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Medicinal Chemical Studies on Antiplasmin Drugs. I. Establishment of the Screening Assay Method for Antiplasmin Drugs and the Superiority of *trans*-4-Aminomethylcyclohexanecarboxylic Acid (Tranexamic Acid[1]) to *cis*-Form[2]

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The new caseinolytic assay method for antiplasmin activity of synthetic compounds was established by introducing the determination of ID_{50}. *trans*–Form isomer of 4–aminomethylcyclohexanecarboxylic acid (tranexamic acid) was shown to have very potent antiplasmin activity whereas the *cis*–form isomer was little effective. The antiplasmin activity of tranexamic acid was 5—7 times greater than that of ε–aminocaproic acid.

Since 1957, ε–aminocaproic acid (ε–ACA)[4–6] has found its wide–spread applications to the clinical fields concerning various diseases associated with increased plasmin activity in blood. Okamoto, *et al.*,[7,8] after their introduction of ε–ACA, succeeded in selecting 4–aminomethylcyclohexanecarboxylic acid (AMCHA) as a more potent antiplasmin drug. The compound used by them was presumably a mixture of stereoisomers as there should be *cis* and *trans* forms in 1,4–disubstituted cyclohexane derivatives. Our group succeeded in the separation of these two forms and assignment of their configurations as reported in the following paper.[9]

This report deals with the establishment of the assay method for antiplasmin activity, by which we determined the antiplasmin activity of AMCHA isomers and are attempting to search for more potent drugs.

Okamoto, *et al.*,[10] with co–operation with us, reported that *trans*–form of AMCHA showed a very potent antifibrinolytic activity *in vitro* and *in vivo*. We also preliminarily communicated the chemical data of the two isomers and the superiority of *trans*–form to *cis*–form in antiplasmin activity.[11]

Materials and Methods

Human Plasminogen——Euglobulin solution was used as plasminogen preparation. Euglobulin fraction was obtained from human citrated blood according to the method of Norman[12] and dissolved in

1) It is the name submitted by the World Health Organization as an international non–proprietary name. WHO Chronicle, **20**, 216 (1966).
2) A part of this work was presented by S. Okamoto, K. Kinjo, S. Oshiba, M. Mangyo, M. Shimizu, S. Sato, and U. Okamoto at the International Society of Haematology, Stockholm, Sweden, in September, 1964.
3) Location: *Minamifunabori–cho, Edogawa–ku, Tokyo.*
4) Mitsubishi Kasei Kogyo Co., Ltd., refer to British Patent 707 693 (1957), field in 1953.
5) S. Okamoto, *Keio J. Med.*, **8**, 211 (1959).
6) T. Abe and A. Sato, *Keio J. Med.*, **8**, 219 (1959).
7) S. Okamoto and U. Okamoto, *Keio J. Med.*, **11**, 105 (1962).
8) S. Oshiba and S. Okamoto, *Keio J. Med.*, **11**, 117 (1962).
9) T. Naito, A. Okano, S. Kadoya, T. Miki, M. Inaoka, R. Moroi, and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), in press.
10) S. Okamoto, S. Sato, Y. Takada, and U. Okamoto, *Keio J. Med.*, **13**, 177 (1964).
11) M. Shimizu, T. Naito, A. Okano, and T. Aoyagi, *Chem. Pharm. Bull.* (Tokyo), **13**, 1012 (1965).
12) P.S. Norman, *J. Exptl. Med.*, **106**, 423 (1957).

phosphate buffer saline (pH 7.4) to a volume equal to 25% of the original blood volume. The resultant euglobulin solution was lyophilyzed after centrifugation at about 10000 rpm for 30 minutes to remove insoluble materials. The lyophilyzed preparation can be stocked without any loss of activity at least for 6 months in a cold room.

Streptokinase——A commercial preparation, Varidase (Lederle Laboratories Division) was used. The activity was taken to be that indicated on the vials. A stock solution of streptokinase containing 20000 units/ml of phosphate buffer saline (pH 7.4) was stored in a refrigerator and diluted with the same buffer before use.

Casein——A commercial milk casein was purified by the method of Norman.[12] The purified casein produced blank value below 0.100 at 280 mμ, after deproteinization of 2 ml of 2% casein solution by adding 2 ml of 1.7 M perchloric acid.

Thrombin——Thrombin was a commercial preparation of Mochida Pharmaceutical Co., Ltd., Tokyo.

Fibrinogen——Bovine fibrinogen (Armour Pharm. Co.) was obtained from Daiichi Pure Chemicals Co., Ltd., Tokyo.

Buffer——Phosphate buffer saline was used, which contained 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of KH_2PO_4 and 8 g of NaCl per 1 liter at pH 7.4. The buffer prepared was autoclaved at 120° for 10 minutes and stored in a refrigerator.

