Biochemical Pharmacology, Vol. 19, pp. 2083–2090. Pergamon Press. 1970. Printed in Great Britain

COMPARATIVE ACTION OF VARIOUS KININOGENASES ON CRUDE HORSE PLASMA SUBSTRATES

POLINA BUDNITSKAYA, ELVIRA GAPANHUK and OLGA B. HENRIQUES*

Institute Gamaleya, Moscow, U.S.S.R.

(*Received* 19 *September* 1969; *accepted* 9 *January* 1970)

Abstract—The kininogenase activity of trypsin, plasmin, plasma kallikrein and heated *Bothrops* venom was compared, using fresh, heated and heat-acid-denatured horse plasma as source of kininogen. The venom kininogenase was found to have the highest activity on fresh horse plasma, followed by plasmin and trypsin which were equally active, and plasma kallikrein which was half as active as plasmin on these substrates. Plasmin and trypsin released more kinin from heat-treated than from fresh plasma whereas kallikrein released half as much as it liberates from fresh plasma. On heat-acid-denatured plasma equal activity was found for plasmin, trypsin and *Bothrops* kininogenase while the activity of plasma kallikrein was one tenth of that of plasmin or trpysin. None of the enzymes studied released additional kinin from any of the substrates after activation with trypsin or plasmin. After activation with plasma kallikrein, plasmin, trypsin and *Bothrops* kininogenase released additional kinin from the three substrates used. The results are discussed pointing out the possible importance of plasmin in the kinin system. Plasmin can replace trypsin in the determination of plasma kininogen by the method of Diniz *et al.*[13]

THE RELEASE of kinin in plasma is generally attributed to plasma kallikrein. Already in 1950 however, Beraldo[1] has shown that incubation of plasmin with plasma globulins resulted in the release of vasoactive substance. Later other authors gave some evidence that plasmin is involved in the release of kinin in plasma.[2, 3] More recently it has been shown that plasmin is able to release kinin from purified plasma kininogen[4, 5] as well as from heat treated plasma.[5, 6] Therefore it seemed important to compare the kininogenase activity of this enzyme on crude plasma substrates with that of other kininogenases.

The investigation reported here is concerned with the estimation of kinin released from fresh horse plasma, heat-treated plasma and plasma denaturated by heat in acid medium[7] by plasmin, plasma kallikrein, trypsin and *Bothrops* venom kininogenase.

MATERIALS AND METHODS

Bradykinin, synthetic, was kindly supplied by Sandoz Ltd., Basle. Loss of bradykinin in the diluted solutions was prevented by the addition of oxalic acid in the final concentration of 10^{-3} M.[8]

Trypsin, crystalline, Spofa, Czeckoslovakia, was used. One mg trypsin hydrolyzed 106 μmoles of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) per min when

* Present Address: Wolfson Laboratories, G.I. Unit, Dept. of Medicine Western General Hospital, Edinburgh, EH4 2XU.

assayed by the method of Brown.[9] The solutions of trypsin were always prepared immediately before use in 10^{-2} M hydrochloric acid.

Bothrops jararaca venom was obtained from Instituto Butantan, Brazil. The venom was dissolved in 0·9% NaCl, was heated to 90°, immediately refrigerated, centrifuged and the concentrated solution was kept frozen while not in use.

Plasma kallikrein was obtained from horse plasma as described previously,[5] exception made of rechromatography on DEAE-cellulose.

Plasmin was obtained from pre-plasmin prepared according to Robbins and Summaria[10] with the following minor modifications. Fraction B (Cohn's II–III) paste, from which γ-globulins were previously separated, was suspended in 0·05 N H_2SO_4 and stirred for 4 hr in the cold (4°). Precipitation of pre-plasmin at pH 6·0 and storing the mixture for 48–72 hr at 4°, instead of 18 hr as recommended by Robbins and Summaria.[10] The precipitate of pre-plasmin was collected by centrifugation and suspended in water to which 1 M HCl was added carefully to pH 3·7.

Pre-plasmin was activated with 500 NIH-units of streptokinase (Kabikinase, AB Kabi, Stockholm, Sweden) per mg protein of pre-plasmin. Activation was proceeded with for 15 min at room temperature. Proteolytic activity of the plasmin obtained was determined by the method of Kunitz[11] on casein and the units of activity were calculated according to Robbins and Summaria.[10]

The plasmin activity of the crude extract, the water solution in pH 3·7 and the final product were respectively 0·25, 1·3 and 6 casein units per mg of protein.

