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SEPARATION OF KININ-FORMING FACTORS IN HUMAN PLASMA

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SUMMARY.—The kinin-forming proteases in human serum were studied. Two serum kallikreins, *i.e.* enzymes acting directly and specifically on kinin-yielding globulin (kininogen), were separated by ion-exchange chromatography. They were differentiated from plasmin by their high ratio kinin-forming/esterolytic activity, by the absence of fibrinolytic activity, and by a lower Michaelis–Menten constant for the hydrolysis of an arginine ester. Their relationship to enzymes which induce kinin formation mainly by activating serum prekallikreins, was studied.

PLASMA kinins are released from α_2-globulins called kininogens by the action of endopeptidases collectively described as kininogenases. The principal kininogenase in human plasma is the highly specific enzyme plasma kallikrein. In normal physiological conditions, this enzyme circulates in blood as the inactive precursor prekallikrein which can be activated by the enzymic activators clotting Factor XII (Hageman Factor; Margolis, 1958) and Permeability Factor/Dilution (PF/Dil; Mason and Miles, 1962). The fibrinolytic protease plasmin can activate prekallikrein, and also liberate kinin from the substrate kininogen.

Like plasmin, kallikreins from plasma and tissues hydrolyse simple esters of arginine, particularly when the α-NH_2 group of the latter is acylated. Kallikreins do not act on numerous other substrates of plasmin, such as fibrinogen, fibrin, ACTH, antihaemophilic globulin (factor VIII), casein or protamine. The number and the detailed chemical and functional properties of human plasma kallikreins and of their activators are still not certain. Kagen, Leddy and Becker (1963), Kagen (1964) and Webster (1968) detected in human plasma only one enzyme which acted directly on kininogen, and could therefore be regarded as kallikrein. Other recent studies suggest that human plasma contains at least two kallikreins (Armstrong and Mills, 1964; Moriya, Yamazaki and Fukushima, 1965; Vogt, 1966; Oates and Melmon, 1966; Eisen, 1966).

However, some of the described kinin-forming factors could be activators of prekallikrein and not plasma kallikrein itself since the test systems used for assay did not always determine whether the enzyme under study liberated kinin directly from the substrate kininogen, or whether it acted by converting prekallikrein to kallikrein. Such an ambiguous situation arises when the investigated enzyme is mixed with substrates like fresh plasma, serum, or its pseudoglobulin fraction, which contain prekallikreins.

In vivo assays of kinin-forming activity, such as those based on increased vascular permeability (Miles and Miles, 1952), vasodilatation (Sarnoff, Sussman and Sarnoff, 1958) or lowering of the blood pressure (Frey, Kraut and Werle, 1950) also do not distinguish clearly between plasma kallikreins and activators of their precursors. Moreover, some of the *in vivo* effects may be produced by plasma proteases which do not form kinins. For example, the activated first component of complement (C_1'-esterase) increases vascular permeability through release of histamine.

The activity of plasma kallikrein and other kininogenases can be specifically measured only by the release of kinins from substrates free of prekallikreins, *e.g.* plasma heated at 60° for 1–3 hr, or purified kininogen.

In view of the divergent findings, the present experiments were carried out to study the number and some enzymic properties of kininogenases separated by ion-exchange chromatography of human serum.

MATERIALS AND METHODS

The following commercial preparations were used: diethyl-aminoethyl (DEAE) cellulose (pre-swollen, water content 75 per cent; Guri). N-Benzoyl-l-arginine ethyl ester (BAEe), l-lysine ethyl ester (LEe) and N-acetyl-l-tyrosine ethyl ester (ATEe) all from British Drug Houses. Alcohol dehydrogenase from yeast and Nicotinamide adenine dinucleotide (NAD) from Boehringer. Bovine fibrinogen (Armour). Soya bean trypsin inhibitor (SBTI; Worthington Biochem. Corp.); Synthetic bradykinin (Sandoz).

The following materials were generously provided: Trasylol (proteolytic inhibitor from bovine lung, 5128 inhibitor units/mg., and hog pancreatic kallikrein, 210 Frey units/mg. from Bayer, Leverkusen). Human plasmin (glycerol-activated; 10·4 Remmert-Cohen units/ml., 3·6 units/mg. protein; Michigan Dept. Health). Purified human permeability factor (PF/Dil.; Dr. B. Mason, Lister Institute, London).

