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RELEASE OF KININS FROM FRESH PLASMA UNDER VARYING EXPERIMENTAL CONDITIONS*

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Abstract—The study of release of kinins from fresh plasma under varying experimental conditions was undertaken, using plasma of guinea pigs, dogs, rats, rabbits and human, in presence of preservatives of the released activity (8-hydroxyquinoline and/or versene). Considerable species differences were found in the amounts of released kinins by different agents: acetone, glass treatment, trypsin and chymotrypsin. Chymotrypsin was able to release kinin only from fresh guinea pig plasma, though it has been found to reduce the BKg from dog blood when injected i.v. When incubated with fresh plasma, trypsin was able to release only part of the whole activity, losing this capacity when the plasma was previously treated with glass. Acetone drastically reduced to 20 per cent the previous stock of BKg, with a corresponding release of activity, although when incubated with glass treated fresh plasma of the guinea pigs, no further reduction of the BKg could be observed. A similar observation was made with chymotrypsin, which did not reduce any further the BKg content after the glass treatment in fresh plasma of the guinea pig. A simplified version of the scheme of release of kinins from fresh plasma is presented.

THE MECHANISM of spontaneous release of bradykinin and related polypeptides from fresh plasma has attracted much interest owing to the possibility that such endogenous materials might participate in physiological and pathological conditions. We knew from the beginning that bradykinin can be released from fresh plasma by trypsin and by some snake venoms.[1–3] We also stressed that trypsin and the venom of *B. jararaca* had a different mode of action when the substrate (plasma or globulin fraction) was used in the native state or after denaturation, e.g. by heating in presence of a 1 per cent solution of acetic acid.[4, 5] This led to the assumption that trypsin itself or the venom of *B. jararaca* might act upon fresh plasma by activating the system responsible for the spontaneous release of bradykinin.[5, 6]

This system which is activated in fresh plasma by glass contact,[7, 8] dilution,[9, 10] acetone[11] or peptone[12] is probably a complex one involving at least two enzyme systems: (a) the plasminogen-plasmin system and (b) an endogenous kininogenin, also called 'plasma-kallikrein'. Along that line, there are indications that one enzyme system could act upon the other to produce the final effect, namely release of the kinin from the precursor present in the α_2-globulin fraction of normal plasma. Therefore, if it can no longer be assumed that plasmin is the only final agent which could be activated under these conditions, we are left with very little basis for understanding the mechanism by which kinins are spontaneously released and more information is needed to clarify the mechanism by which kinins are released in fresh plasma.

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In this paper we use the generic term *kininogenin* to indicate any protease that is able to release bradykinin or related peptides from the α_2-globulin fraction (bradykininogen) of fresh plasma. A preliminary report of part of this work has already been presented.[13]

METHODS†

Release of kinins. All blood samples were collected into siliconized syringes on sodium oxalate and centrifuged in siliconized or polyethylene tubes. The separated plasmas were collected with siliconized pipettes and kept in silicone coated or polyethylene flasks for the duration of the experiment. To test the release of kinin 0·5 ml of plasma was mixed with 0·2 ml *Tris* (pH 7·5) buffer and 0·01 ml of a solution of 8-HQ (100 mg/ml). Eventually, as indicated in the experiments, 8-HQ was replaced by 0·01 ml of a solution of EDTA (100 mg/ml). The releasing agent, either trypsin, chymotrypsin or acetone, was added to a final volume of 1 ml. The activity was estimated directly from the sample, after the indicated intervals of incubation, upon the guinea pig ileum, in the usual way.[14]

Kininase activity. All assays of destruction of kinin were done with synthetic bradykinin (BRS 640, Sandoz, Basel). The residual activity after incubation with plasma was estimated upon the guinea pig ileum, using as standard an acidified solution of synthetic bradykinin, containing 1 μg/ml. The kininase activity was quantitatively measured as the time (in minutes) for 50 per cent inactivation of the added bradykinin. The incubating mixture had the following compositions: (a) 0·5 ml of plasma; (b) 0·2 ml of *Tris* (pH 7·5) buffer; (c) 0·1 ml of synthetic bradykinin solution (1 μg/ml); to this mixture, the protecting agent, either 8-HQ (1 mg/ml) or EDTA (1 mg/ml) was added as indicated, and then saline added to make the final volume equal to 1 ml. Before the experiment, each plasma sample was treated with glass powder 60 min before the incubation, to avoid any spontaneous release of kinin during the experiment.