Inhibitors——ε-ACA and the two stereoisomers of AMCHA (*cis*-AMCHA and *trans*-AMCHA) were synthesized in our laboratory.

Measurement of Proteolysis——An euglobulin solution was added to a casein solution and the final volume of the mixture was adjusted to 1.9 ml with the phosphate buffer saline (PBS). After preincubation at 37° for 3 minutes, 0.1 ml of a streptokinase solution was added to initiate the reaction. At a proper incubation period at 37°, 2 ml of 1.7M perchloric acid was added to the reaction mixture, allowed to stand at room temperature for about 1 hr, and centrifuged. The extinction of the clear supernatant solution was read at 280 mμ. An enzyme blank was treated similarly with exception that the streptokinase solution was added after addition of perchloric acid. The rate of proteolysis was represented as an increase in the extinction at 280 mμ.

Measurement of Inhibitory Effect——Inhibitory effects of test compounds on the proteolysis by the actions of plasminogen and streptokinase were measured by a modification of the Norman's procedure.[12] An inhibitor was mixed at its various concentrations with the mixture of euglobulin and casein solution, and the caseinolytic reaction was carried out by adding streptokinase as described above. The reaction system in detail is described in the next section, Experimental. The rate of inhibition was calculated by comparison with the control. The determination of the inhibitor concentration which produces 50% inhibition, referred to as ID_{50}, was performed according to the method of Treffers[13] and Aoyagi and Mizuno.[14] The ID_{50} was read from a graph obtained by taking inhibitor concentration plotted on a logarithmic scale as abscissa and probit of percent inhibition as ordinate (see Fig. 8). In this plotting a linear relationship was observed between inhibition percent and inhibitor concentration.

Antifibrinolytic Assay——Antifibrinolytic effects of inhibitors were assayed according to the fibrin-clot lysis time assay of Okamoto and Okamoto[7] and to the fibrin-plate assay of Okamoto, *et al.*[15] In the lysis time assay, antifibrinolytic activity of an inhibitor was presented as the concentration of the inhibitor which increased lysis time by two times; and in the fibrin-plate assay, it was presented as the concentration of the inhibitor which reduced lyzed area to 50% of the control.

Experimental

Studies on Assay Conditions of Inhibitory Effects of the Test Compounds

1) **Plasminogen**——Casein digestion by the actions of plasminogen and streptokinase proceeded sigmoidally as shown in Fig. 1. In the assay system for antiplasmin activity of the test compounds, 0.7 unit of plasminogen was used. This is the amount present in about 0.5 ml of serum. One unit of plasminogen was defined as the amount of the proenzyme which catalyzed hydrolysis of 1 μmole of N-tosyl-L-arginine methylester per minute at 37° after its complete activation to plasmin with streptokinase at 37° for 1 hr. The method of determination of plasminogen will be reported elsewhere.[16]

2) **Substrate Concentration**——Casein was digested in the plasminogen-streptokinase system at various casein concentrations. In the presence of 0.7 unit of plasminogen per ml of the reaction mixture, the maximum rate was observed at the casein concentration of 1—1.5% and an apparent substrate inhibition was observed at higher casein concentrations (Fig. 2).

13) H.P. Treffers, *J. Bacteriol.*, **72**, 108 (1956).
14) T. Aoyagi and D. Mizuno, *J. Gen. Microbiol.*, **20**, 180 (1959).
15) S. Okamoto, U. Takada, and U. Okamoto, *Nihon Seirigaku Zasshi*, **27**, 207 (1965).
16) Y. Abiko, M. Iwamoto, and M. Shimizu, *J. Biochem.*, in press.

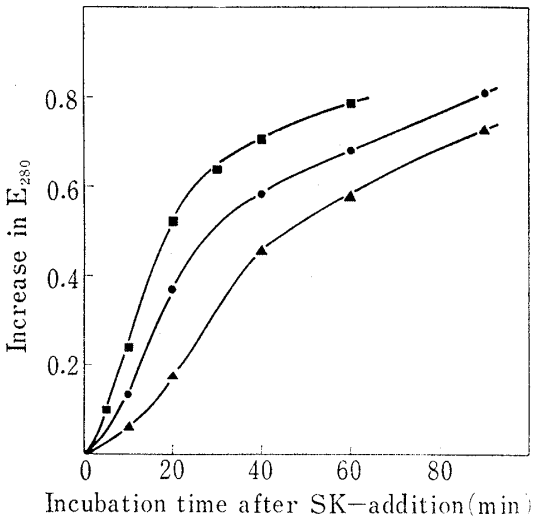

Fig. 1. Time Course of Casein Digestion by Plasminogen–SK System

An euglobulin solution was added to 6 ml of 2% casein solution and diluted with PBS to a final volume of 11.4 ml. After preincubation at 37° for 5 minutes, 0.6 ml of streptokinase (SK) solution (2,000 units/ml PBS) was added to the mixture and incubated at 37°. At proper time intervals, each of 2 ml of the reaction mixture was withdrawn and added to 2 ml of 1.7M perchloric acid. After 1 hour-standing at room temperature the mixture was centrifuged and the extinction of the supernatant was read at 280 mμ. (▲) 0.35 unit plasminogen/ml reaction, (●) 0.7 unit plasminogen/ml reaction, (■) 1.4 unit plasminogen/ml reaction.