Non-contact serum was prepared, avoiding contact with glass. Blood freshly collected by venepuncture from volunteers was rapidly centrifuged, and the plasma separated and allowed to clot. Non-contact plasma was prepared by the same procedure, but heparin 5 U/ml. was added to the blood. Heating of this plasma at 60° for 60 min. destroys the precursors of the kinin-forming enzymes, the bulk of their inhibitors and the kinin-digesting carboxypeptidase N; a large proportion of the substrate kininogen is preserved.

Anion exchange chromatography of deionized human serum was carried out with DEAE-cellulose (Peterson and Sober, 1962) in columns 32 × 2 cm. The columns were equilibrated with 7·5 mM-sodium phosphate buffer pH 8·0, conductivity 1·3 m-mhos/cm. Elution of the protein was started with the same buffer flowing at a rate of 30–40 ml./hr from a reservoir of 500 ml. A concentration gradient was produced by pumping into this reservoir a limiting buffer of 12·5 mM-phosphate + 1 M-NaCl (conductivity 60 m-mhos/cm.). Fractions (5–10 ml.) were collected by a volume-controlled collector.

Protein concentrations were measured by the method of Lowry, Rosebrough, Farr and Randall (1951), and by the optical density of fractions at 280 mμ. (E^{280}_{cm} = protein concentration (mg/ml); Dixon and Webb, 1966).

Kinins were assayed on the isolated rat uterus suspended in a 5 ml. bath in de Jalon's solution. An automatic biological assay apparatus was used with an iso- or auxotonic writing lever (Eisen, 1963).

Plasma kallikrein and other kininogenases were measured as follows: 0·01–0·2 ml. of chromatographic fraction or purified enzyme, 0·2 ml. of heated plasma substrate, and buffer to 0·6 ml. were mixed and incubated at 37°, and the kinin formed measured as bradykinin after 7 min. Incubation for 7 min. was sufficient to detect even low concentrations of kininogenase. Results were not calculated for 1 min. periods because no proof had been obtained that the kinin formation from 0–7 min. proceeded at a constant rate.

Kininogenase + activator of prekallikrein was measured by using as substrate untreated non-contact plasma, instead of heated plasma, and estimating the kinin formed after 6 min. at room temperature. This incubation period and temperature reduced the influence of the kallikrein- and kinin-inactivating factors in unheated plasma. If the ratio:

$$\frac{\text{effect on unheated plasma}}{\text{effect on heated plasma}}$$

produced by a fraction was higher than the ratio produced by a pure kininogenase, the fraction was assumed to contain activator of prekallikrein.

Kininogen content. Fractions or heated plasma were incubated with high concentrations of hog pancreatic kallikrein (18 Frey units/ml.), and the maximum kinin level measured (usually attained in 2–5 min.).

Esterolytic activity: 1. Fractions or enzymes were incubated with BAEe or ATEe, and alcohol dehydrogenase and NAD, in sodium pyrophosphate–glycine buffer pH 8·7 with 0·075 M semicarbazide (Trautschold and Werle, 1961). The ethanol released by esterolysis was thus rapidly dehydrogenated. The concomitant reduction of NAD to NADH was continuously recorded at 340 mμ in a Unicam SP 800 spectrophotometer. 2. Hydrolysis of BAEe at pH 8·0 was measured at 253 mμ according to Schwert and Takenaka (1955).

The inhibitors Trasylol and SBTI were incubated with enzymes for 5 min. before substrates, buffers, etc., were added.

Fibrinolytic factors. The presence of plasmin and plasminogen activator was studied by applying 0·02 ml. of fractions to bovine fibrin plates (0·4 per cent), or mixing 0·2 ml. of fraction with 0·2 ml. of bovine fibrinogen (0·3 per cent) and 0·04 ml. of thrombin (20 U/ml.), and observing the lysis. Plasmin alone was measured on fibrin plates in which the contaminating plasminogen had been destroyed by heating at 85° for 30 min. (Astrup and Mullertz, 1952).

Electrophoresis of fractions was carried out on cellulose acetate strips 12 × 2·5 cm., in barbitone buffer, pH 8·6, 0·07 M, and applying 0·5 mAmp/cm. strip width.

