Analysis of Fibrinolysis by the Use of Epsilon-Aminocaproic Acid: Preliminary Report

By Katsuhiro Fukutake, Keiji Shida, Toyome Arakawa and Katsuji Kato

PLASMIN, a proteolytic and fibrinolytic enzyme of blood, is present normally as an inactive precursor, plasminogen, in the circulating blood. In various pathologic conditions plasminogen may convert into active plasmin in vivo. Recently it has been reported by Okamoto et al. that e-aminocaproic acid (ACA) has a potent inhibitory activity in the dissolution of fibrin by plasmin when activated by streptokinase (SK) in plasma. It is one of the purposes of this communication to determine the effect of this compound upon the fibrinolytic process.

The present report is concerned particularly with re-examination of the theories which have been proposed to explain the mechanism of fibrinolysis. Evidence obtained here indicates that certain newly mentioned products, metafibrin and fibrinolysopeptide, may be split off from fibrin in the early phase of the fibrinolytic process.

MATERIALS

Human plasminogen was prepared by the following procedure: human plasma was treated with \( \frac{1}{10} \) volume of \( 1 \) M BaCl\(_2\), diluted to 10 times the original volume with distilled water, and adjusted to pH 5.2 with 1 M acetic acid. The precipitate was dissolved and fractionated at 25 to 29 per cent saturated ammonium sulfate; the precipitate was dissolved again and dialysed. The final product was kept at \(-20^\circ\) C.

The bovine fibrinogen employed was Cohn's fraction I (Armour); Varidase, in place of streptokinase (Lederle), and tosylarginine methyl ester and lysine ethyl ester (H. M. Chemical Co.) were used. \( \epsilon \)-ACA was prepared by Daiichi Seiyaku Co. and commercial thrombin was made by Mochida Seiyaku Co. in Tokyo.

RESULTS

Nitrogen content of the TCA-supernate in the fibrinolytic process.—The results measured by Kjeldahl-Nessler's method demonstrated a significant increase of the nitrogen content in the trichloracetic acid-supernate during the entire fibrinolytic process (fig. 1). Although clot lysis time determined macroscopically was 7.5 minutes at 37 C., it showed no definite influence on the curve of splitting nitrogen.

Effect of \( \epsilon \)-ACA upon clot lysis time.—Fibrinolytic activities of human plasmin activated by SK were determined by Loomis' method at 37 C. with various concentrations of \( \epsilon \)-ACA. Potent inhibitory activities of \( \epsilon \)-ACA were revealed at concentrations over \( 1 \times 10^{-4} \) M. (fig. 2), but no effect on clotting time was observed at any concentration of this acid.

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Fig. 1 (top, left).—Nitrogen contents of the TCA-supernate in the proteolytic process. Several tubes, each containing 0.5 ml. of 2 per cent fibrinogen solution, are incubated at 37°C, after adding 0.2 ml. of SK-activated human plasmin. At several incubation periods, 1.0 ml. of 20 per cent TCA solution is added to each tube and centrifuged at 3000 rpm. The nitrogen content of the supernate has been measured by Kjeldahl-Nessler’s method. The expression “mg/dl” means mg. per cent.

Fig. 2 (top, right).—Inhibitory effects of \( \varepsilon \)-ACA on clot lysis time.

Fig. 3 (bottom).—Effect of \( \varepsilon \)-ACA on fibrinolytic process after clot lysis.

Effect of \( \varepsilon \)-ACA on fibrinolytic process after clot lysis.—On the other hand, \( \varepsilon \)-ACA appears to be a rather weak inhibitor of the subsequent digestion of fibrin, as exemplified by the splitting off of a substance having an absorption band at 276 m\( \mu \) (fig. 3). However, slight inhibiting effects on this process are noted at concentrations higher than 0.3 M.

Absorption curves of the TCA-supernate before and after clot lysis.—The absorption curves of the TCA-supernate before and after clot lysis were determined in the area of ultraviolet wave lengths. It was found after one hour of lysis (fig. 4) that the supernate showed marked absorption of 276 m\( \mu \), but very little absorption either before or at the time of lysis.
These data, in addition to the results of figure 5, which show the lag phase of ultraviolet absorption in the early stage of fibrinolysis, indicate the possibility of the existence of two reaction phases in the fibrinolytic process: one, the early phase indicated by clot lysis and producing some peptide-like substances without ultraviolet absorption; the other, the late phase splitting off a peptide-like hydrolysate with ultraviolet absorption.

