

# Studies on the Prekallikrein (Kallikreinogen)—Kallikrein Enzyme System of Human Plasma

## II. EVIDENCE RELATING THE KAOLIN-ACTIVATED ARGININE ESTERASE TO PLASMA KALLIKREIN

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**ABSTRACT** Evidence is presented in this paper that the kaolin-activated arginine esterase of plasma is related to plasma kallikrein activity. Such a relationship is based on studies that (1) establish a constant ratio of esterase activity on various synthetic substrates for the kaolin-activated arginine esterase, purified kallikrein(s), and preparations obtained during the fractionation procedure; (2) exclude other known plasma and tissue arginine esterases; (3) confirm the requirement for factor XII in the activation of the enzyme precursor; and (4) show similarities in behavior between the plasma esterase and purified kallikrein(s) toward a variety of inhibitors.

Based on this probable identification, evidence is provided that the concentration of active factor XII determines the rate of activation of plasma kallikreinogen, and that the activation may be blocked by polybrene. Once activated, plasma kallikrein is rapidly inactivated by the naturally occurring plasma inhibitor, but the inhibition is incomplete. Acid or chloroform treatment of plasma rapidly inactivates the plasma inhibitor without affecting the concentration of plasma kallikreinogen.

Another plasma arginine esterase with properties suggestive of permeability factor is activated by factor XII in the presence of synthetic substrates, but only at low ionic strength. The data suggest that this enzyme is closely related to plasma kallikrein and that it arises from a common precursor.

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## INTRODUCTION

The appearance of large amounts of arginine esterase activity in human plasma after exposure of the latter to kaolin (1) prompted the purification and identification of the enzyme(s) involved. As shown in the preceding paper (2), this study resulted in the isolation of three enzymatically active fractions that, while exhibiting some differences in chemical and physical properties, possessed a similar biologic activity to that described for plasma kallikrein. Since the relation of these three fractions to each other, or their identification as separate entities remains to be clarified, the total enzymatic activity will henceforth be referred to as plasma kallikrein.

The studies reported in this paper further establish the relationship of the kaolin-activated arginine esterase activity of plasma with plasma kallikrein. This result was accomplished (a) by demonstrating the similarity of the former with purified kallikrein preparation, both with respect to substrate specificity and inhibition by natural (3) and synthetic inhibitors; and (b) by distinguishing the kaolin-activated plasma arginine esterase activity from other known basic amino acid esterases in plasma and tissues.

Since kaolin is a known activator of Hageman factor (factor XII), experiments also were performed to clarify the role of this factor in the activation of plasma kallikreinogen, the naturally occurring inactive precursor of plasma kallikrein. Some insight into the nature of this interaction stemmed from the observation of Margolis (4) who demonstrated that a substance producing pain and acting on smooth muscle was released by the action



of glass on plasma, however not if factor XII was absent. Webster and Ratnoff (5) then suggested that Hageman factor may be responsible for activation of kallikrein from an inactive plasma precursor, kallikreinogen. Our observations not only confirm the importance of the activation of Factor XII in the conversion of kallikreinogen to kallikrein, but describe some of the kinetics involved in this interaction.

Another plasma globulin, PF/dil, or permeability factor, first described by MacKay, Miles, Shacter, and Wilhelm (6) in guinea pigs has been tentatively identified in human serum (7) and found to increase capillary permeability in guinea pigs. This enzyme is also activated by active factor XII (8), possesses arginine esterase activity, and releases kinins from normal plasma. Unlike kallikrein, it does not release kinins from plasma heated at 56°C for 2 hr (9), apparently requiring a heat labile plasma component as well. Furthermore, it has been observed (10) that the permeability-producing activity of PF/dil is completely inhibited by heparin in a concentration of 500  $\mu$ /ml, whereas kallikrein is not; since PF/dil has been proposed (8) as an activator of plasma kallikrein, the possible relationship of PF/dil to the kaolin-activated arginine esterase was also explored in this investigation.

## METHODS

**Enzymes and activators.** Human thrombin<sup>1</sup> and bovine trypsin, twice crystallized salt free,<sup>2</sup> were used in concentrations as described previously (11). Human plasmin was prepared by activation of human plasminogen by streptokinase; the plasmin was isolated by precipitation at pH 2 with 1.0 M NaCl and stored at -20°C at pH 2 in concentrations of 100 casein units/ml before use (12). Urinary kallikrein was a partially purified human preparation obtained through the kindness of Dr. Marion E. Webster and contained 5.6 Frey units/mg. Streptokinase was a highly purified preparation (600 U/ $\mu$ g N) obtained through the courtesy of Lederle Laboratories, Pearl River, N. Y. Thromboplastic extract (Simplastin),<sup>3</sup> kaolin NF colloidal,<sup>4</sup> and ellagic acid<sup>5</sup> were used.

Assay of enzyme activity was performed with substrates noted in the previous communication (2); final concentration of substrate was 0.015 M, except when otherwise indicated. Measurement of esterase activity was accomplished by quantification of the methanol released from the substituted basic amino acid methyl esters, using a modification (13) of the procedure of Siegelman, Carlsen, and Robertsen (14). In this method the methanol released is oxidized to formaldehyde by potassium permanganate and, after the removal of excess permanganate by sodium sulfite, the

formaldehyde is coupled with chromotropic acid to yield a purple-colored compound; this absorbed maximally at 580 m $\mu$  and followed Beer's law to an absorbancy reading of 0.900 in a Beckman spectrophotometer. By reference to a standard methanol curve, which was checked frequently by running known methanol concentrations along with test solutions, the micromoles of ester hydrolyzed were calculated and expressed as  $\mu$ moles hydrolyzed/ml plasma per hr. Activation studies were performed by adding plasma to kaolin (final concentration 10 mg/ml) or to ellagic acid (final concentration  $5 \times 10^{-5}$  M), for time indicated in text.