Bradykininogen determinations. All determinations of the bradykininogen (BKg) content were done using trypsin as the releasing agent, acting upon denatured plasma, according to the technique described by Diniz *et al.*[15] in current use in this laboratory (Diniz and Carvalho;[16] Corrado *et al.*[17]).

The amounts of BKg indicated are given in terms of μg of synthetic bradykinin. The estimations of activity were done upon the guinea pig ileum, as indicated before.

Sensitization of the gut by chymotrypsin. In the experiments in which the releasing effect of chymotrypsin was studied, pre-treatment of the guinea pig ileum with chymotrypsin was necessary for reproducibility, since Edery[18] has shown that chymotrypsin increases the sensitivity of the preparation to bradykinin and related peptides. This was confirmed, and after several additions of increasing doses of chymotrypsin to the bath, the ileum showed the highest level of sensitivity to bradykinin. In some preparations in spite of having atropine and anti-histaminics in the bath, the addition of a large dose of chymotrypsin elicited a slow type of contraction, with persistent tone after several washings of the gut. Though α-, β-, γ- and δ-chymotrypsin were utilized, the effects observed were more pronounced with δ-chymotrypsin.

† The following abbreviations are used: *Tris*—tris(hydroxymethyl)aminomethane; 8-HQ—8-hydroxyquinoline; EDTA—ethylenediaminetetracetic acid (versene); BAEE—benzoyl-arginine ethyl ester; TAME—tosyl-arginine-methyl ester; SBI—soya bean trypsin inhibitor; BKg—bradykininogen.

RESULTS

Inhibition of kininase activity in plasma, by 8-HQ and EDTA

Though 8-HQ (1 mg/ml) was found to protect bradykinin when incubated with plasma of most species, it was less effective with guinea pig plasma. EDTA (versene) was less effective than 8-HQ in many species, such as the rat, the guinea pig, the horse and the cow (Table 1). The figures in the Table indicate the mean of 4 samples, of the times for 50 per cent inactivation of the added bradykinin. In some experiments, in which chymotrypsin was tested upon the guinea pig plasma, a combination of 8-HQ + EDTA gave better results than either inhibitor alone.

TABLE 1. INHIBITION OF KININASE ACTIVITY OF PLASMA BY 8-HYDROXYQUINOLINE AND EDTA

Animal	Control		8-HQ		EDTA	
	Mean	Range	Mean	Range	Mean	Range
Rat	0·5	0–1	>60*	—	3·7	1·5–6·5
Cat	0·5	0–1	>60*	—	>60*	—
Guinea pig	0·5	0–1	9	8–10	0·5	0–1
Dog	1·2	0–2	>60*	—	>60*	—
Cow	3·5	2–6	>60*	—	43	13–60
Horse	0·8	0–2	>60*	—	34	22–40
Rabbit	0·5	0–1	>60*	—	>60*	—
Man	3·2	2–5	>60*	—	>60*	—

Legend: The figures indicate the time (in min) for 50 per cent consumption of incubated bradykinin. Incub. mixture: (a) 0·5 ml of plasma; (b) 0·2 ml of *Tris* buffer pH 7·5; (c) synthetic bradykinin, 1 μg (0·1 ml); (d) 8-hydroxyquinoline (5-HQ) 1 mg/ml and EDTA 1 mg/ml; (e) saline up to 1 ml. The values represent the means of 4 plasma samples. Each sample was treated with glass powder 60 min before incubation to avoid spontaneous kinin liberation.

* The whole bradykinin activity was present after 60 min incubation.