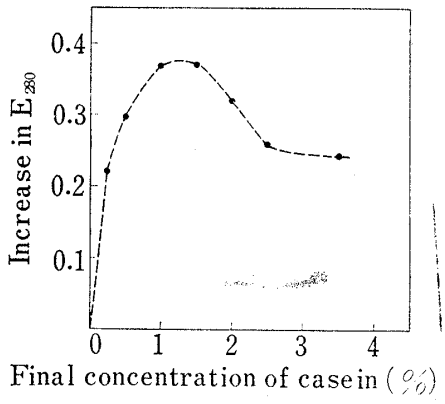

Fig. 2. Effect of Casein Concentration on the Rate of Caseinolysis by Plasminogen–SK System

Euglobulin (0.7 unit plasminogen) was added to 1 ml of a casein solution of various concentrations and diluted with PBS to a final volume of 1.9 ml. After preincubation at 37° for 3 minutes, 0.1 ml of SK solution (2,000 units/ml PBS) was added to the mixture and incubated at 37° for 20 minutes. After incubation 2 ml of 1.7M perchloric acid was added and the mixture was centrifuged after 1 hour-standing at room temperature. The extinction of the supernatant was read at 280 mμ against each enzyme blank.

3) **Concentration of Streptokinase**——Casein was digested in the plasminogen–streptokinase system at the constant concentrations of casein and plasminogen and at various concentrations of streptokinase. As shown in Fig. 3, in the presence of 0.7 unit of plasminogen per ml and at the casein concentration of 1%, the maximum rate was obtained at the streptokinase concentration of 200 units/ml.

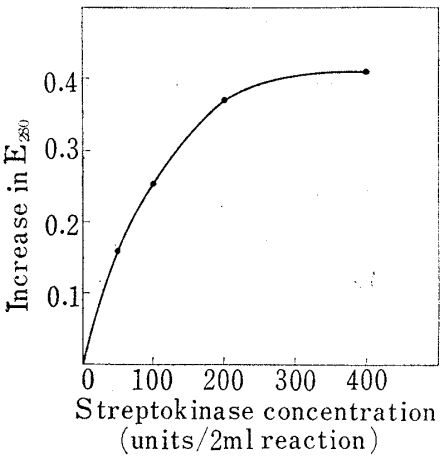

Fig. 3. Effect of Streptokinase Concentration on the Rate of Caseinolysis by Plasminogen–SK System

Euglobulin (0.7 unit plasminogen) was added to 1 ml of 2% casein solution and diluted with PBS to a final volume of 1.9 ml. After preincubation at 37° for 3 minutes, 0.1 ml of SK solution of various concentrations (500—4,000 units/ml PBS) was added, and the mixture was incubated at 37° for 20 minutes. After incubation 2 ml of 1.7M perchloric acid was added, and centrifuged after 1 hour-standing at room temperature. The extinction of the supernatant was read at 280 mμ against each enzyme blank.

Time courses of caseinolysis initiated by addition of streptokinase at various concentrations (Fig. 4) showed a lag in the early stage of the reaction in all cases examined, indicating that the activation of plasminogen by streptokinase was time-dependent (that is, enzymatic in nature) in these cases. When casein was added to plasmin previously activated from plasminogen by streptokinase at 25° for 30 minutes, the caseinolytic reaction was found to proceed without lag phase (Fig. 5).

4) **Reaction Time**——As shown in Figs. 1 and 4, the reaction curve of casein digestion in the plasminogen–streptokinase system was sigmoidal. The rate of caseinolysis was dependent on the amount of plasminogen only in the early or middle stage of the reaction, and after a long period of incubation the amount of acid-soluble products reached the same maximum level, which was independent on the amount of plasminogen added (Fig. 1). As shown in Fig. 6, inhibitory effect of *trans*-AMCHA on caseinolysis in the plasminogen–streptokinase system was found to be strong especially in the early stage of the reaction, and the

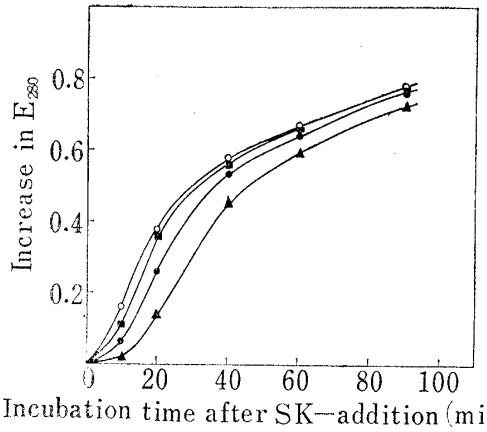

Fig. 4. Time Courses of Caseinolysis by Plasminogen–SK System at Various SK Concentrations

Reaction systems were similar to that described in Fig. 3. (▲) 50 units SK/2 ml reaction, (●) 100 units SK/2 ml reaction, (■) 200 uints SK/2 ml reaction, (○) 400 units SK/2 ml reaction.