**Miscellaneous agents and reagents.** The buffer was 0.1 M sodium phosphate, pH 7.6, in 0.15 M sodium chloride, except where otherwise noted. *Chloroform-treated plasma* was prepared by adding equal volumes of chloroform and plasma at 2°C, shaking for 1 min, and centrifuging at 2°C for 5 min at 2000 g; the top two-thirds of the upper layer was removed and used for the experimental observations. *Acid-treated plasma* was prepared by adding one volume 1/6 N HCl (final pH 2), then allowing the mixture to incubate 25°C for 15 min before neutralizing with 1 volume of 1/6 N NaOH and 1 volume of 0.1 M phosphate buffer, pH 7.6, in 0.15 M NaCl. *Defibrinated plasma* was prepared by adding bovine thrombin<sup>6</sup> (final concentration 1 U/ml) and winding out fibrin. The plasma was then incubated at 37°C for 30 min to destroy residual thrombin; such plasma had no demonstrable arginine esterase activity before kaolin activation. Inhibitors were as described in the preceding paper (2) and their concentrations are indicated in the text. *Plasma samples* for these studies were single specimens obtained from blood drawn directly into plastic syringes. The specimens were transferred to siliconized tubes containing 1 volume 3.8% sodium citrate for 9 volumes blood, and centrifuged at 2000 g for 15 min at 5°C. After separation, the plasma was studied immediately or frozen in siliconized tubes until assayed.

## RESULTS

**Observations during the purification of plasma kallikrein and comparison of its esterase activity with that of the kaolin-activated plasma enzyme.** The purification of plasma kallikrein reported in the preceding paper was initiated by screening various plasma fractions prepared by the method of Cohn et al. (15) for their kaolin-activated arginine esterase activity. As previously discussed, the early stages of purification (Table I, preceding paper) were attended by an increase in spontaneous activation of the enzyme with a consequent decrease in the amount of precursor that could be activated after exposure to kaolin. Thus in a typical purification procedure, the following percentage of total activity, i.e. that observed in the presence of kaolin, was present in the spontaneously active form: Cohn fraction IV<sub>1</sub>, 29.0%; saline supernatant, 22.6%; crude ceruloplasmin-containing precipitate, 64%; and CM-Sephadex peaks A + B, 100%. Moreover, the ratios of substrate hydrolysis to the one observed for TAME (henceforth referred to as substrate ratios) of the different preparations at various stages of purification were

<sup>1</sup> Obtained through the courtesy of Dr. Kent Miller; it was provided as a highly purified preparation containing 1600 NIH U/ml.

<sup>2</sup> Mann Research Laboratories, Inc., New York.

<sup>4</sup> Mallinckrodt Chemical Works, St. Louis, Miss.

<sup>3</sup> Warner-Chilcott Laboratories, Morris Plains, N. J. A 20-determination vial was dissolved in 4-ml distilled water before use.

<sup>5</sup> K-K Laboratories, Long Island City, N. Y.

<sup>6</sup> Parke Davis and Co., Detroit, Mich.



TABLE I  
Hydrolysis of Various Arginine and Lysine Esters by  
Plasma and Tissue Basic Amino Acid Esterases

Enzyme preparation	Source	Substrate/TAMe			
		Ratio:	BAMe	AAMe	TLMe ALMe
Kaolin-activated arginine esterase	Human plasma		0.89	0.66	0.22 0.15
Thrombin	Human plasma		1.00	1.30	1.70 0.40
Plasmin	Human plasma		1.01	0.49	1.48 2.35
Trypsin	Bovine pancreas		0.18	0.51	1.00 0.73
Kallikrein	Human urine		0.81	0.07	0.25 0.23

essentially constant, no difference being observed between the spontaneously activated enzyme and that activated by kaolin. The hydrolysis of four substituted arginine and lysine esters at a single substrate concentration, under similar test conditions, is shown for the kaolin-activated plasma arginine esterase in Table I. When these substrate ratios are compared with the ratios determined for the three purified kallikrein preparations (Table III, preceding paper), no substantial difference is observed with respect to the arginine substrates. The slight differences observed in comparing the lysine substrates are probably due to the small degree of the hydrolysis which yields a proportionately greater error in the determination.

*Comparison of kaolin-activated plasma arginine esterase with other basic amino acid esterases.* The substrate/TAMe ratios observed for the kaolin-activated plasma arginine esterase and for purified plasma preparations of human thrombin, human plasmin, bovine trypsin, and urinary kallikrein are indicated in Table I. Distinct differences existed between these various enzymes. The kaolin-activated enzyme is primarily an arginine esterase and it contrasts with plasmin, which hydrolyzes lysine esters most readily. The kaolin-activated arginine esterase also differs from thrombin that displays its highest catalytic activity toward TLMe, from trypsin that has little affinity for BAMe, and from human urinary kallikrein that did not hydrolyze AAMe appreciably.

Since active PTA (factor XI) is a known arginine esterase and this enzyme would be activated by kaolin in plasma, it was important to exclude this entity as responsible for kaolin-activated arginine esterase activity. This was accomplished by demonstrating that plasma severely deficient in factor XI showed a kaolin-activated arginine esterase activity comparable in amount to normal plasma (1).