Substrates. Plasma was obtained from horse blood collected in siliconed containers (Silicone GKI, USSR) over potassium oxalate (3g/l. blood); after separation by centrifugation at about 5000 rev./min. in polyethylene tubes, it was immediately used.

Heat-treated horse plasma. Part of the fresh plasma was heated at 56° for 3 hr to get rid of pre-kallikrein and kallikrein[12] and then dialyzed against 0·9% NaCl.

Acid-heat-denatured plasma was obtained in the conditions of the test for kininogen determination described by Diniz *et al.*[13]

Kininogen determination was essentially made according to the method described by Diniz *et al.*[13] with the modification used by Henriques, Picarelli and F. de Oliveira.[14] Heating with acid was excluded when using normal or heat-treated plasma. The time of incubation of substrates with trypsin and venom kininogenase was 30 min. With plasma kallikrein however, 45 min incubation were needed and with plasmin 150 min, to achieve maximal kinin release. *o*-phenantroline was added to all incubates in the final concentration of 10^{-3} M[15] and 3×10^{-3} M with incubates of fresh plasma, to prevent the action of kininase present in plasma or kallikrein preparation. The relative potency of the incubates was calculated from the response to single and double doses[16] of both synthetic bradykinin and aliquots. Controls were always made, incubating the plasmas with all the reagents, except enzyme and the enzymes with all the reagents except substrate.

Protein in enzyme solutions were determined by the biuret reaction or the method of Lowry, Rosenbrough, Farr and Randall.[17]

RESULTS

Effect of concentration of enzymes. All the experiments were made using 0·2 ml of fresh, heat-treated (HT) or heat-acid-denatured (HAD) plasma in the incubation mixture. In the experiments made in order to find the optimal concentration of

enzyme, it has been observed that a large excess of plasma kallikrein or heat-treated venom may be used without inactivation of the kinins released. On the other hand, partial destruction of the kinins formed is observed when high concentrations of trypsin or plasmin are employed. In the conditions of our experiments plasmin has been found to be most effective at a concentration of 3 casein units (500 μg) with the substrates used, (Fig. 1) while with trypsin the plateau of activity is reached with 200 μg, a lower yield being observed with larger amounts of the enzyme, as previously reported by Dinzi and Carvalho.[7]

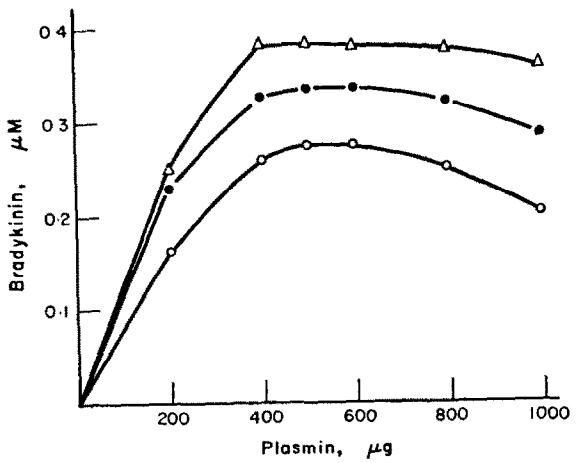

FIG. 1. Release of kinin from fresh plasma —○—, heat-treated plasma —●— and heat-acid-denaturated plasma —△— following incubation with different concentrations of plasmin. Incubates: 0·2 ml plasma substrate + 0·5 ml 0·2 M Tris buffer, pH 7·8 + 0·2 ml *o*-phenantroline 1×10^{-2} M + plasmin, at 37° for 150 min. Total volume of the incubate: 2 ml. Samples of the incubates were tested on the guinea pig ileum.

The esterase activity of the enzymes on *p*-toluenesulfonyl-L-arginine methyl ester (TAME), expressed as μmoles/min/mg protein, were 106 for trypsin, 0·35 for the venom, 0·015 for plasma kallikrein and 0·11 for plasmin.