RESULTS

Chromatography of untreated human serum on DEAE-cellulose

The kininogenases recovered from different sera varied in potency, but their distribution in relation to the eluted proteins was similar in all experiments. Fig. 1 presents the results of a typical experiment. A first peak of kininogenase activity was associated with the protein which passed through DEAE-cellulose without being bound, and was collected with buffer of the initial conductivity (1·3 m-mhos/cm.). Electrophoresis on cellulose-acetate membranes showed that this peak contained mainly γ-globulin. These findings were similar to those of Kagen *et al.* (1963) and Kagen (1964) who thought that this γ-globulin kininogenase was plasma kallikrein.

A second peak of kininogenase activity was eluted at a conductivity of 2–4 m-mhos/cm., and was followed at a conductivity of 7–9 m-mhos/cm. by the bulk of the anionic proteins bound by DEAE-cellulose. The second kininogenase peak had the electrophoretic mobility of a β-globulin.

To ensure that this second serum kininogenase was not merely the tail-end of the kininogenase in the unbound proteins, buffer of starting conductivity was in some experiments pumped through the column until the peak of unbound protein

was followed by several protein-free fractions (Fig. 1); only then was the gradient of ionic concentration commenced.

It was also possible that the heated plasma substrate still contained remnants of prekallikrein. Fractions might then have formed kinin by activating this prekallikrein, rather than by acting on kininogen directly. To exclude this possibility, heated plasma was shaken with kaolin 20 mg./ml., and then separated. The kaolin would activate and adsorb remaining prekallikrein. However,

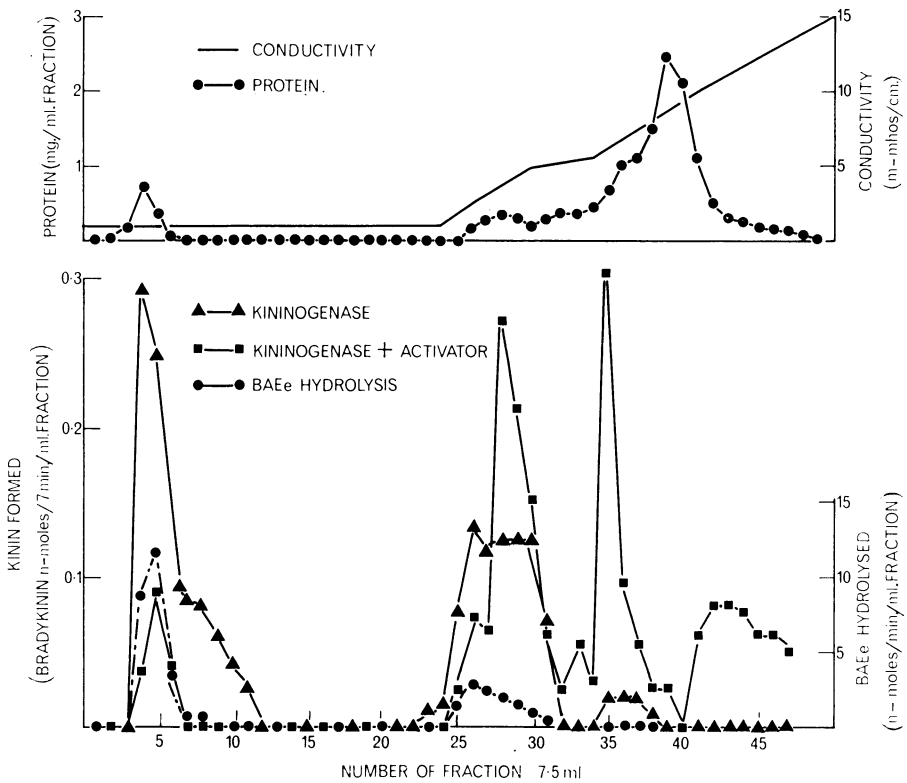

FIG. 1.—Chromatography of 4 ml. of deionized human serum on DEAE-cellulose in a column 23 × 2 cm. Starting buffer 7·5 mM sodium phosphate pH 8·0. Concentration gradient with 12·5 mM phosphate + 1M-NaCl, pH 8·0 commenced after fraction14. Kinin-forming activities were measured at pH 8·0, BAEe hydrolysis at pH 8·7.

treatment with kaolin did not induce kinin formation in heated plasma, and did not abolish its response to the kininogenases in the first and second peak. The finding of a second kininogenase in human serum, which was anionic at pH 8, did not agree with Kagen's (1964) report that this kinin-forming factor behaved like the β-globulin PF/Dil., in that it activated prekallikrein but did not attack kininogen directly.