**Effect of e-ACA on the esterolytic action of plasmin.**—The esterase activity of plasmin for tosylarginine methyl ester and lysine ethyl ester is not inhibited by the addition of e-ACA at the concentration of 0.01 M, although these synthetic compounds are excellent substrates for the determination of plasmin activity (figs. 6 and 7).

This finding may offer a proof that fibrinolysis as a measure of clot lysis is not identical with the esterolytic process, which may be concerned in the later phase of the fibrinolytic phenomenon.

**Fig. 4 (top, left).**—Absorption bands of the TCA-supernate before and after clot lysis.

**Fig. 5 (top, right).**—Ultraviolet absorptions in the TCA-supernate during the fibrinolytic process.

**Fig. 6 (bottom, left).**—Effect of e-ACA on the esterase activity of plasmin for tosylarginine methyl ester.

**Fig. 7 (bottom, right).**—Effect of e-ACA on the esterolytic action of plasmin for lysine ethyl ester.
Fibrinolysis and metafibrinolysis.—The foregoing results favor the view that the fibrinolytic process may involve at least two phases of reaction, namely, fibrinolysis and metafibrinolysis.

Fibrinolysis is indicated simply by clot lysis, in which plasmin splits off fibrinolysopeptide and metafibrin from fibrin. In the phase of metafibrinolysis, metafibrin is broken down to metafibrinopeptide and other hydrolysates.

The hypothetical scheme proposed here is diagrammed in figure 8.

Nature of metafibrin.—Electrophoretic pattern of metafibrin. In order to confirm the existence of metafibrin, electrophoretic and chromatographic studies were done. Purification of the material will be performed in the future.

Paper electrophoreses were carried out at pH 10, 8.6, 4.6 and 3. In all patterns only one spot of metafibrin was shown which could be distinguished from other materials, such as fibrin, fibrinogen and hydrolysate of metafibrinolysis, on the basis of its mobility (fig. 9). However, the material tested here is in itself not pure; it is the reaction mixture with monochloracetic acid added at the point of clot lysis time.

N-terminal amino acid of metafibrin. N-terminal amino acids of metafibrin (a TCA-precipitate at the end point of fibrinolysis) were determined by the DNP method using 2,4-dinitrofluorobenzene. As observed by two phase paper chromatography, metafibrin has tyrosine and aspartic acid as N-terminal amino acids in the ether-soluble fraction (fig. 10); the quantitative ratio of tyrosine and aspartic acid in the eluate from paper with 5 per cent bicarbonate is 1:2 by the determination of optical density at 360 mμ.

It is presumed, therefore, that four molecules of N-terminal glycine are lost from fibrin during fibrinolysis, and that N-terminal aspartic acid appears in the metafibrin formed.

Nature of fibrinolysopeptide.—Electrophoretic pattern of DNP-fibrinolysopeptide. Paper-electrophoresis analyses of DNP-fibrinolysopeptide were performed at pH 10, 8.6, 4.6 and 3. The pattern obtained at pH 4.6 presents two yellow spots in which one has the same mobility as fibrinopeptide A,

![Diagram](image-url)
Table 1.—Rf of DNP-Amino Acids in Two Phase Paper Chromatography

<table>
<thead>
<tr>
<th>DNP-amino acid</th>
<th>1.5 M Phosphate buffer (pH 6.0)</th>
<th>Toluene solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0.37</td>
<td>0.09</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.31</td>
<td>0.09</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.41</td>
<td>0.36</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.04</td>
<td>0.80</td>
</tr>
<tr>
<td>DNP-OH</td>
<td>0.41</td>
<td>0.23</td>
</tr>
<tr>
<td>DNP-NH₂</td>
<td>0.09</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Constitution of toluene solution; toluene : glycolmonochlorohydrin : pyridine : 0.8N NH₄OH = 5:3:1:3. Ammonium solution is discarded from the toluene mixture after the saturation of this compound.

while the other does not move under the electrophoretic condition of 7 mA for 3 hours (fig. 11).