Time course of kinin release with the various enzymes. With HAD-plasma it was found that maximal kinin liberation was observed already after 30 min of incubation of the substrate with the *Bothrops* kininogenase and trypsin and after 45 min with plasma kallikrein while an incubation period of 150 min was required when plasmin was used as kininogenase (Fig. 2). Similar results were obtained when fresh plasma or HT-plasma were used as substrates.

Kininogen content of fresh, HT- and HAD-plasma as determined with various kininogenases. Table 1 shows the release of kinin from fresh, HT- and HAD-plasma by trypsin, heat-treated *Bothrops* venom, plasma kallikrein and plasmin. It can be seen that trypsin released the same amount of kinin from HT- (0·40 μmoles as bradykinin) or HAD-plasma (0·42) and only three fourths of this amount from fresh plasma (0·30). Plasmin also released less kinin from fresh plasma (0·29) than from the treated ones. Whereas trypsin released practically the same amount of kinin from HT-plasma or HAD-plasma, plasmin released more kinin from HAD-plasma (0·45) than from

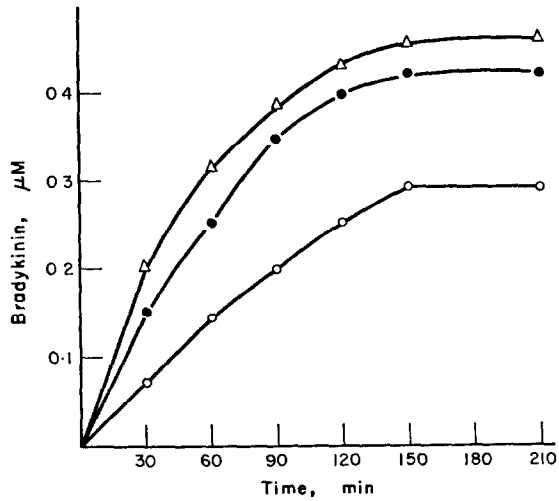

FIG. 2. Time course of kinin release from plasma —○—, heat-treated plasma —●— and heat-acid-denaturated plasma —△— following incubation with 500 μg plasmin (3 casein units). Incubates as in Fig. 1. Samples were tested on the guinea pig ileum at the end of the various periods of time.

TABLE 1. KININOGEN CONTENT OF EQUINE PLASMA SUBSTRATES AS DETERMINED BY TRYPSIN, *BOTHROPS* KININOGENASE PLASMA KALLIKREIN AND PLASMIN

	Trypsin	*Bothrops* kininogenase	Plasma kallikrein	Plasmin
	activity expressed in μmoles of bradykinin/0·2 ml plasma			
Fresh plasma	0·30 ± 0·020	0·84 ± 0·019	0·14 ± 0·011	0·29 ± 0·015
Heat treated plasma	0·40 ± 0·014	0·49 ± 0·013	0·09 ± 0·012	0·38 ± 0·013
Heat acid denaturated plasma	0·42 ± 0·019	0·40 ± 0·011	0·05 ± 0·008	0·45 ± 0·016

0·2 ml of each plasma substrate were incubated at 37° for 30 min with trypsin (200 μg) or *Bothrops* kininogenase (165 μg), 45 min with plasma kallikrein (340 μg) and 150 min with plasmin (500 μg), at pH 7·8 (Tris buffer), in the presence of 3×10^{-3} M *o*-phenantroline in fresh plasma incubates and 1×10^{-3} M in the other ones. The amounts of enzymes are expressed in μg of protein and their activities on *p*-toluenesulfonyl-L-arginine methyl ester were 106 for trypsin, 0·35 for *Bothrops* kininogenase, 0·05 for plasma kallikrein and 0·11 for plasmin, respectively, expressed in μM/min/mg protein. The kinins released were measured as bradykinin on the guinea pig lieum. Each value represents the average of ten independent determinations.

HT-plasma (0·38), this difference being significant with $P < 0·01$. Venom kininogenase on the other hand released two times more kinin from fresh plasma (0·84 μmoles) than from HAD-plasma (0·40) or from HT-plasma (0·49). Plasma kallikrein released less kinin than any of the kininogenases described above from fresh plasma (0·14 μmoles), HT- (0·09) or HAD-plasma (0·04). The findings corresponding to kallikrein and plasmin are presented in Figs. 4 and 5 respectively.