Studies were also conducted on the hydrolysis of acetyl tyrosine methyl ester (ATMe) by plasma, but

since no enhancement was observed after kaolin activation, the increased arginine esterase activity could not be ascribed to C'1 esterase.

*Independence of the kaolin-activated arginine esterase activity from other known major arginine esterases in plasma.* The other major arginine esterases in plasma, i.e. thrombin and plasmin, were directly compared by separate or simultaneous activation of siliconized plasma with kaolin, thromboplastin-calcium, or streptokinase. As shown in Table II, the exposure of normal siliconized plasma to kaolin resulted in an arginine esterase activity comparable in amount to that activatable from prothrombin and plasminogen. However, the esterase activities were independent as evidenced by the additional hydrolysis produced by any combination of these three enzymes. Under the conditions of these experiments, the amount of hydrolysis observed was not strictly additive, for at the concentration of substrate employed, none of these enzymes was saturated; this allowed for enzyme competition for the substrate.

*Activation of kaolin-activated plasma arginine esterase by Hageman factor.* Kaolin was added to mixtures of normal and Hageman factor-deficient plasma and after 1 min, the esterase activity of the mixtures were then measured. The data shown in Fig. 1 were derived from studies with CBZ-arginine methyl ester, though similar observations were made with TAMe and BAMe. Note that the level of esterase activity in the factor XII-deficient plasma was very low and corresponded to that

TABLE II  
Arginine Esterase in Normal Siliconized Plasma\*

Activator	Enzyme	$\mu\text{M TAMe}$ HYD/ml plasma per hr.
Kaolin, 10 mg/ml	Kallikrein	77.5
Streptokinase, 10,000 U/ml	Plasmin	96.0
Thromboplastin/Ca <sup>++</sup>	Thrombin	78.0
Kaolin plus streptokinase	Kallikrein plus plasmin	158.0
Kaolin plus thromboplastin/Ca <sup>++</sup>	Kallikrein plus thrombin	137.0
Streptokinase plus thromboplastin	Plasmin plus thrombin	125.0

\* Experimental technique as follows: To 0.2 ml normal human siliconized defibrinated plasma was added either 10 mg solid kaolin, 0.2 ml streptokinase (10,000 U/ml), 0.4 thromboplastin extract in 0.040 M CaCl<sub>2</sub> or mixtures of any two of these, and sufficient buffer (0.1 M phosphate, pH 7.6, in 0.15 M NaCl) to make a final volume of 0.8 ml. After shaking and incubating for precisely 1 min at 25°C, 1.4 ml of ice cold 0.05 M TAMe was added and assay performed as indicated in Methods.



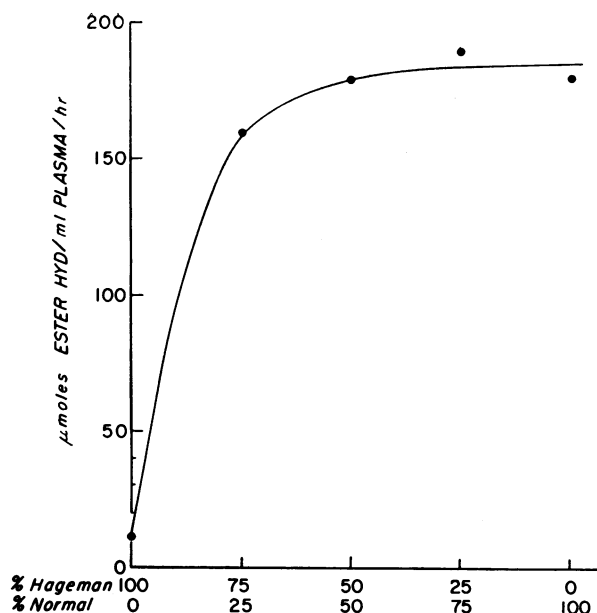


FIGURE 1 Effect of addition of normal to Hageman factor-deficient plasma on kaolin-activated arginine esterase. Mixtures of normal human siliconized plasma and Hageman-deficient siliconized plasma were made in the proportions as indicated on the abscissa. 0.2 ml of the plasma mixture was incubated with 2.0 ml of 0.015 M CBZ-AMe in buffer containing 10 mg kaolin. Ester hydrolyzed after 15 min incubation at 37°C was measured; results expressed as  $\mu$ moles hydrolyzed/ml plasma per hr (ordinate).

found in normal plasma before activation (1); in addition, that relatively small amounts of normal plasma were sufficient to restore the esterase activity in Hageman-deficient plasma to levels characteristic of kaolin-activated normal plasma. It will be noted that the mixture of 25% normal-75% Hageman-deficient plasma hydrolyzed 160  $\mu$ moles CBZ-AMe/ml plasma per hr. The amount of normal plasma in this mixture when tested separately hydrolyzed only 44  $\mu$ mole/ml plasma per hr, which is equivalent to 25% of the value shown for the 100% normal plasma (Fig. 1). This experiment, therefore, suggested that Hageman factor-deficient plasma contained normal levels of the inactive precursor of the arginine esterase (kallikreinogen), but that Hageman factor was required for its elaboration.

Using normal plasma treated with chloroform, so as to remove the plasma kallikrein inhibitor (see below), a kinetic study of the activation of the enzyme by ellagic acid was performed. Ellagic acid was chosen in preference to kaolin because activation is much slower and ellagic acid is soluble. Shown in Fig. 2 is the time dependence for the appearance of active enzyme. Kinetic analysis of the data, shown in the insert, reveals that the time dependence is a linear function of the log of maximum activity divided by the amount of unactivated

enzyme. This finding indicates that activation under these conditions follows pseudo first-order kinetics with kallikreinogen in excess, and suggests that the formation of the active arginine esterase from its plasma precursor is proportional to the concentration of activated Hageman factor.