Little or no kininogenase was found in the main peak of protein bound to DEAE-cellulose. This peak contained kininogen from which pancreatic kallikrein could release kinin equivalent in some cases to 1 n-mole of bradykinin per ml. of fraction (0·16 n-mole/mg. protein).

Similar results were obtained with ion-exchange chromatography of plasma. However, the separation of proteins was less satisfactory.

These experiments thus confirm that human serum contains at least two kininogenases: one which is cationic at pH 8 and not bound by DEAE-cellulose, and a more anionic kininogenase which is bound by DEAE-cellulose.

Chromatography of sera treated with acetone

Incubation with acetone is one of the oldest procedures for isolating plasma kallikrein (Frey *et al.*, 1950) and is still extensively used for this purpose (Webster and Pierce, 1961; Moriya, 1965; Webster, 1968). Its use is based on the fact that it destroys the plasma factors which inactivate kallikrein. The ensuing slow activation of plasma prekallikrein requires Factor XII (Eisen and Keele, 1960; Webster and Ratnoff, 1961).

Pre-treatment of serum with acetone, accordng to Moriya (1965), was used in the hope that it would lead to a more complete activation of the prekallikreins, and less decay of kallikreins during ion exchange chromatography.

The results may be summarized as follows: 1. Kininogenases of higher purity were obtained; the activity per mg. protein was much higher in both peaks. Esterolytic potency per mg. protein was only slightly increased (Table I). 2. The second peak of kininogenase appeared as a broad shoulder. It was followed by the main body of anionic protein which contained a separate, fairly high peak of esterolytic activity, but very little or no kininogenase.

TABLE I.—*Kininogenase Activity and BAEe-hydrolysis in Serum, Acetone Treated Serum, Plasmin and Pancreatic Kallikrein*

	Kininogenase n-moles Bk/mg./7 min.		BAEe hydrolysed n-moles/mg./7 min.		Kininogenase × 10^3 / BAEe-hydrolysis	
Kinin-forming peak	1st	2nd	1st	2nd	1st	2nd
Serum	0·4	0·96	70	140	5·7	6·8
Acetone-treated serum	4·0	3·7	105	147	38·1	24·6
Plasmin	7·2		2180		3·3	
Pancreatic kallikrein	3465		156000		22·2	

Figures give n-moles of bradykinin (Bk) released or BAEe-hydrolysed in 1 min./mg. of protein in serum fractions or enzyme preparations. The right hand part of the table shows the ratios between the two activities.

Presence of enzymes which activate prekallikreins

It was possible that in addition to kininogenases, the kinin-forming peaks contained also activators of a precursor of plasma kallikrein, or that one of the enzymes detected had both these actions. To detect the presence of activators, fractions were incubated not only with enzyme-free heated plasma (P_{60}), but also with fresh non-contact plasma (P) or serum in which they could activate the precursors of kinin-forming enzymes as well as attack kininogen directly (Fig. 1). Although in P an enzyme may induce kinin formation in two ways, the total effect may be smaller than in P_{60}, since heating plasma also destroys the inactivators of enzymes and of kinins. When the ratios of kinin formed by different fractions in fresh and in heated plasma (P/P_{60}) are compared, fractions containing both activator and kininogenase will be relatively more effective on fresh plasma, and

thus produce higher P/P_{60} ratios, than will fractions containing only kininogenases. The first and second kininogenase peaks in any chromatographic run produced similar P/P_{60} ratios. These ranged from 0·7–2·8, which was of the same order as the ratios produced by the pure kininogenase, hog pancreatic kallikrein (0·8–1·0), and much lower than the ratios obtained with the activator human PF/Dil. (> 30).

These experiments do not support the view that the kinin-forming activity of the second peak is entirely due to activation of prekininogenases. However, in acetone-treated sera and in some untreated sera, this second peak was broad. As the fraction number advanced, the effect on P increased and the effect on P_{60}