Release of kinins from fresh plasma of different species

In order to detect any correlation between the amounts of kinins released by enzymes (trypsin and chymotrypsin), and under the action of glass powder or by acetone treatment, the plasma of different species of animals and man were submitted to the indicated experimental conditions. All plasma samples contained 1 mg/ml of 8-HQ and in the case of guinea pig, in some experiments, 1 mg/ml of EDTA was added. Fig. 1 compares the amounts of kinin released from plasma of different species by the agents indicated: chymotrypsin (C), acetone (A), glass (G) and trypsin (T). The actual amounts released are indicated in the upper part of the graphs in μg/ml, and the percentages in the lower section of Fig. 1. There were surprisingly large differences between species and between the potencies of different kinin-releasing agents.

Of all plasmas studied, only guinea pig plasma released kinin under the action of chymotrypsin. The demonstration of a release of kinin by chymotrypsin was only made possible in the presence of 8-HQ or of 8-HQ + EDTA, since in the absence of any protective agent the released material was very quickly destroyed by chymotrypsin. Acetone in the amount of 10 per cent (v/v) was very active upon the plasma of the guinea pig and of the rabbit, but rather ineffective upon dog's plasma. Glass treatment was more effective upon the plasma of the rat and less so on the plasma of

guinea pig and almost inactive upon the plasma of the rabbit and the dog. From these experiments upon fresh plasmas, no obvious correlation could be found between the different treatments. Trypsin worked better than acetone upon the plasma of the dog, the rat and the man, but less so upon the plasmas of the guinea pig and of the rabbit.

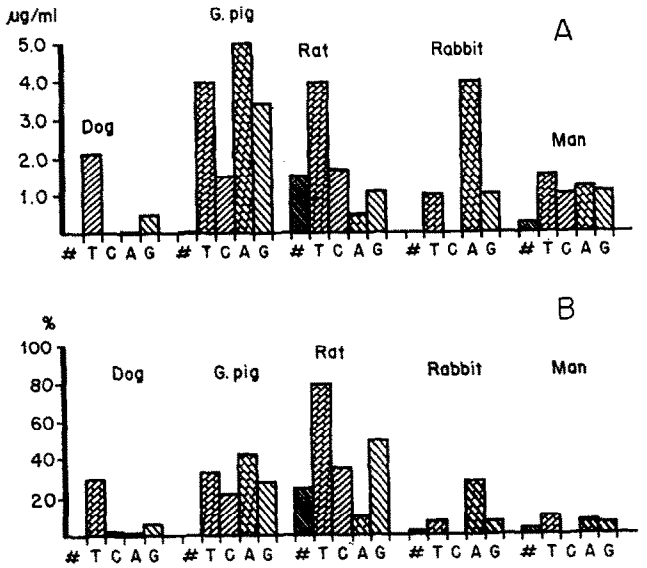

FIG. 1. Comparative release of kinins from fresh plasma of several species by different agents: # = control; T = trypsin; C = Chymotrypsin; A = acetone; G = glass treatment. (A) Actual amounts of released activity in μg/ml of synthetic Bradykinin(BRS 640); (B) released activity calculated in percentages of the total BKg previously contained in the samples.

Release of kinin by chymotrypsin. The most surprising effect of the above experiments was the capacity of chymotrypsin to release kinin from fresh guinea pig plasma. A series of experiments were done to establish the conditions in which the phenomenon could be observed in a reproducible way. We had primarily to decide whether the release was a real one and not due to an effect of sensitization of the guinea pig ileum, by the enzyme, since according to Edery[18] chymotrypsin is a powerful sensitizer of the gut to the action of such polypeptides of the bradykinin family. Fig. 2A shows this effect of δ-chymotrypsin upon the guinea pig ileum, Fig. 2 C, D give the assay of the released kinin after incubation of fresh guinea pig plasma with 40 μg and 80 μg respectively of the same preparation of chymotrypsin, after the gut had been maximally sensitized to the enzyme. Fig. 2B gives the spontaneous release without addition of the enzyme. Table 2 gives a summary of all experiments of release of kinins by chymotrypsin from guinea pig plasma, in μg of synthetic bradykinin, by the two concentrations of δ-chymotrypsin.