Further evidence that activated factor XII is responsible for conversion of the arginine esterase to an active form was suggested by experiments employing hexadimethrine (polybrene) and chloroform-treated plasma. As shown on the left of Fig. 3, polybrene, a known inhibitor of activated Hageman factor, blocked the ellagic acid-mediated activation when added before activation but had no effect on the enzyme after activation. Trasylol, a potent inhibitor of plasmin and of pancreatic kallikrein, in a concentration of 200 KI units/ml plasma, proved to have an action similar to polybrene (Fig. 3, right); it was a much more potent inhibitor of the activation process than of the active enzyme, although it did inhibit the latter by approximately 25%, a figure that compares well with the 30-35% inhibition of the three purified plasma kallikrein preparations by Trasylol (Table III, preceding paper).

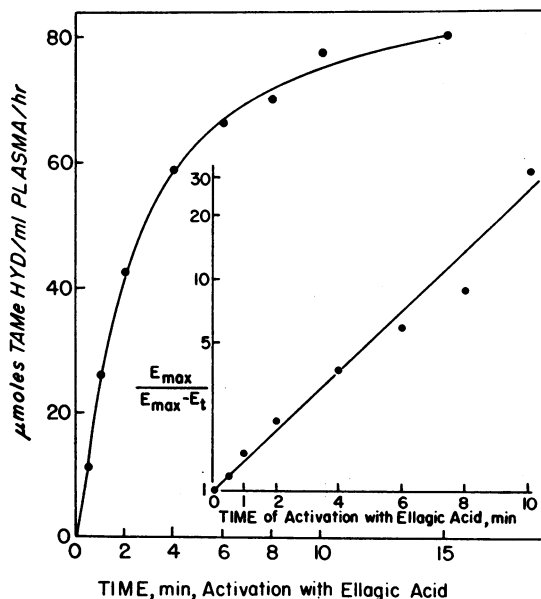


FIGURE 2 Kinetics of ellagic acid activation of plasma arginine esterase. Equal volumes of ellagic acid ( $10^{-4}$  M) in 0.1 M phosphate, pH 7.6, 0.15 M NaCl and chloroform-treated plasma were mixed and incubated at 25°C. At various times, 0.4 ml of activation mixture was added to 1.6 ml ice cold 0.015 M TAME in buffer containing a final concentration of 20  $\mu$ g polybrene/ml. Esterase activity assayed as indicated in Methods. Kinetic analysis of the data is shown in insert, where abscissa is time of activation and, on the ordinate,  $E_{max}$  = enzyme activity after 15 min incubation and  $E_t$  = enzyme activity at the various times of observation.



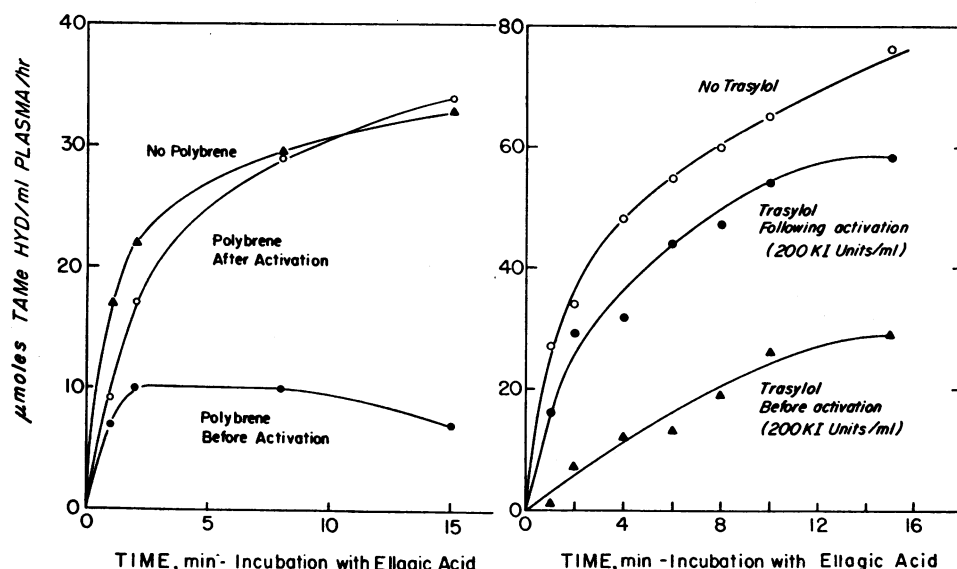


FIGURE 3 Effect of polybrene (left) and Trasylol (right) on the course of ellagic acid activation of plasma arginine esterase and on the fully activated enzyme. For control observation, equal volumes of ellagic acid  $10^{-4}$  M in 0.1 M phosphate, pH 7.6, 0.15 M NaCl and chloroform-treated plasma were mixed and incubated at  $25^{\circ}\text{C}$ . At various times 0.4 ml of the activation mixture was removed and added to 1.8 ml buffer containing TAME (final concentration in reaction mixture, 0.015 M). To study effects of polybrene and Trasylol on activation reaction, these inhibitors were incorporated into the activation mixture and incubated for various times *before* addition of substrate as above. To study their effects on the active enzyme, polybrene and Trasylol were incorporated into the substrate solution and added at various times *after* the incubation. Final concentration in the assay mixture was polybrene, 20  $\mu\text{g}/\text{ml}$ , and Trasylol, 200 U/ml. Esterase activity (ordinate) measured as noted in Methods; time of incubation (abscissa).