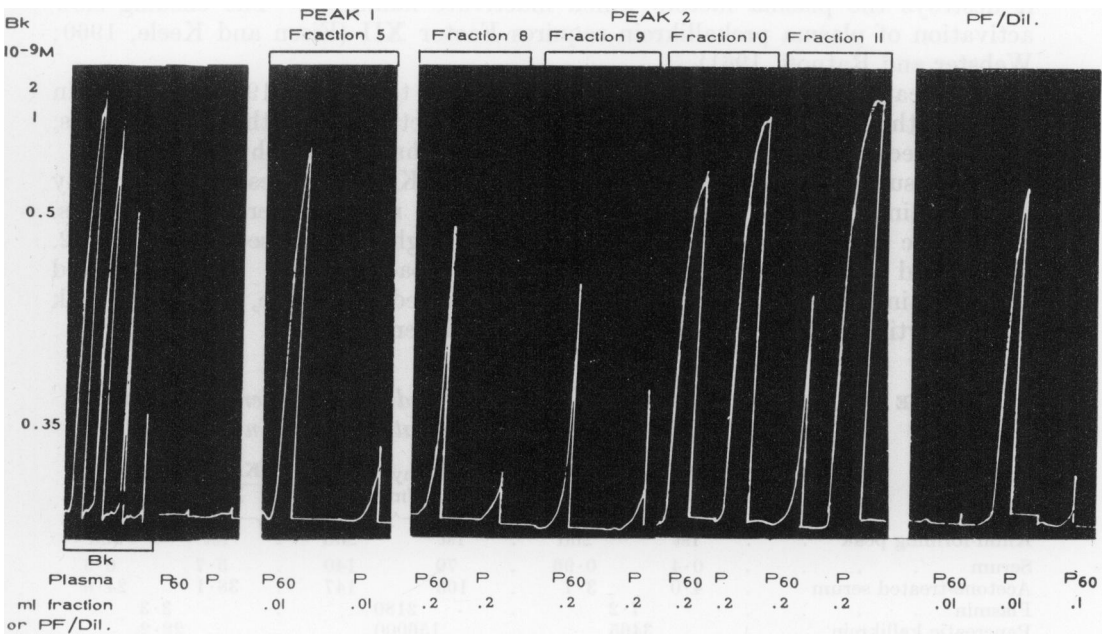

FIG. 2.—Kinin formation by fractions from acetone-treated serum. Rat uterus in 5 ml. bath. Bk = responses to 0·35, 0·5, 1 and 2 × 10^{-9} M bradykinin (recorded at slower speed). To measure kininogenase, 0·2 ml. of heated non-contact plasma (P_{60}) was incubated with the specified volume of fraction or human permeability factor (PF/Dil.), and buffer added to 0·6 ml; 0·2 ml. of the mixture was tested after 7 min. at room temperature. To measure kininogenase + activator, P_{60} was replaced by untreated non-contact plasma (P); 0·2 ml. was tested after 7 min. at 37°.

decreased (Fig. 2). Thus only the early fractions of this peak acted like kininogenases; later fractions appeared to contain mainly activator.

Hydrolysis of benzoyl-arginine ethyl ester (BAEe)

All fractions with potent or moderate kininogenase activity also split esters. In some experiments, the fractions containing the bulk of anionic proteins hydrolysed BAEe at considerable rates, although no kininogenase activity was detected. As Table I shows, the ratios kininogenase/BAEe-hydrolysis of the kinin-forming fractions from acetone-treated sera were higher and comparable to the ratio found

with the specific kininogenase pancreatic kallikrein. The ratio produced by a non-specific kininogenase (glycerol-activated human plasmin) was considerably lower.

The hydrolysis of arginine esters offers a convenient way of measuring the potency of kallikreins, and Trautschold, Fritz and Werle (1966) have recently recommended this activity as the basis for kallikrein units. The value of such a basis is however limited by the fact that arginine esters are split by numerous plasma and tissue proteases, which are not always readily distinguished from kallikreins and other kininogenases.

The kinetics of BAEe hydrolysis by fractions were therefore studied and compared with those of plasmin. Differences in these characteristics would help