Additional increase in the kinin activity of the enzyme–substrate incubate by subsequent exposure to a second kininogenase. Table 2 shows the amount of kinin released from fresh, HT- and HAD-plasma after superimposed incubation with the kininogenases

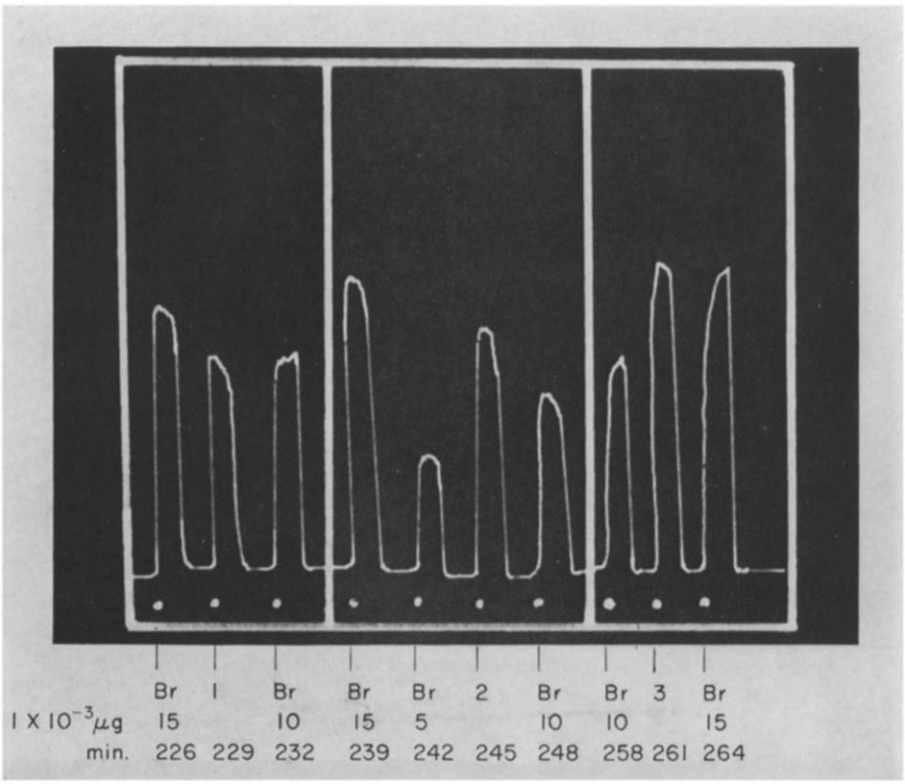

FIG. 3. Kinin release from plasma substrate by plasma kallikrein. Incubation conditions as in Fig. 1. Incubate 1— fresh plasma; 2— heat-treated plasma; 3— heat-acid-denaturated plasma; respectively 0·05, 0·05 and 0·10 ml of those incubates were used on the ileum; Br—synthetic bradykinin.