*Comparison of the action of inhibitors on kaolin-activated arginine esterase and purified plasma kallikrein preparations.* Since DFP at a concentration of  $10^{-4}$  M was found to completely inhibit all three purified plasma kallikrein preparations, a study of the effect of DFP on the ellagic acid-activated arginine esterase in chloroform-treated plasma was undertaken. DFP, at a concentration of  $2.5 \times 10^{-4}$  M, produced a logarithmic decline in esterase activity with a half-life at  $37^{\circ}\text{C}$  of 20 min (Fig. 4). The exponential decrease was characteristic of a pseudo first-order reaction in which the DFP is in excess and the decay proportional to the concentration of active enzyme. No change was noted in enzymatic activity by the incubation procedure alone. That the reaction with DFP was at the active site of the enzyme was confirmed by demonstrating substrate protection: DFP, in a final concentration of  $5 \times 10^{-4}$  M, inhibited 50% of the esterase activity of the enzyme in 30 min; however, complete protection against DFP inactivation was afforded by TAME and BAME when the latter were present in a final concentration of  $5 \times 10^{-3}$  M.

Since the inactivation of the active arginine esterase by DFP was slow ( $t_{1/2}$  at  $25^{\circ}\text{C}$  was 40 min) relative to

the rate of activation of the plasma precursor by ellagic acid ( $t_1 = 3$  min), the effect of DFP on the activation reaction could be studied. When DFP  $2.5 \times 10^{-4}$  M was preincubated with plasma for 1 hr at  $25^{\circ}\text{C}$ , before activation, no effect on the subsequent course of activation was noted; the activation followed first-order kinetics with a rate identical to that of the control sample.

Other agents affected the kaolin-activated arginine esterase in a manner similar to their effects on the three purified enzymes. Heparin (500–1000 U/ml) failed to inhibit the purified enzymes or the kaolin-activated esterases; the same was true for the potent trypsin inhibitors, ovomucoid (200  $\mu\text{g}/\text{ml}$ ) or tosyl lysyl chloromethyl ketone ( $3 \times 10^{-3}$  M). Diphenyl carbamyl fluoride ( $6 \times 10^{-3}$  M) inhibited the kaolin-activated arginine esterase 79%, which is essentially the same as that observed for the three purified enzymes (70–79%). Soybean trypsin inhibitor (200  $\mu\text{g}/\text{ml}$ ), which inhibited kallikreins I and II completely, but inhibited kallikrein III only 35%, inhibited the kaolin-activated enzyme 53%, a finding consistent with plasma containing a mixture of different types of plasma kallikrein.

Observations on the naturally occurring plasma in-



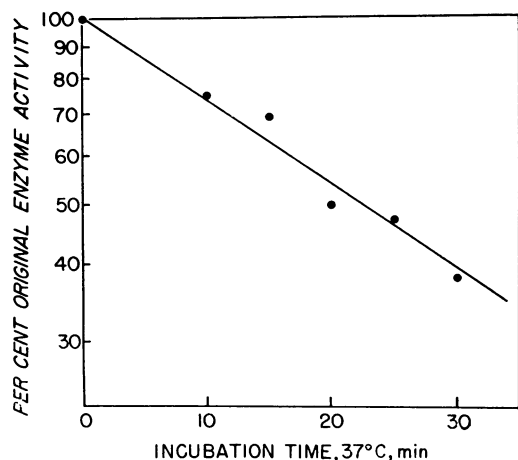


FIGURE 4 Effect of DFP on ellagic acid-activated arginine esterase. Equal volumes of ellagic acid- ( $10^{-4}$  M) and chloroform treated plasma were mixed and incubated at  $37^{\circ}\text{C}$  for 30 min. Diisopropylfluorophosphate ( $5\text{ }\mu\text{l}$  in isopropyl alcohol) was added to give a final concentration of  $2.5 \times 10^{-4}$  M). At various times  $0.4\text{ ml}$  of this incubation mixture was added to  $1.8\text{ ml}$  BAME (final concentration  $0.015\text{ M}$ ) containing polybrene (final concentration  $20\text{ }\mu\text{g/ml}$ ). Esterase activity was determined as indicated in Methods. A control, with  $5\text{ }\mu\text{l}$  of isopropanol added, showed no decline of activity at  $37^{\circ}\text{C}$  for 30 min.

hibitor of the kaolin-activated enzyme were also made. As displayed in Fig. 5, when the time course of activation was observed following the addition of kaolin to normal plasma, maximal activation of the esterase was achieved after 1 min of exposure to kaolin. Thereafter, there is a rapid decay of activity to a level about 50% of its maximum value; the most likely explanation for this decay is the presence of a plasma inhibitor, for following chloroform or acid treatment of the plasma, procedures known to inactivate inhibitors, no significant loss in esterase activity occurred (Fig. 5).

*Spontaneous activation of arginine esterase activity at low ionic strength and the relation of this activity to the kaolin-activated esterase.* In the course of studying the effect of ionic strength on the kaolin-activated arginine esterase of plasma, it became clear that there was a closely related arginine esterase activated by a somewhat different mechanism. At low ionic strength, arginine esterase activity of equal magnitude to the kaolin-activated enzyme developed spontaneously in normal, but not in Hageman-deficient plasma. The spontaneous activation was shown to be dependent on the presence of both Hageman factor and substrate, but did not require kaolin or ellagic acid. Like the kaolin-activated enzyme, the activation was first order and was blocked by polybrene.