Negative results with chymotrypsin were obtained in fresh and denatured plasma of the dog, hamster, rabbit and human, and also with the denatured plasma of the guinea pig.

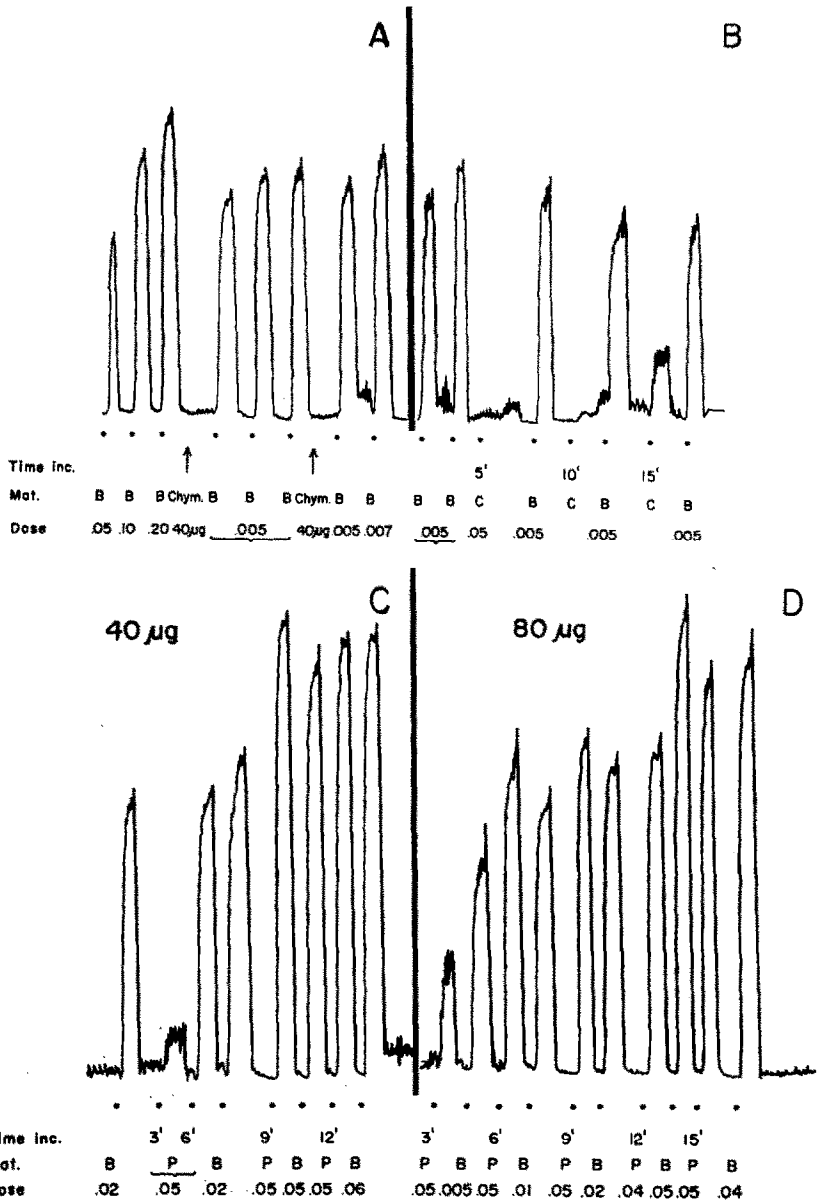

FIG. 2A. Sensitizing effect of δ-chymotrypson upon bradykinin elicited contractions on the guinea pig ileum: synthetic bradykinin (B) and δ-chymotrypsin (Chym) at the indicated doses.

B. Spontaneous liberation of kinin by incubating fresh guinea pig plasma in presence of 8-HQ at 37°. At the indicated time interval an aliquot of the control mixture (C) was added to the isolated organ bath.

C and D. Release of kinin from guinea pig fresh plasma by 40 and 80 µg/ml of chymotrypsin respectively. An aliquot of 0·05 ml was tested after incubation of the mixture during the indicated time interval: P (probe).