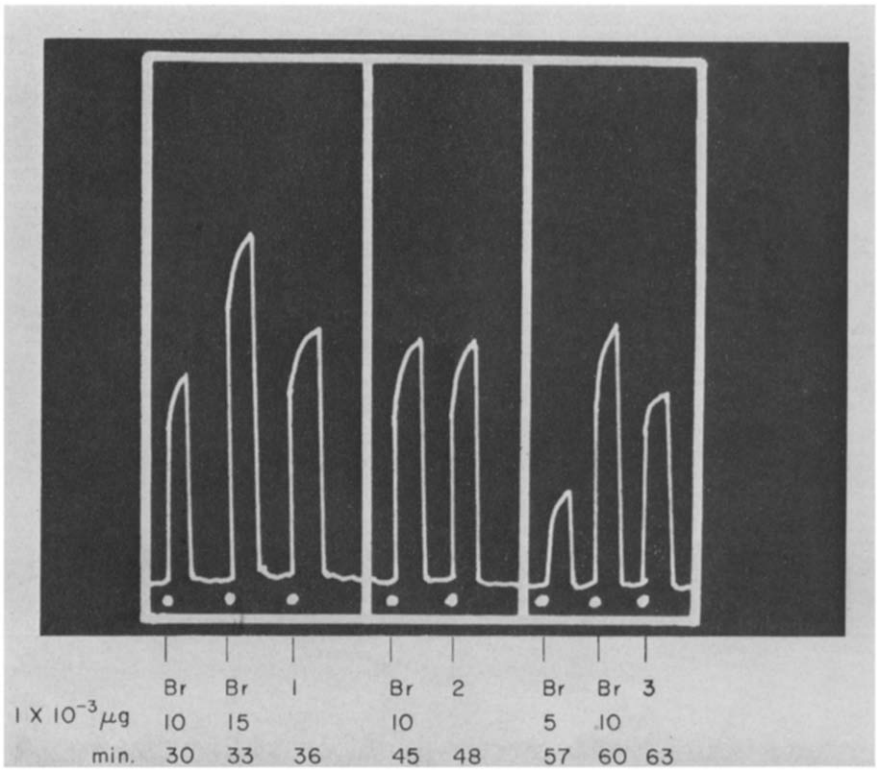

FIG. 4. Kinin release from plasma substrates by plasmin (3 casein units). Incubation as in Fig. 1. Incubate 1—fresh plasma; 2—heat-treated plasma; 3—heat-acid-denaturated plasma; respectively 0·30, 0·20 and 0·07 ml of those incubates were used on the guinea pig ileum. Br—synthetic bradykinin.

TABLE 2. ADDITIONAL INCREASE IN THE KININ ACTIVITY OF THE ENZYME SUBSTRATE INCUBATE BY SUBSEQUENT EXPOSURE TO A SECOND KININOGENASE

Kininogenases		Fresh plasma	Heat treated plasma	Heat acid denaturated plasma
First	Second			
Trypsin +	Trypsin	0·29 ± 0·018	0·42 ± 0·016	0·42 ± 0·013
	Bothrops kininogenase	0·75 ± 0·017	0·42 ± 0·018	0·42 ± 0·015
	Plasma kallikrein	0·30 ± 0·018	0·41 ± 0·016	0·43 ± 0·012
	Plasmin	0·35 ± 0·013	0·41 ± 0·016	0·43 ± 0·012
Bothrops kininogenase +	*Bothrops* kininogenase	0·80 ± 0·016	0·47 ± 0·018	0·40 ± 0·015
	Trypsin	0·77 ± 0·018	0·47 ± 0·015	0·41 ± 0·011
	Plasma kallikrein	0·78 ± 0·013	0·47 ± 0·016	0·41 ± 0·012
	Plasmin	0·80 ± 0·011	0·46 ± 0·015	0·41 ± 0·016
Plasma kallikrein +	Plasma kallikrein	0·15 ± 0·012	0·09 ± 0·010	0·042 ± 0·011
	Trypsin	0·31 ± 0·015	0·40 ± 0·016	0·42 ± 0·013
	Bothrops kininogenase	0·79 ± 0·013	0·47 ± 0·012	0·41 ± 0·013
	Plasmin	0·30 ± 0·013	0·40 ± 0·015	0·43 ± 0·011
Plasmin +	Plasmin	0·30 ± 0·012	0·39 ± 0·016	0·43 ± 0·011
	Trypsin	0·30 ± 0·013	0·40 ± 0·011	0·43 ± 0·015
	Bothrops kininogenase	0·78 ± 0·011	0·41 ± 0·013	0·42 ± 0·012
	Plasma kallikrein	0·29 ± 0·015	0·40 ± 0·010	0·42 ± 0·012

The incubation mixtures with the first kininogenase were the same as described in Table 1 as well as the time and condition of incubation. At the end of the first period of incubation the second kininogenase was added and the mixtures were again incubated for the period of time found optimal for each kininogenase (see Table 1). Each value represents the average of eight independent determinations.

studied. It can be seen that after incubation with trypsin or plasmin only venom kininogenase was able to release additional kinin and only from fresh plasma. No other enzyme released additional kinin from any of the substrates after trypsin or plasmin.

After incubation with kallikrein additional kinin was released from the substrates with the three other enzymes, in an amount sufficient to reach the level of kinin that each enzyme released from the corresponding substrate when incubated alone.

DISCUSSION

The data here presented show that plasmin is a much more potent kininogen than kallikrein, a finding which may be relevant since these kininogenases are the only two which so far have been demonstrated to be present in plasma. In fact plasmin seems to play a much more important role in the kinin system than it has been attributed to it. This enzyme releases twice as much kinin from fresh plasma, four times more from HT-plasma and ten times more from HAD-plasma than plasma kallikrein. Its smaller potency on fresh plasma, compared to HT- and HAD-plasma is probably

due to the presence of antiplasmin in fresh plasma.[18] Plasma possesses also antikallikrein activity.[19, 20] However the isolation and study of serum kallikrein inhibitors has shown that their antikallikrein activity is much weaker than their antiplasmin activity,[21] at least in the rabbit.