As shown in Fig. 6, this low ionic strength-substrate activation was inhibited progressively by increasing ionic strength. The active enzyme differed from the

kaolin-activated enzyme by a very high BAME/TAME ratio (approximately 3:1), as compared with the latter which is close to unity (Table III). The enzyme activated at low ionic strength was also strikingly inhibited by  $500\text{ U/ml}$  heparin. These properties suggest that the low ionic strength-substrate-activated enzyme may be PF/dil (see Discussion). From the data displayed in Fig. 6 and Table III, it may be seen that at low ionic strength, BAME/TAME ratio was intermediate between the two enzymes (i.e., low ionic strength-substrate and kaolin-activated enzymes) and suggestive of a mixture of these two forms. In the presence of kaolin, the percent of increase in the amount of activity attributable to the kaolin-activated enzyme increased with increasing phosphate concentration. That the effect is due to an increasing ionic strength and not to phosphate was shown by duplicating these results by increasing the sodium chloride concentration (Fig. 6). Nor was it due to anion inhibition, since chloride, bromide, and sulfate also produced the same effect.

It is evident from the data shown in Figure 6 that there is a reciprocal relationship between the two enzymatic activities. With increasing sodium chloride concentration, the low ionic strength-substrate-activated enzymatic activity fell with a reciprocal rise in the kaolin-activated enzyme, the latter being measured in the pres-

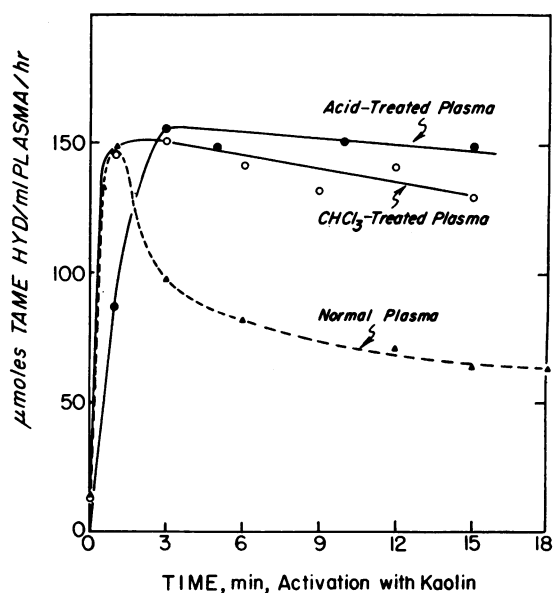


FIGURE 5 Effect of naturally occurring plasma inhibitor on kaolin-activated arginine esterase. Solid kaolin (final concentration  $10\text{ mg/ml}$  assay mixture) was added to normal siliconized plasma, acid-treated plasma, or chloroform-treated plasma. At various times after incubation at  $25^{\circ}\text{C}$ ,  $0.2\text{ ml}$  of plasma was removed and added to  $2.0\text{ ml}$  TAME (final concentration  $0.015\text{ M}$ ). Esterase activity was determined according to Methods.



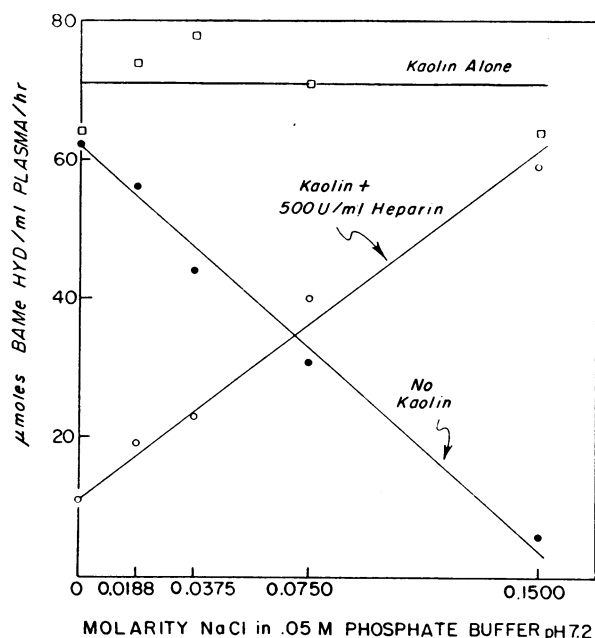


FIGURE 6 Effect of ionic strength on arginine esterases and heparin inhibition. Normal human siliconized plasma (0.2 ml) was added to 2.0 ml BAME (final concentration 0.015 M) in 0.05 M phosphate buffer, pH 7.2, containing various concentrations of NaCl. For study of kaolin activation, kaolin (10 mg/ml) was incubated with the plasma for 1 min at 25°C before adding substrate. For studying the effects of heparin, final concentration of 500 U/ml heparin included in the substrate solution.

ence of heparin in order to exclude any activity attributable to the other enzyme. However, the total activity measured at any ionic strength in the presence of kaolin, but without heparin, remained the same.

In order to explore further the possible interrelationship between the enzymes activated at low and high

ionic strength, a study of the activation of these enzymes in chloroform-treated plasma was undertaken. When chloroform-treated plasma was incubated at 25°C, spontaneous activation occurred slowly over a period of 1–2 hr in the absence of kaolin: at high ionic strength (0.1 M phosphate, 0.15 M chloride), arginine esterase activity evolved reaching half of its total kallikrein activity in 50 min, and the ratio of BAME/TAME was consistently slightly less than 1.0; whereas at low ionic strength (0.05 M phosphate), the activity evolved at a similar rate, but the BAME/TAME ratio, initially 2.31, dropped progressively over the 50 min period to a value of 0.95.