The fresh guinea pig plasma previously submitted to the glass treatment, was also unable to yield any kinin activity by incubation with chymotrypsin.

Release of kinin from fresh plasma, by trypsin. Since there are indications that trypsin releases bradykinin from fresh plasma by activating the endogenous kininogenin (Hamberg and Rocha e Silva[5]), the experiments presented in Table 3 were done in order to test whether the treatment with glass would change the amounts of bradykinin released by trypsin from guinea pig plasma. Table 3 indicates that this is the

TABLE 2. RELEASE OF KININS FROM FRESH GUINEA PIG PLASMA BY CHYMOTRYPSIN

	Kinins released (μg/ml) Time of incubation			
	3′	6′	9′	12′
Control plasma	0	0	0·02	0·1
+ Chymotrypsin, 40 μg	0·05	0·27	1·08	1·34
40 μg	0·02	0·86	1·16	1·0
40 μg	0·07	0·58	0·87	0·87
80 μg	0·54	2·7	1·9	1·08
80 μg	0·54	0·58	2·0	1·75
Plasma treated with glass				
+ Chymotrypsin, 40 μg	0	0	0	0
80 μg	0	0	0	0

The figures indicate μg/ml of Bradykinin ('Sandoz', BRS 640).
Activity estim. directly on guinea pig ileum.

case since incubation of fresh plasma with increasing amounts of trypsin (up to 1 mg/ml) released only part of its activity as measured in percentage of the BKg previously present. In a plasma which contains as much as 10 μg/ml of BKg, calculated as synthetic bradykinin, not more than 2·4 μg/ml or 24 per cent of the total could be released by incubation with as much as 1 mg of trypsin. What was more interesting was the fact that after the glass treatment, even those small amounts were no longer released, as shown in Table 3.

TABLE 3. RELEASE OF KININS FROM FRESH GUINEA PIG PLASMA BY TRYPSIN

	Kinins released (μg/ml) Time of incubation			
	1′	4′	10′	16′
Control plasma	0	0	0·2	0·3
Trypsin, 50 μg	0·3	0·8	0·8	0·8
100 μg	1·2	1·2	1·2	1·0
500 μg	1·2	1·6	1·6	1·6
1 mg	2·0	2·4	2·4	—
Plasma treated with glass	0	0	0·1	0
Trypsin, 50 μg	0	0	0·1	0
100 μg	0	0	0·1	0
500 μg	0	0	0	0
1 mg	0	0	0	0

The figures indicate μg/ml of Bradykinin ('Sandoz', BRS 640).
Activity estim. directly on guinea pig ileum.

The BKg content was estimated in normal fresh plasma in comparison with the residual amounts in the plasmas submitted to the action of increasing concentrations of trypsin, before and after the glass treatment. Fig. 3 shows that after reaching a stable level following the glass treatment, a further incubation with trypsin will reduce only slightly the residual BKg estimated in the plasma samples.

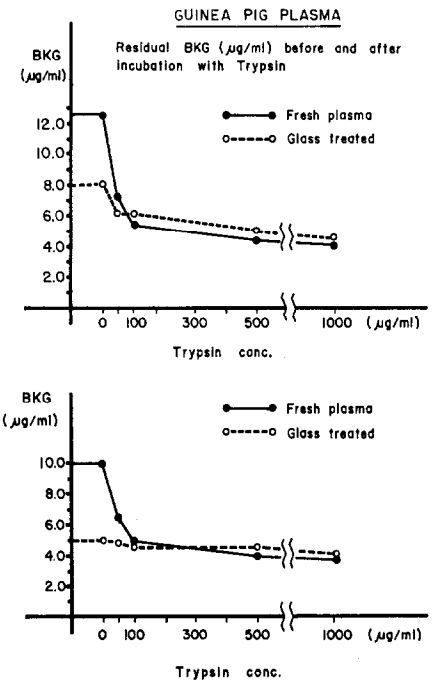

FIG. 3. Residual BKg (μg/ml) in guinea pig plasma, before and after the treatment with glass. The amounts of BKg are in equivalents of synthetic bradykinin (μg/ml).

