e-Aminocaproic Acid: an Inhibitor of Plasminogen Activation*

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(Received for publication, October 23, 1958)

Human and animal sera contain a globulin, plasminogen, which in the presence of activators is rapidly converted to plasmin, a proteolytic enzyme active at neutral hydrogen ion concentrations. Activators specific for plasminogen include streptokinase and staphylokinase of bacterial origin, urokinase and plasma activator found in body fluids, and fibrinokinese derived from tissues. Trypsin, an activator of other enzyme systems, will activate plasminogen, and plasminogen may undergo spontaneous activation. Streptokinase, urokinase, and trypsin activate plasminogen by a first order enzymatic reaction that involves the release of trichloroacetic acid soluble moieties (1), and spontaneous activation involves a similar process (2).

Plasmin acts on a number of substrates among which are casein, fibrin, fibrinogen, gelatin, protamine-heparin complex, and certain synthetic substrates containing lysine and arginine esters. Arginine and lysine esters are competitive inhibitors of the proteolytic activity of plasmin (3) as well as of the activator activity of urokinase, streptokinase, and trypsin (1). Numerous inorganic and organic inhibitors of plasmin activity have been described (4, 5) and often the same substances, which include toxic phosphorus compounds (6) also inhibit trypsin. However certain quaternary amines and laurylamine (5, 7), in high concentration, inhibit only plasmin and in low concentration possess the peculiar property of enhancing both the actions of plasmin and trypsin.

Hitherto, because of limitations of technical method, infrequent distinction has been made between plasmin inhibitors and inhibitors of plasminogen activation. The present communications describes the effects of e-aminocaproic acid, a newly described "plasmin inhibitor" (8), as an inhibitor of plasminogen activation and also of plasmin. The results indicate that the primary action of e-aminocaproic acid is to inhibit the activation of plasminogen, but that it also possesses, depending upon its concentration, the dual property of either inhibiting or enhancing the action of plasmin.

EXPERIMENTAL

Materials and Methods

E-Aminocaproic Acid—6-Amino hexanoic acid had a melting point of 208° (uncorrected) and on analysis was chemically pure.

* This work was supported by grants from the National Heart Institute, United States Public Health Service, Bethesda, Maryland, and Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

† Kindly supplied through Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, New York.

2 Armour Research Division, Chicago, Illinois.

3 Human plasma Fraction III was obtained from E. R. Squibb and Sons through the courtesy of the American Red Cross.

4 Worthington Biochemical Corporation, Freehold, New Jersey.

5 Obtained through the courtesy of Dr. J. Ploug, Leo Pharmaceuticals, Copenhagen, Denmark.

6 Hammarsten quality casein, Nutritional Biochemicals, Cleveland, Ohio.

Human Plasminogen—A bovine plasma Fraction III preparation, containing 2.35 casein units of plasminogen per mg. of tyrosine (see below) was used as a source of bovine plasminogen. Activation with streptokinase was performed in the presence of trace amounts of a human plasminogen preparation (0.02 casein units per casein unit of bovine plasminogen).

Human Plasminogen—This was prepared by Kline's modification (9) of Christensen and Smith's (10) procedure from human plasma Fraction III. The plasminogen preparations contained 100 to 150 casein units per mg. of tyrosine. These preparations have been characterized (11).

Human Plasmin—Human plasmin was prepared by spontaneous activation of human plasminogen (100 casein units per mg. of tyrosine) in 50 per cent glycerol (2). The activity of this preparation was 200 casein units per mg. of tyrosine.

Trypsin—This was a salt free preparation crystallized two times.

Urokinase—This contained 5100 Ploug units per mg. dry weight (12).

Fibrinokinase—Prepared by the method of Astrup and Stern- dorff (13) this preparation, by the fibrin plate assay, showed an activity comparable to a urokinase solution containing 5.25 Ploug units per ml. or 84 Ploug units per mg. of tyrosine.

Streptokinase—A highly purified preparation which was biophysically homogeneous was used. It contained 100 streptokinase units per mg. of nitrogen.

Phosphate Buffer—At 0.1 M, pH 7.6, this buffer was used in the assay procedures unless otherwise indicated.