*Attempts to activate plasma kallikrein by other agents.* Based on its arginine esterase activity, this enzyme system could also be slowly activated by acid treatment of plasma, similar to that observed for chloroform (see above). Attempts to activate the enzyme with white cell or platelet sonicates were unsuccessful. No activation was observed upon the addition of plasmin or trypsin to plasma, but naturally occurring inhibitors may have prevented an effect in these experiments. Experiments were also performed to try to demonstrate enzyme activation by antibody-antigen reactions. Rabbit antiovalbumin serum (1.4 mg/ml) was added to equal volumes of purified ovalbumin (140 μg/ml). The resulting antibody-antigen complex was added to 5 volumes of siliconized human plasma, but no arginine esterase activity evolved during 30 min of incubation. Similar experiments in which purified rabbit antidinitrophenol antibody was reacted with dinitrophenol coupled to albumin, also failed to activate plasma kallikreinogen.

*Lack of effect of hydrocortisone on the plasma kallikreinogen-kallikrein system.* Hydrocortisone was added to plasma in concentrations ranging from  $6.8 \times 10^{-6}$  M to  $6.8 \times 10^{-7}$  M; these concentrations failed to inhibit the activation of plasma kallikreinogen by ellagic acid,

TABLE III  
Chemical Properties of Arginine Esterases\*

DEAE cellulose peak	Ratio:	Substrate/TAME				% Inhibition			Pan- creatic trypsin inhibitor
		BAME	AAME	TLMe	ALMe	Soy bean trypsin inhibitor	Plasma 25°C	TrasyloI	
						200 μg/ml	20 min	1000 U/ml	200 μg/ml
I	0.85	0.81	0.31	0.37		98	50	32	34
II	0.73	0.67	0.35	0.35		91	44	35	38
III	1.02	0.79	0.30	0.35		35	0	7	8

\* The ratio of the three kallikreins were compared at substrate concentrations of 0.015 M. The inhibitors were added to substrate solutions so that the final concentrations were as listed in the table in a final concentration of 0.05 M TAME. The amount of normal siliconized human plasma used gave a final dilution in the assay mixture of 1:11. The buffer was 0.1 M phosphate, pH 7.6, 0.15 M NaCl in all experiments.



nor was any effect noted on the degree of inhibition by plasma of the ellagic acid- or kaolin-activated arginine esterase. In addition, in studies with a purified preparation of plasma kallikrein, no effect on the latter's arginine esterase activity was discernible in the presence of hydrocortisone at concentrations similar to those used in plasma.

## DISCUSSION

The studies described in this report provide evidence that the arginine esterase activity appearing in plasma after the addition of kaolin is probably identical with plasma kallikrein. Four lines of evidence have been obtained to support this conclusion: (1) purification data and similarities in esterase activity between purified kallikrein and the kaolin-activated esterase; (2) evidence that the activity cannot be ascribed to other known plasma or tissue arginine esterases; (3) the requirements of Hageman factor for the activation; and (4) similarities in behavior of the plasma enzyme and purified kallikrein toward inhibitors. It is recognized that this evidence is indirect and that any of the above criteria by itself is insufficient for establishing the identity of the kaolin-activated arginine esterase as plasma kallikrein. However, the inability to distinguish the plasma esterase from purified kallikrein by all of these methods allows one to conclude that there is a high probability that the enzymatic activities are identical. Henceforth, for the purposes of this discussion, the kaolin-activated arginine esterase will be referred to synonymously with plasma kallikrein.

The purification of plasma kallikrein described in the preceding communication (2) was undertaken so as to establish the identity of the kaolin-activated arginine esterase as plasma kallikrein, an enzyme system recently reviewed by Webster and Innerfield (16). Gradual "spontaneous" activation of the esterase occurred during purification, presumably due to removal of inhibitors and to slow activation of Hageman factor, despite precautions to minimize contact. Nevertheless, no differences were ever noted in substrate ratios between the "spontaneously" activated and kaolin-activated enzymes, and the latter (Table I) compared favorably even with the most highly purified kallikrein preparations obtained (2).

A large number of plasma proteolytic enzymes are capable of hydrolyzing basic amino acid esters; these include thrombin (17), plasmin (18), clotting factors X and XI, C'1 esterase, kallikrein (19), and PF/dil (20). The ratios of activity toward five substituted esters (Table I) established a significant difference between the kaolin-activated enzyme and other plasma and tissue enzymes. Although the ratios of activity toward specific substrates depend both on  $K_m$  and  $V_{max}$ , never-

theless, this qualitative technique is sufficiently discriminating to distinguish between bovine and human thrombin (11). Concerning the other coagulation factors capable of hydrolyzing arginine esters, only PTA (XI) would be activated in calcium-poor plasma as used here, and PTA was excluded by experiments reported elsewhere (1). In other experiments, no appreciable hydrolysis of such C'1 esterase substrates as ATMe was observed after kaolin activation of plasma.

The data in Table II confirmed the independence of the kaolin-activated plasma esterase from the coagulation and fibrinolytic enzyme systems. Furthermore, these experimental results provide information on the magnitude of plasma kallikreinogen; note that the arginine esterase activity resulting from kaolin activation is of the same order as that which can be derived from complete activation of plasminogen or prothrombin, indicating that the kallikrein system is one of the major proteolytic enzyme systems of plasma.