The lower yield of kinin activity with plasma kallikrein could also be explained on the basis of the smaller activity of the kinin released. However it has been shown that bradykinin is the kinin formed when plasma kallikrein acts on plasma γ-globulins,[22] and preliminary work in our laboratory has shown that bradykinin is the main kinin released from HAD-plasma by plasma kallikrein. Table 2 shows clearly that all the enzymes superimposed to kallikrein increased the yield of kinin from the various plasmas to the same level obtained when only those enzymes were incubated with the crude substrates. This observation shows that plasma kallikrein has activated only part of the available kininogen in plasma. This may be explained by the findings of Henriques *et al.*,[5, 23] Jacobsen,[24] and Yano *et al.*[25] who found that kininogen 2 does not react with plasma kallikrein.

The smaller yield of kinin from HT- and HAD-plasma when activated with plasma kallikrein shows that part of the specific kininogen for this enzyme was inactivated. Jacobsen[24] has shown that kininogen 1 is inactivated by heating. That denaturation of plasma proteins renders it less sensitive to kallikrein has previously been shown by Werle and Preisser.[26]

From Table 2 we can see that heat denaturation renders the plasma less sensitive to snake venom and more available to trypsin, as previously observed by Habermann *et al.*,[27] a fact that can be attributed to the inactivation of trypsin inhibitors in HT- and HAD-plasmas.

Our data differ from those recently reported by Back and Steger[28] who found that trypsin released much more kinin from Greenbaum's kininogen[29] than plasmin. The time course of kinin release from human kininogen observed by Back and Steger[28] was also different from the one observed for the crude plasma substrates used here. While with trypsin the plateau of kinin release was reached at 30 min incubation with any of the crude substrates, the maximum activity with plasma kallikrein was obtained at 45 min and with plasmin only at the end of 150 min. The kininogen content of the various plasma substrates determined with plasmin as compared with plasma kallikrein was twice as high in fresh plasma, four times higher in HT-plasma and ten times higher in HAD-plasma while Back and Steger observed almost the same release of kinin with both enzymes when activating Greenbaum's kininogen.

Back and Steger[28] also found that the kininogen in plasma which had already been completely activated by plasmin can be activated further by trypsin. Table 2 shows that no additional activation of substrate from fresh, HT- or HAD-horse plasma was observed with trypsin after activation of the substrate with plasmin. All these differences with the observations of Back and Steger[28] may perhaps be explained by the fact that those authors used human plasmin, human plasma kallikrein, human kininogen and human plasma whereas our results are for human plasmin but horse plasma kallikrein and horse plasma substrates.

Our results have also some bearing on the measurement of kininogen by the method of Diniz *et al.*[13] Kininogen has been determined in the blood of animals in various experimental conditions[7, 30–36] and in humans in various physiological conditions.[7, 37, 38] All the authors mentioned used trypsin as kininogenase and denaturated

the plasma by heat in acid medium previous to the determination of kininogen, in order to destroy endogenous plasma kallikrein, kininase and trypsin inhibitors, measuring the kinin released from kininogen on the isolated guinea pig ileum.[7] The method is simple and convenient but it may be questioned whether it would give the real picture of the kininogen existing in plasma which could be used by plasma kininogenases since kallikrein has been considered to be the main plasma kininogenase and in this method trypsin is used as kininogenase while we know that plasma kallikrein does not release kinin from kininogen 2.[5, 23–25] Also it is difficult to interpret changes in kininogen level in blood plasma in various conditions when denatured protein is used as substrate and trypsin as kininogenase, unless it were demonstrated that the amount of kininogen contained in the denatured substrate is the same or proportional to that of fresh plasma and that either trypsin or endogenous plasma kininogenase release the same amount of kinin from this substrate. Our data show that plasmin, also an endogenous kininogenase, releases from fresh, HT- or HAD-plasma the same amount of kinin as trypsin when using horse plasma. Further experiments should be done to find out if this observation can be extended to human plasma.

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