Proteolytic Activity—Such activity was determined by a modification of the Remmert and Cohen (14) casein assay. Modification of the substrate concentration gave superior reproducibility. The casein concentration in the digestion mixture was 2 per cent instead of the customary 4 per cent. A unit of activity released 180 μg. of tyrosine per hour; thus our unit is approximately 50 per cent of that originally described. Plasminogen was converted to plasmin by streptokinase, 1000 units per ml., a sufficiently large amount to insure almost instantaneous activation, and the plasmin was assayed by the same method.

Fibrin Plate Test—The fibrin plate assay (15) was modified so...
that a 0.2 per cent solution of bovine fibrinogen was used. The fibrinogen was contaminated with bovine plasminogen, thereby rendering the fibrin plate susceptible to the action of plasminogen activators; this system has been widely used for the assay of plasminogen activators (16). The product of two perpendicular diameters of the lysed zone was used as a measure of proteolytic or activator activity, but the lysed area is not directly proportional to the amount of enzyme employed. A linear relationship is seen when the logarithm of the enzyme concentration is plotted against the logarithm of the activity (17).

Streptokinase cannot be directly assayed on bovine plasminogen, since bovine plasminogen preparations do not contain proactivator (16, 18). Before the assay of the streptokinase solutions they were mixed with 0.01 casein unit of human plasminogen per ml. to supply proactivator (this trace amount of added plasminogen was insufficient to affect the plate assay, which at its lower limit is sensitive to 0.1 casein unit of plasmin per ml.).

**Kinetic Studies in Presence of ε-Aminocaproic Acid—**Studies on the activation of plasminogen in the presence of ε-aminocaproic acid involved the determination of the amount of plasmin activated in a given period of time. ε-Aminocaproic acid and the activator being studied interfered with the plasmin assay and had to be removed before assay. The activation reaction was stopped by lowering the pH in the activation mixture to 2.0 at which pH plasmin is stable. The ε-aminocaproic acid was removed by dialysis in the cold against 0.01 N hydrochloric acid. The activator was removed or denatured by precipitation of the plasmin with 1 M sodium chloride at pH 2.0 (18), and the washed precipitate was dissolved in distilled water and then assayed.

**Determination of Esterase Activity—**This was done by the hydrolysis of benzoyl arginine methyl ester (3). Of the material to be assayed, 0.2 ml. was added to 0.2 ml. of buffer plus 6 ml. of benzoyl arginine methyl ester (0.5 mg. per ml. in phosphate buffer, 0.05 M, pH 7.0). After 2 minutes and 32 minutes of incubation at 37°C, 3-ml. specimens of the digestion mixture were withdrawn and immediately added to 0.1 ml. of 10 per cent acetic acid, thus stopping the digestion process by lowering the pH. The optical density of the 32-minute specimen was read in the Beckman spectrophotometer at wave length 258 mμ, (19) with the 2-minute specimen used as blank value. Plasminogen was assayed after conversion to plasmin.

**RESULTS**

**Inhibition of Plasminogen Activation by ε-Aminocaproic Acid—**Fig. 1 shows that ε-aminocaproic acid inhibited the enzymatic process (1) involved in plasminogen activation by streptokinase rather than the proteolytic activity of plasmin itself. The experimental conditions were bovine plasminogen, 3.2 casein units per ml.; streptokinase, 1000 units per ml.; and trace amounts of human plasminogen to provide proactivator. In one series, varying concentrations of ε-aminocaproic acid were added at the start of the activation period (30 minutes at 37°C), and in the other series ε-aminocaproic acid was added at the end of the activation period and immediately before plasmin assay. The results in Fig. 1 and in later figures have been expressed as percentage inhibition of control activity.

The addition of ε-aminocaproic acid, at the end of the activation period, did not affect the assay values for plasmin (bottom line of Fig. 1), but its addition at the start of the activation period caused an inhibition of the activation process, the degree of which was a function of the added ε-aminocaproic acid concentration (top line of Fig. 1). Precisely similar findings were recorded in another experiment, in which human plasminogen was substituted for bovine plasminogen.

**Fibrin Plate Assay—**The effect of ε-aminocaproic acid upon trypsin and plasmin and upon the plasminogen activators, streptokinase, fibrinokinase, and urokinase, was studied by means of the fibrin plate test. The respective enzyme concentrations were adjusted to give, in the absence of ε-aminocaproic acid, roughly similar zones of plate lysis. Fig. 2 demonstrates the degree of inhibition of each enzyme (in per cent) plotted against the concentrations of added ε-aminocaproic acid.