Apparently the latter spot represents fibrinolysopeptide in comparison with the pattern of fibrinopeptides, although the TCA-supernate containing both fibrinopeptides and fibrinolysopeptide was used for the electrophoretic analysis. The other patterns, except those at pH 4.6, do not show any separation of the two varieties of peptides.

Following electrophoresis, the paper patterns were stained with ninhydrine. The third spot, which has no N-terminal amino acid, appeared on the cathode side near the original line. This is fibrinopeptide B.

N-Terminal amino acid of fibrinolysopeptide. Because of the difficulty in obtaining a sufficient amount of fibrinolysopeptide free of fibrinopeptide, the DNP-amino acids from the hydrolysate of the TCA-supernate at the end point of fibrinolysis were determined. The results in figure 12 show two N-terminal amino acids of glycine and glutamic acid in the ether-soluble fraction. The glycine terminal here supposedly comes from fibrinolysopeptide, while glutamic acid represents the terminal of fibrinopeptide A.
Fig. 11 (at left).—Schematic electrophoretic pattern of fibrinolysopptide at pH 4.6 with acetate buffer. (A) fibrinopeptide A; (B) fibrinopeptide B; (C) fibrinolysopptide. Solid spots represent DNP-amino acids.

Fig. 12 (at right).—N-terminal amino acids of fibrinolysopptide by Sanger’s DNP-method. (1) DNP-NH₂, (2) DNP-OH, (3) glycine, (4) glutamic acid.

Fig. 13.—Scheme of N-terminal amino acids of metafibrin and fibrinolysopptide.

DISCUSSION

During the formation of fibrin by thrombin, fibrinopeptide is removed from fibrinogen to become a fibrin monomer. Lorand¹² calculated that about 3 per cent of fibrinogen was rendered trichloracetic acid-soluble. This amount represents a removal of 8000 to 9000 Gm. material per mole of fibrinogen.

Lorang, Middlebrook, Bettelhein and Bailey,² in determining N-terminal amino acids of fibrinogen and fibrin, found that the former contains two molecules of tyrosine and two of glutamic acid, while the latter contains four of glycine and two of tyrosine as N-terminal amino acids. Apparently two molecules of N-terminal glutamic acid are lost following the thrombin action, forming N-terminal glycine groups. Recent experiments of Blombäck and Yamashina⁵ are generally in agreement with these conclusions.

It was found by Bettelhein and Bailey¹ that there were actually two kinds
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of fibrinopeptides; one, fibrinopeptide A, moved faster than the other, fibrinopeptide B, in the electric field at pH 4.6.

Furthermore, these observers found that the N-terminal amino acid of fibrinopeptide A was glutamic acid, but fibrinopeptide B had no N-terminal amino acids. Blombäck et al. also observed similar composition of N-terminal amino acids in fibrinopeptide A and B, separated by the fractionation procedure using Dowex 50 × 2.

Recently Laki demonstrated that both of these fibrinopeptides contained arginine as the C-terminal end group. With these findings it is possible to visualize that the bonds which are split by thrombin are those between arginine and glycine in the fibrinogen molecule.

According to the results here observed, two reaction phases in the fibrinolytic process may be proposed: fibrinolysis and metafibrinolysis; plasmin splits off fibrinolysopeptide and metafibrin from fibrin in fibrinolysis. Although we are still carrying out the purification studies on these materials, the preliminary data thus far indicate that fibrinolysopeptide has N-terminal glycine, and that metafibrin has tyrosine and aspartic acid as N-terminal amino acids; the ratio of tyrosine and aspartic acid is 1:2, quantitatively.

Therefore, it is presumed that four molecules of N-terminal glycine are removed from fibrin by the action of plasmin, and at the locations of separation four molecules of aspartic acid appear as N-terminals of metafibrin. The split peptide is naturally fibrinolysopeptide itself having N-terminal glycine (fig. 13).

SUMMARY

From the foregoing analysis of the inhibitory action of ε-aminocaproic acid in the fibrinolytic process, it is evident that the process involves two reaction phases, fibrinolysis and metafibrinolysis.

Fibrinolysopeptide and metafibrin are split off from fibrin in fibrinolysis. The former is a peptide with N-terminal glycine, while the latter is a mono-chloracetic acid-soluble metaprotein having both aspartic acid and tyrosine as N-terminal amino acids.

REFERENCES

FIBRINOLYSIS ANALYSIS WITH L-AMINOCAPROIC ACID

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