Action of glass powder on the BKg content of fresh plasma (guinea pig)

The results present in Table 4 are in agreement with those presented by Diniz and Carvalho.[16] Prolonged glass treatment will not reduce by more than 30–40 per cent

TABLE 4. BRADYKININOGEN (BKG) CONTENT IN FRESH GUINEA PIG PLASMA BEFORE AND AFTER GLASS TREATMENT

Exp. No.	BKg in plasma (μg/ml)			
	Before	After 1 min	After 30 min	After 60 min
I	10·0	7·2	5·9	5·7
II	11·1	8·2	7·9	6·7
III	7·0	6·3	4·6	5·5
IV	5·5	6·0	4·6	4·3
V	17·0	12·0	7·2	10·0
Averages	10·12	7·94	6·04	6·44

Each sample contained 3 ml fresh plasma and 0·2 g glass powder.
BKg in μg/ml synthetic bradykinin ('Sandoz', BRS 640).

the BKg content of fresh plasma. After 30 min shaking with glass powder, the residual BKg attained a minimum that was not reduced by a further 30 min shaking with the glass powder.

Action of acetone on the BKg content of guinea pig plasma

In contrast to what happened in the previous section with the glass powder treatment, incubation of plasma with acetone (10 per cent in vol.) drastically reduced the bradykininogen content of plasma to very low levels. As shown in Fig. 4, the treatment of acetone upon fresh guinea pig plasma reduced the BKg content from a normal average value of 8·6 μg/ml to the low level of 1·4 μg/ml. The addition of

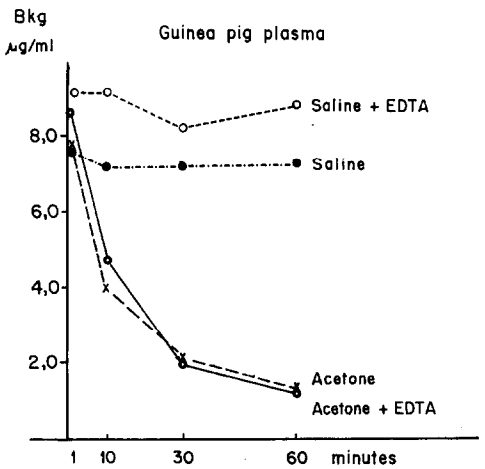

FIG. 4. Residual BKg (μg/ml) in guinea pig plasma, after incubation with saline, saline + EDTA and shaking with 20% (v/v) of acetone, without and with EDTA.

1 mg/ml of EDTA did not change significantly the result after 60 min of the acetone treatment, though the presence of EDTA might have protected BKg against spontaneous destruction in fresh plasma, without acetone (Fig. 4). Therefore, the acetone treatment is able to destroy more than 80 per cent of the previous BKg store of fresh guinea pig plasma.

However, when the plasma was submitted to acetone after previous treatment with glass powder, there was no further drop in BKg after it had been reduced to the 40 per cent level by the glass treatment. In the experiments in Table 5 a treatment of fresh plasma with glass powder for 60 min completely destroyed the capacity of acetone to further reduce the BKg content.

The result of this experiment combined with that of the previous one indicates that shaking with glass removes an ingredient (or factor) which interacts with acetone for the release of kinins.

Action of chymotrypsin on the BKg content of guinea pig plasma

When chymotrypsin is injected into dogs a drastic reduction of the BKg content of blood plasma was observed without signs of any effect upon the blood pressure. When incubated *in vitro* with the fresh plasma of the guinea pig chymotrypsin releases kinin activity as shown in a preceeding paragraph. Drastic reduction of the BKg

TABLE 5. ACTION OF ACETONE AFTER A PREVIOUS 60 MIN TREATMENT WITH GLASS POWDER (FRESH GUINEA PIG PLASMA)