Concentrations of ε-aminocaproic acid which inhibited the three plasminogen activators had little or no effect on plasmin and trypsin activities. There was a 60-fold difference between the concentrations of ε-aminocaproic acid causing 50 per cent inhibition in the two test systems, the figures being 0.5 μ for trypsin and approximately 0.008 μ for the activators.

**Kinetics of Inhibition of Proteolytic Activity—**The kinetics of the inhibition of the proteolytic activity of plasmin by ε-aminocaproic acid were studied, with casein as a substrate. Fig. 3 shows the inhibition of plasmin activity (0.40 casein unit per ml. in digestion mixture) at two concentrations of ε-aminocaproic acid. The casein concentration was varied 7-fold. The data are plotted with the reciprocal of the substrate concentration.
EFFECT OF \(\epsilon\)-AMINO CAPROIC ACID ON PLASMIN, TRYPSIN AND PLASMINOGEN ACTIVATORS.

**FIG. 2.** Fibrin plate assays. The concentrations of materials tested were adjusted to give equal zones of lysis in the absence of \(\epsilon\)-aminocaproic acid. Marked differences are apparent between the inhibitory effect exerted by \(\epsilon\)-aminocaproic acid on streptokinase (SK), fibrinokinase (FK), and urokinase (UK) on the one hand and plasmin and trypsin on the other.

**CAPROIC ACID.**

![Graph](image)

**INHIBITION OF TRYPSIN ACTIVITY BY \(\epsilon\)-AMINO CAPROIC ACID**

![Graph](image)

**INHIBITION OF TRYPsin ACTIVITY by \(\epsilon\)-AMINO CAPROIC ACID**

![Graph](image)

**INHIBITION OF TRYPSIN ACTIVITY by \(\epsilon\)-AMINO CAPROIC ACID**

![Graph](image)

**FIG. 4.** Noncompetitive inhibition of trypsin activity by \(\epsilon\)-aminocaproic acid demonstrated on a Lineweaver-Burk plot. \(S\) is expressed in terms of tyrosine for the reasons noted in Fig. 3.

**THE INHIBITORY ACTION OF \(\epsilon\)-AMINO CAPROIC ACID UPON PLASMINOGEN ACTIVATION**

![Graph](image)

**FIG. 5.** Lineweaver-Burk plots demonstrating that the inhibitory action of \(\epsilon\)-aminocaproic acid upon urokinase and streptokinase was competitive in nature. CAS. U., casein units.

The highest plasminogen concentration was 22 casein units per ml., and the concentration was varied 7-fold. The plasmin formed was assayed by casein hydrolysis after removal of \(\epsilon\)-aminocaproic acid and urokinase, and complete recovery of plasminogen and plasmin was demonstrated by tyrosine assays. The results plotted in the double reciprocal manner fulfill the Lineweaver-Burk criteria for competitive inhibition.

Fig. 5B illustrates a precisely similar experiment except that streptokinase was used as an activator. The plasminogen concentration in this experiment varied from 6 to 48 casein units per ml. and the streptokinase concentration was 50 units per ml. The results, presented in a double reciprocal plot, show that the \(\epsilon\)-aminocaproic acid inhibited plasminogen activation by streptokinase in a competitive manner.

Because of technical reasons the plasminogen concentrations in the latter two experiments were high, and since \(\epsilon\)-aminocaproic acid was a competitive inhibitor, a correspondingly high inhibitor concentration had to be used in order to demonstrate the inhibitory action. However, complete inhibition resulted...
with lower concentrations of e-aminocaproic acid when lower concentrations of plasminogen and activator were used (Fig. 2).

The demonstration that trypsin activity as measured by proteolytic assay was inhibited noncompetitively by e-aminocaproic acid (Fig. 4) strongly suggested that e-aminocaproic acid would inhibit the activation of plasminogen by trypsin in a similar fashion. The data illustrated in Fig. 6 provide confirmation of this hypothesis. It was impracticable to test the activator properties of plasmin in this manner, since the activation reaction proceeds so slowly that reliable kinetic data cannot be obtained. Nevertheless, since plasmin activity, measured by proteolytic assay, was inhibited noncompetitively by e-aminocaproic acid (Fig. 3), there can be little doubt that a similar relationship would hold for plasmin activator activity.