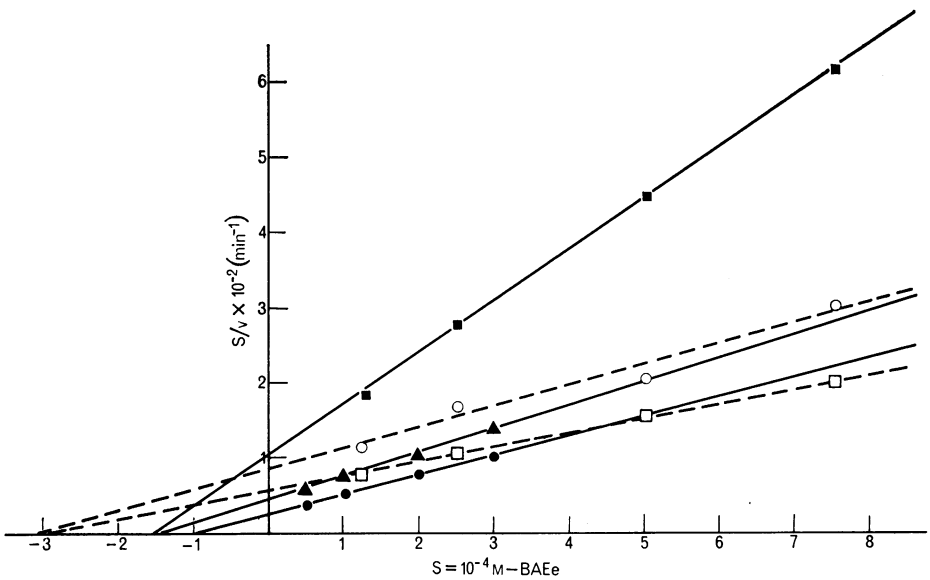

FIG. 3.—To determine the Michaelis–Menten constant (Km), a constant concentration of fraction or of plasmin was incubated at pH 8·7 and 25° with several concentrations of the substrate BAEe (S, abscissa). The dehydrogenation of the ethanol released from BAEe was continuously recorded (see Materials and Methods). The readings from the 4–8th min. after addition of enzyme were used for calculation of the rate of BAEe splitting (v). S/v (ordinate) was plotted against S according to Hanes (1932), and the regression line Y calculated by the method of least squares. The intercept of Y with the abscissa gives −Km.

▲——▲ First Kininogenase Peak
$Y = 0{\cdot}447 + 0{\cdot}314x$
$K_m = 1{\cdot}40 \times 10^{-4}$

●——● Second Kininogenase Peak
$Y = 0{\cdot}258 + 0{\cdot}258x$
$K_m = 1{\cdot}00 \times 10^{-4}$

■——■ Late Esterolytic Peak
$Y = 1{\cdot}038 + 0{\cdot}688x$
$K_m = 1{\cdot}51 \times 10^{-4}$

○——○ Plasmin (20 mμ/ml.)
$Y = 0{\cdot}855 + 0{\cdot}282x$
$K_m = 3{\cdot}03 \times 10^{-4}$

□——□ Plasmin (40 mμ/ml.)
$Y = 0{\cdot}572 + 0{\cdot}194x$
$K_m = 2{\cdot}95 \times 10^{-4}$

differentiation between kallikreins, plasmin and other proteases which attack arginine esters.

The esterolytic factors detected in the fractions hydrolysed BAEe at pH 8·7 with Michaelis–Menten constants (Km) lying between 1–2 $\times$ 10^{-4} M (Fig. 3). No clear difference between the first and second kininogenase, and the late esterolytic peak was found. The Km of human glycerol-activated plasmin was consistently higher (3 $\times$ 10^{-4} M).

Hydrolysis of acetyl-tyrosine ethyl ester (ATEe) and of lysine ethyl ester (LEe)

The first component of complement is converted into an esterolytic enzyme (C'1-esterase) by some of the procedures which activate plasma prekallikrein. C'1-esterase splits ATEe somewhat faster than BAEe and other arginine esters. To check whether any of the BAEe hydrolysis by serum fractions was due to C'1-esterase, their action on ATEe was tested.

LEe is hydrolysed by trypsin and plasmin. No hydrolysis by kallikreins from plasma or tissues was found (Webster and Pierce, 1961; Trautschold and Werle, 1961).

None of the fractions acted on ATEe or on LEe. The experiments allowed the conclusion that BAEe was hydrolysed at least 20 times faster than LEe or ATEe.

Fibrinolytic activity

The content of fibrinolytic factors in the serum fractions was examined on standard bovine fibrin clots and on unheated and heated fibrin plates. No active plasmin was detected in any of the fractions.

Effects of inhibitors

Oates and Melmon (1966) thought that the anionic kallikrein found in human plasma was secreted by kallikrein-producing tissues. It seemed therefore of interest to determine whether the second peak of kininogenase activity was, like the cellular kallikreins, effectively inhibited by the proteolytic inhibitor from bovine lung (Trasylol), and not affected by SBTI. Table II, A and B, shows that both kininogenases in human plasma were far more resistant to Trasylol, and far more readily inhibited by SBTI, than was pancreatic kallikrein.