Exp. No.	BKg content (μg/ml)		
	Before glass treat.	After glass treat.	After glass + acetone treat.
I	7·2	4·6	5·3
II	10·0	5·5	5·1
III	6·0	4·6	3·8
IV	6·7	5·0	5·1
V	11·0	5·9	5·2
VI	13·3	7·8	7·7
Averages	9·03	5·6	5·4

The figures indicate μg/ml of synthetic Bradykinin ('Sandoz', BRS 640).
After 60 min shaking with glass powder, the samples were further treated with acetone.

content of fresh guinea pig plasma was observed by incubation of plasma with 40 μg–80 μg, as indicated in Fig. 5. However, when the plasma was previously treated with glass powder, no further reduction in BKg could be observed even with the highest concentration (80 μg/ml) as indicated in Fig. 5. The analogy of this phenomenon with what happens when the plasma is treated with acetone before and after the glass treatment is manifest.

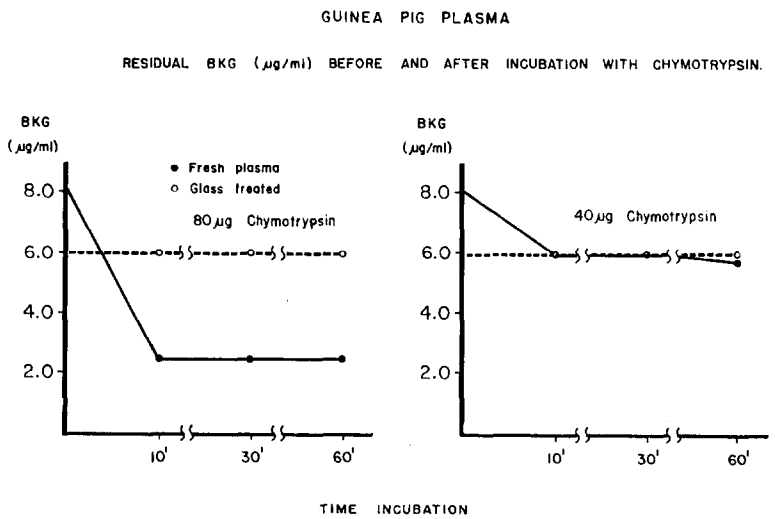

FIG. 5. Residual BKg (μg/ml) in guinea pig plasma after incubation with 40 μg and 80 μg of chymotrypsin, for the indicated lengths of time.

DISCUSSION

The experiments presented in this paper were all done on fresh plasma and differ from others presented in many publications on the same subject by the fact that the main deductions have been taken from estimations of the total and residual BKg.

Experiments in which the only estimated parameter is the released activity, are open to the criticism that we never know the amount of substrate actually offered to the releasing agent. We have shown in Fig. 1A and B, that the percentage of activity released calculated from the amount of BKg available in the control samples, can be very small, though the amounts of activity released were large enough to be estimated upon the biological preparation.

Furthermore, in many experiments described on this subject, no protecting agent (anti-kininase) has been utilized; in others, protecting agents of low potency as it is the case, for instance, with EDTA in the guinea pig, the rat or the rabbit. The advantage of 8-HQ in many circumstances has been repeatedly emphasized[14, 19] and better so, the association of 8-HQ and EDTA as used in some experiments with the guinea pig plasma.

The basis of the deduction from the consumption of BKg, as previously done in papers from this laboratory[16, 17] and from others[20] is open to criticism since destruction of BKg might not be parallel to the release of active kinins. This is particularly true as concerns chymotrypsin, since it might reduce very drastically the stock of BKg, without producing any detectable biological effect, as has been shown in our laboratory after injections of large doses of chymotrypsin in the dog. Apparently, the guinea pig so far is the only animal in which we might observe any effect of chymotrypsin as due to the release of kinins from fresh plasma.