As previously suggested (4, 5), Hageman factor was required, as shown by the findings that low arginine esterase activity in normal plasma increased in specimens collected in glass as compared with siliconized equipment (1), and large amounts appeared after the addition of kaolin or ellagic acid. This need was substantiated by the experiment which demonstrated that kaolin was unable to generate arginine esterase activity in Hageman-deficient plasma (Fig. 1).

The kinetic study with ellagic acid (Fig. 2) clarifies the role of Hageman factor in the activation of plasma kallikreinogen, for it demonstrates that the rate of activation of the latter is proportional to the concentration of activated factor XII. Furthermore, it was possible to establish that hexadimethrine (polybrene), a known inhibitor of factor XII (21), can be used to inhibit the activation of plasma kallikreinogen, but has little to no effect on plasma kallikrein itself (Fig. 3). Trasylol, a polypeptide lung inhibitor with known inhibiting action toward trypsin and pancreatic kallikrein (22), proved to be a much more efficient inhibitor of the activation step than it had on plasma kallikrein itself (Fig. 3). This latter finding is in agreement with previous observations (2) in which Trasylol and pancreatic trypsin inhibitor, which are probably identical (23), were shown to be weak inhibitors of purified plasma kallikrein.

In agreement with Webster and Pierce (18), who tested the effect of inhibitors on plasma kallikrein in a bioassay system, it was found that DFP inhibited the kaolin-activated plasma arginine esterase (Fig. 4) in a manner and extent analogous to our observations on purified kallikrein preparations (2). As might be expected from studies on other proteolytic enzymes, the inhibition of plasma kallikrein by DFP could be blocked



by the substrates BAME and TAME. These characteristics place plasma kallikrein in the class of proteases with an active site containing serine: in this group are plasmin, thrombin, trypsin, chymotrypsin, and elastase (24).

The potent trypsin inhibitors ovomucoid and TLCK showed virtually no effect, either on purified kallikrein preparations, or on the kaolin-activated arginine esterase. Diphenyl carbamyl fluoride, a chymotrypsin and trypsin inhibitor, inhibited the purified and plasma enzymes equally well. The partial inhibition of the kaolin-activated plasma esterase by soybean trypsin inhibitor was consistent with a mixture of the three kallikrein types in plasma.

The studies on the naturally occurring plasma inhibitor to the kaolin-activated arginine esterase (Fig. 5) were in agreement with those described for the plasma kallikrein inhibitor (3, 25). The kallikrein inhibitor was missing in hereditary angioneurotic edema and unstable below pH 5.5 (acid treatment of plasma removed the inhibitor), destroyed by exposure to alcohol at low temperature (all inhibitor activity disappeared during initial alcohol fractionation), and incomplete both on the plasma esterase and the purified kallikrein preparations. In addition, chloroform treatment of plasma removes the inhibitory activity completely without altering the enzymatic activity formed by kaolin or ellagic acid. The action of the naturally occurring inhibitor of plasma kallikrein (25) was apparent after kaolin activation of plasma kallikrein. The inactivation was time-dependent but was sufficiently delayed to allow for assay of the maximum activity 1 min after activation. The decay curve was asymptotic with a maximum of 60–70% inhibition, even after an hour's incubation. Part of the kallikrein may exist in a form not susceptible to inhibition by the plasma inhibitor; in this respect, it should be noted that kallikrein III was not inhibited by the plasma inhibitor (2).

The studies at different ionic strengths (Table III and Fig. 6) may shed light on the activation of PF/dil and the relation of the latter enzyme to plasma kallikrein. At low ionic strength an arginine esterase evolved in the presence of substrate, yet only in the absence of kaolin or ellagic acid. The activation was slow, required activated factor XII, and was inhibited by polybrene. This enzyme differed from plasma kallikrein; it had a much higher BAME/TAME ratio of activity and was inhibited by heparin (500 U/ml plasma). The activation at low ionic strength is reminiscent of the activation of PF/dil which also requires factor XII (8) and where dilution of plasma may achieve similar conditions (6). Moreover, PF/dil is inhibited in the guinea pig permeability test by concentrations of heparin (10) similar to those which inhibited the esterase activity. If the low

ionic strength-activated enzyme is PF/dil, then the reciprocal relationship, as shown in Fig. 6, suggests that PF/dil and kallikrein arise from a common precursor (kallikreinogen) and are related forms but with different properties, or that the former is a precursor or intermediate form of plasma kallikrein. The experiments involving activation of these enzymes in chloroform-treated plasma suggest the conversion of the PF/dil-like enzyme to kallikrein. Presumably, in the absence of inhibitor and at low ionic strength, the PF/dil-like enzyme was formed first and then converted to kallikrein. At higher ionic strength, only kallikrein activity was observed. We recognize that this is only one possible interpretation of the data, and confirmation of our hypothesis requires the isolation and characterization both of PF/dil and of the presumed common precursor.

Finally, our experiments with hydrocortisone failed to confirm the conclusions of Cline and Melmon (26) who, using a bioassay, observed that plasma kallikrein was inhibited by hydrocortisone ( $10^{-6}$  M) and suggested that this finding may account for the anti-inflammatory effect of steroids. Davies, Holman, Johnston, and Lowe (27), using the rat uterus bioassay, also failed to find any inhibition of kinin release from plasma by guinea pig serum kallikrein in the presence of the synthetic corticosteroid, paramethasone ( $2.5 \times 10^{-6}$  M). The explanation for these divergent observations remains unclear.

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