1).

In conclusion, the recommended procedure for determination of total kininogen in plasma was as follows: Plasma (0.2) was added to 1.8 ml of 0.03 N HCl solution, and kept at 37° for 15 min. Then, the pretreated plasma was neutralized by 0.05 ml of 1 N NaOH and added to 0.5 ml of 0.2 M Tris buffer (pH 7.8). Incubation started after addition of 200 μg trypsin and was terminated 30 min later by adding 5 ml of boiled absolute ethanol (70°). After immersion of the tubes for 10 min in a water-bath at 70°, the mixture was centrifuged at 1000 *g* at 20°. The supernatant was transferred into 50-ml round-bottom flasks and evaporated to dryness under reduced pressure. The dried residue was dissolved in 2 ml saline and assayed for bradykinin on rat uterus.

The kininogen concentrations in the circulating blood were markedly influenced by the hematocrit value. When hemodilution occurs, kininogen levels may be decreased without consumption. Hemoconcentration also may cause the increased levels of kininogen. It was reported that kininogen levels, which were expressed in terms of μg BK released/mg of plasma protein, will reflect free kinin levels in plasma and show the real consumption [30].

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