Enhancing Effect of e-Aminocaproic Acid on Proteolytic Activity—The experiments thus far described have revealed that e-aminocaproic acid, in concentrations exceeding 0.03 M, acted as a noncompetitive inhibitor of the proteolytic activities of trypsin and plasmin. However, there were indications that e-aminocaproic acid in certain lower concentrations would increase the plasmin activity above that found in the control solution containing no e-aminocaproic acid. Plasmin assays were made in the presence of varying concentrations of e-aminocaproic acid with three different substrates: casein, fibrin, and benzoyl arginine methyl ester. The plasmin concentration was 2.4 casein units per ml., and the e-aminocaproic acid concentration was varied from 5 X 10^{-4} M to 2 M; in the actual digestion mixture the e-aminocaproic acid concentration was varied from 1.6 X 10^{-4} M to 1 M, the dilution depending upon the assay system. The results are presented in Fig. 7, where the increase (or decrease) in activity (measured in per cent of the control value) is plotted against the e-aminocaproic acid concentration in the digestion mixture.

With all three substrates enhanced proteolytic activity was demonstrated with a peak value at an e-aminocaproic acid concentration of 10^{-3} M. The peak percentage of enhancement was approximately the same for each substrate, and the range over which an increase was found was roughly the same for benzoyl arginine methyl ester and casein. The range of e-aminocaproic acid concentrations which caused increased proteolysis on the fibrin plate was somewhat narrower than for casein and benzoyl arginine methyl ester, but here the true concentration of the enzyme-e-aminocaproic acid mixture varied, since the lysed area increased during the incubation period, thus diluting the solution which was being assayed.

Effect on Solubility of Plasminogen and Plasmin—An incidental observation was made in the course of this investigation, namely, that addition of e-aminocaproic acid increased the solubility of plasminogen and plasmin.10 Ordinarily plasminogen prepared by acid extraction, and plasmin prepared from this plasminogen, were but sparingly soluble at neutral pH, but when e-aminocaproic acid was present in the solvent, even in low concentrations, plasminogen and plasmin formed a clear solution. It was also noted that when precipitation of plasminogen or plasmin by 1 M NaCl at pH 2.0 was attempted, an e-aminocaproic acid concentration of as little as 0.01 M was sufficient to prevent complete precipitation. e-Aminocaproic acid also increased the solubility effect on casein in the presence of 5 per cent trichloroacetic acid.

10 F. B. Abild and J. J. Hagan (personal communication) have made similar observations with regard to lysine and certain other basic amino acids.

**FIG. 6.** A Lineweaver-Burk plot illustrating that the inhibitory effect of e-aminocaproic acid upon the activation of plasminogen by trypsin is noncompetitive in nature. Cas. U., casein units.

**FIG. 7.** e-Aminocaproic acid in low concentration enhances the action of plasmin upon benzoyl arginine methyl ester (BAMe), casein and fibrin (plate test).

This effect was not seen when the concentration of e-aminocaproic acid was 0.2 M or less; however, at higher concentrations, prior dilution of the solution allowed for complete precipitation.

**DISCUSSION**

Numerous substances have been described as inhibitors of plasmin (3-7), but in most instances no distinction has been made between inhibition of the enzyme itself and inhibition of the activation process. Such a distinction is now possible because of advances in purification of the enzyme and its precursor, increasing knowledge of the activation process, and a better understand...
standing of the effects of plasmin and plasminogen activators on their substrates.

It was originally claimed that ε-aminocaproic acid in low concentrations was a powerful inhibitor of plasmin activity (8). However, the present studies reveal that the action of ε-aminocaproic acid on the plasminogen-plasmin system is complex and that although one of its actions is to inhibit plasmin, its effect upon the activation process is the more striking. These studies suggest that previous descriptions of plasmin inhibitors may have been incomplete, and particularly that the quaternary amines and laurylamine, which exhibit properties apparently similar to those of ε-aminocaproic acid, may also exert their primary effect upon the activation process (5, 7). Indeed a review of analytical methods used in these publications in the light of more recent findings would suggest that these experiments would be better interpreted as illustrating inhibition of plasminogen activation rather than inhibition of plasmin action. Support for this view is given by the observation by Bjerrehuus (21) who found that the quaternary amines apparently inhibited urokinase.