DISCUSSION

The present findings strongly suggest that amongst the kinin-forming factors in human plasma which are bound by DEAE-cellulose at pH 8·0 there are one or more kininogenases, *i.e.* enzymes releasing kinins directly from kininogens. This differs from the results of Kagen *et al.* (1963), Kagen (1964) and Webster (1968) who found in these fractions an activator of prekallikrein, but no kininogenase. However, these workers assayed kinin-forming activity by incubating enzymes and substrates in the isolated organ bath and observing the development of kinin for 1 min. In the present study, enzymes and substrates were incubated in higher concentrations, and samples of the mixture applied to the organ bath after 7 min.

The absence of fibrinolytic activity shows that neither of the two kininogenases is plasmin. As postulated by Kagen *et al.* (1963), the kininogenase found in the

TABLE II.—*Inhibition of Kinin-forming Enzymes from Pancreas and Serum by Trasylol and SBTI*

A. BAEe-hydrolysis

	Residual activity (per cent)		
Trasylol IU/ml.	Pancreatic kallikrein	Peak 1	Peak 2
0	100	100	100
0·02	88	—	
0·1	60	—	—
0·14	33	—	—
0·2	18	—	—
0·4	16	—	—
50	—	72·7	78·3
100	—	66·2	54·4
200	—	62·4	45·7
400	—	57·9	35·1
SBTI μg./ml.			
50	100	73·6	40

B. Kininogenase activity

Trasylol IU/ml.	Pancreatic kallikrein	Peak 1	Peak 2
5	6	57	83
10	—	28	57
50	—	11	23
SBTI μg./ml.			
100	100	33	50

γ-globulins may be identified with plasma kallikrein and Vogt's (1966) kininogenase I. The relations of the more anionic kininogenase to kininogenase II (Vogt, 1966) and to " kinin-forming substance " (Armstrong and Mills, 1964) remain to be determined.

The two plasma kininogenases demonstrated in the present work, resembled in their actions on heated and unheated plasma the pure kininogenase pancreatic kallikrein. However, the possibility that one or both of these kininogenases may be activators of prekallikrein as well cannot be fully excluded. Several proteases in plasma and elsewhere, which hydrolyse peptide bonds formed by an arginine or lysine carboxyl group, also appear to be converted from the pre-active to the active state by fission of such a bond (Esnouf and MacFarlane, 1968; Ratnoff and Naff, 1967). Examples are: thrombin, clotting factor X, plasmin, C'1-esterase and trypsin. As a result, many of these proteases activate their own precursors (autocatalysis), and/or the precursors of the other proteases. The distinction between kininogenases and their activators may therefore be in some cases only a matter of degree.

Plasma and tissue kallikreins produce much higher kininogenase/BAEe-hydrolysis ratios than do non-specific kinin-forming proteases like plasmin and trypsin. Such high ratios were obtained with the first and second kininogenase peak from acetone-treated serum, but not from untreated serum. This emphasizes the difficulties of assaying plasma kallikreins by ester hydrolysis. Arginine esters are also split by clotting factors II (thrombin), X, XI, and possibly XII (Esnouf and MacFarlane, 1968), permeability factor (PF/Dil.; Becker, Wilhelm and Miles, 1959), and one or more enzymes of the complement system (Lachmann,

1969). The position is further complicated by the fact that some of these enzymes may also attack kininogens.

Differentiation between some of the proteases acting on arginine bonds may possibly be achieved by kinetic analyses of the reaction. In the present study, the Km of the plasma kininogenases was consistently lower than that of plasmin.

The γ-globulin plasma kallikrein differs from tissue kallikreins by its more cationic character, and by its different response to SBTI, Trasylol, and other proteolytic inhibitors (Frey *et al.*, 1950; Webster and Pierce, 1961). The plasma kininogenase found in the second peak appears to be a β-globulin, but otherwise closely resembles plasma kallikrein; no functional difference has yet been detected. The effects of Trasylol and SBTI argue against the view that this second kininogenase in normal plasma is derived from kallikrein-secreting cells. Secretion from such cells into blood has been convincingly demonstrated in the carcinoid and dumping syndromes (Oates and Melmon, 1966; Zeitlin and Smith, 1966), but not yet in healthy subjects or animals.

Whether the two specific plasma kininogenases can be distinguished by their actions on the human kininogens I and II described by Pierce and Webster (1966), Vogt (1966), and Jacobsen and Kriz (1967), remains to be investigated.

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