As far as the glass or acetone treatment are concerned it is quite conceivable that a reduction of BKg might correspond to the release of active material. If one accepts this view, it is remarkable that prolonged treatment with glass powder is able to remove the whole activity that might be further released by acetone, trypsin or chymotrypsin from fresh plasma, though this treatment is able to remove only 40 per cent of the total available BKg. Margolis and Bishop[21] tried to explain this fact by assuming that the glass treatment would remove one of the components of BKg (Component B) by activation of the Hageman Factor (HF). The insufficiency of this explanation appears very clearly in the experiment with acetone which was able to remove 80 per cent of the previous BKg, but no further reduction was obtained if the plasma had been treated with glass powder.

It appears more likely that the glass would remove the enzyme available in fresh plasma, or a factor that might be important for its activation by acetone, trypsin or chymotrypsin. However, this assumption would not explain why the whole activatable material can be removed by glass in an active form and be unable to reduce more than 40 per cent the total BKg available. As a working hypothesis, we might assume that a change of phase produced by glass would reduce the chances of collision between the enzyme molecule absorbed on glass and the molecule of the precursor (α_2-globulin).

If we exclude plasma kallikrein as acting directly upon BKg, as shown by Henriques *et al.*[22] we are left with two possibilities which appear very appealing for further inquiries.

In the first place, what has been called Hageman factor (HF) has been isolated in highly purified form and was found to be a sialoglyco-protein with pronounced esterase activity when tested upon BAEE and TAME. It was furthermore demonstrated that SBI, ovomucoid, ϵ-aminocaproic acid did not inhibit its esterolytic activity, but the Lima bean trypsin inhibitor and diisopropylphosphofluoridate had a very strong inhibitory effect. For more detailed information about this purified Hageman

factor, see Schoemakers *et al.*[23] Data is lacking about the capacity of this purified HF to release kinins. The possibility of HF to be the factor concerned in the release of kinins from fresh plasma, would account for many peculiarities of the phenomena described in this paper, and its importance would give a new emphasis to the discovery by Margolis[24, 25] of the participation of HF in the process of release of kinins by contact agents, such as glass, kaolin and so forth. If HF is shown finally to release kinins from purified BKg, it could be the main kininogenin present in fresh plasma.

The possibility that HF acts indirectly by activating the plasminogen-plasmin system has been already suggested, since HF appears to induce weak but detectable fibrinolytic effect in fresh plasma (see Eisen[26]). Iatridis and Ferguson[27] found a good correlation between activation of HF and the post-exercise fibrinolytic effect in normal and HF deficient humans, reaching the conclusion that HF converts plasminogen pro-activator into activator. For a general discussion on the arguments in favour and against plasmin being the final agent for the release of kinins, see Eisen[26] and also Hamberg[28]. Anyway, the experiments by Henriques *et al.*[22] greatly increase the evidences in favour of the importance of plasmin activation in the release of kinins from fresh plasma, since it was shown that plasmin, but not plasma-kallikrein, was active in releasing kinins from purified kininogenin, though as shown by Vogt[29] plasmin would act through activation of the same specific endogenous kininogenin (plasma kallikrein).

The following scheme is the simplest one could propose to explain the data available on the release of kinins from fresh plasma:

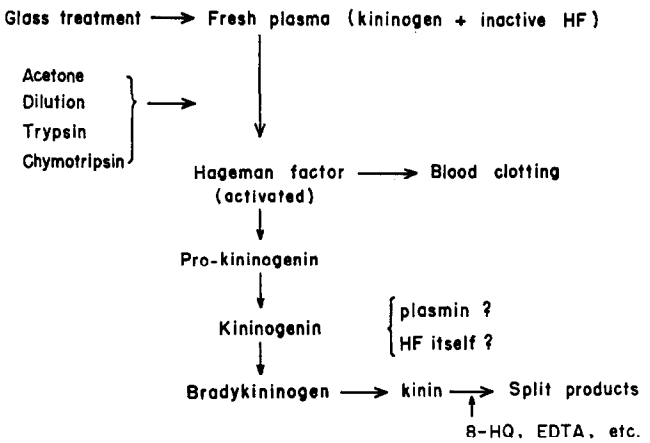

FIG. 6. A simplified scheme of the mechanism of release of kinin from fresh plasma by the indicated agents.

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