The mechanism for the enhanced activity of plasmin observed with certain concentrations of ε-aminocaproic acid is obscure. It is unlikely that the solvent action of ε-aminocaproic acid upon plasmin or some of its substrates will account for this phenomenon, since quaternary amines in similar concentration (10^{-2} M) have been observed to enhance the activity of highly soluble plasmin (7), and the effect was seen with three substrates of different type and solubility characteristics.

It has recently been reported (22) that cholinesterase has increased activity in the presence of short chain quaternary amines. Since it is accepted that plasma cholinesterase has an anionic site and an esteratic site, the authors speculated that the mechanism involved the attraction of the quaternary amine to the anionic site, and the consequent attachment of the substrate to the esteratic site. As with plasmin at higher concentrations, the quaternary amines inhibited the enzyme activity (23), a finding perhaps due to blocking because of the proximity of the esteratic and anionic sites. Noteworthy in this connection is the failure of longer chain quaternary amines to enhance cholinesterase activity. Although we lack similar information concerning the structure of plasmin, analogous mechanisms may be invoked to explain our findings.

Differences exist on a temporal basis between the actions of urokinase and streptokinase on the one hand and trypsin on the other. The first two activators will, under suitable conditions, cause complete activation of plasminogen within a few minutes, whereas activation by trypsin is a process requiring hours. In this respect activation by trypsin is comparable to the autocatalytic activation of plasminogen itself, although this latter mode of activation is even slower than with trypsin. These temporal differences have suggested that activation may take place in at least two ways, a suggestion supported by the fact that study of trypsin activation or autocatalytic activation requires the use of a stabilizing agent of which glycerol has been found to be the most useful (2). In previous communications (1, 2) we have reported that the activation of plasminogen occurs by sequence of the release of trichloracetate acid-soluble moieties, and that it appeared that the amounts of such moieties released varied with the mode of activation. Biophysical evidence (11) tended to support this finding. Thus it is not surprising that an apparently specific inhibitor of the plasminogen activation process should exert a predominant effect upon one class of activators rather than upon the other. Urokinase and streptokinase are both inhibited competitively and at low ε-aminocaproic acid concentration (under the conditions customarily used; however, trypsin and plasmin (the "activator" present during the autocatalytic process) are inhibited noncompetitively and at high ε-aminocaproic acid concentrations. These findings are held to confirm our former suggestion that at least two kinds of active plasmin molecules exist, showing probably identical biochemical activity, but differing molecular sizes. Analogous findings have been reported with regard to the activation of prothrombin, which results in molecules of similar biological activity and varying molecular weight (24).

It has been previously reported (18, 25) that lysine and ornithine serve, respectively, to inhibit the activation of plasminogen by streptokinase and by the plasma activator obtained from human corpses after sudden death from anoxemia. The observation that ε-aminocaproic acid (lysine without the ε-amino group) suppresses competitively plasminogen activation by specific activators (streptokinase, urokinase, and tissue activator) raises considerations concerning the structural requirements involved in the activation site, also with respect to the development of additional competitive inhibitors.

The clear differences existing between the modes of inhibition produced by ε-aminocaproic acid upon activators and upon plasmin can be turned to practical use. Where, as in plasma and serum, both activities may occur together, the use of ε-aminocaproic acid to inhibit plasminogen activator at concentrations insufficient to affect plasmin activity, permits differentiation of their separate effects.11, 12

SUMMARY
1. ε-Aminocaproic acid competitively inhibited the activation of human or bovine plasminogen by streptokinase, urokinase, and probably fibrinokinase, but inhibited plasminogen activation by trypsin noncompetitively.
2. ε-Aminocaproic acid in concentrations exceeding 0.06 M was a noncompetitive inhibitor of the proteolytic activities shown by plasmin or trypsin. In lower concentrations it enhanced the proteolytic action of plasmin.
3. The results support the view that plasminogen activation may occur by two mechanisms yielding plasmins with similar biochemical activities but of different molecular size.

Addendum—In a recent communication to the American Chemical Society ("Streptokinase activation of plasminogen: species specificity and inhibition by ε-aminocaproic acid," Chicago, September 1958), F. B. Ablondi and J. J. Hagan have independently studied the inhibitory effect of ε-aminocaproic acid on plasminogen activation. Their results (personal communication) are qualitatively in agreement with our own.

11 N. Alkjaersig, A. P. Fletcher, and S. Sherry, submitted for publication.
12 S. Sherry, R. I. Lindemeyer, A. P. Fletcher, and N. Alkjaersig, submitted for publication.

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