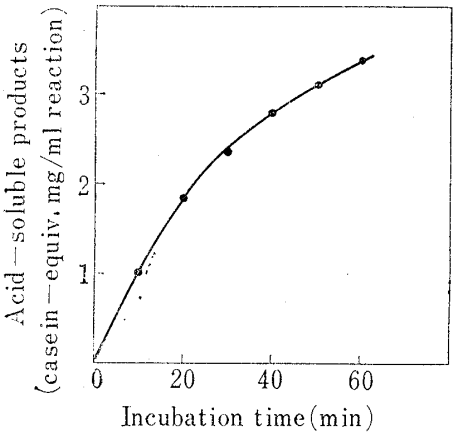

Fig. 5. Time Course of Caseinolysis by Plasmin Previously Activated by SK

Plasminogen was incubated with streptokinase at 25° for 30 minutes and then with casein at 37°. At proper time intervals after addition of casein, each aliquot was withdrawn and mixed with perchloric acid. The deproteinized supernatant was assayed for acid–soluble products by the method of Lowry, *et al.*[17]

longer the reaction period the weaker the inhibitory effect. These results indicated importance of incubation period in the assay reaction.

5) **pH**——Optimal pH of the caseinolytic reaction including activation process was found to be 7.4.

Standard Assay Condition

From the results presented above, the standard assay condition for antiplasmin activity of the test compounds was decided as follows.

A half mililiter of euglobulin solution (0.7 unit of plasminogen) is preincubated with 1 ml of 2% casein solution and 0.4 ml of the phosphate buffer

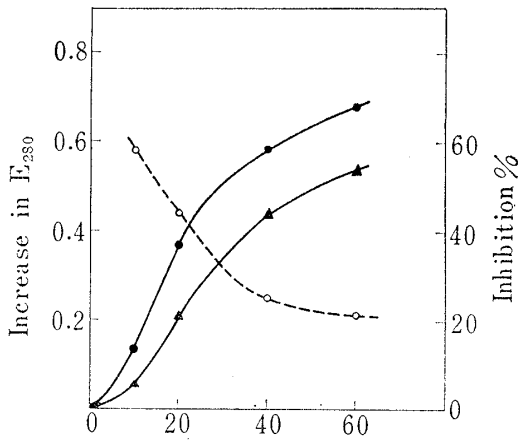

Fig. 6. Effect of *trans*–AMCHA on Caseinolysis by Plasminogen–SK System

Euglobulin (3.5 unit plasminogen) was preincubated with 5 ml of 2% casein solution and with or without 20 μmoles of *trans*–AMCHA in a final volume of 9.5 ml at 37° for 5 minutes. The reaction was initiated by adding 0.5 ml of SK solution (2,000 units/ml PBS), and at proper time intervals 2 ml aliquots were taken and mixed with 2 ml of 1.7M perchloric acid. After 1 hour–standing at room temperature the mixture was centrifuged, and the extinction of the supernatant was read at 280 mμ against an enzyme blank. Final concentration of *trans*–AMCHA in the reaction mixture was 2×10^{-3}M. The inhibition percent was calculated by comparison of the two reactions. (●) Time course of the reaction containing no inhibitor, (▲) time course of the reaction containing *trans*–AMCHA, (···○···) inhibition %.

saline (PBS) containing various amounts of an inhibitor to be tested at 37° for 3 minutes. Then, 0.1 ml of streptokinase solution (200 units/ml PBS) is added and the mixture is incubated at 37° for 20 minutes. After incubation, 2 ml of 1.7M perchloric acid is added, allowed to stand at room temperature for about 1 hr, and centrifuged. The extinction of the clear supernatant is measured at 280 mμ against an enzyme blank in which the streptokinase solution is added after addition of perchloric acid.

The inhibition rates are calculated by comparison with the control run in which the inhibitor is omitted, and the 50% inhibition concentration (ID_{50}) of the inhibitor is obtained graphically as described in Materials and Methods (or see Fig. 8).

Results

Relative Antiplasmin Activities of Some ω-Aminoacids

A series of ω–aminoacids including C_3—C_7 were assayed for their antiplasmin activities by the present method. Fig. 7 shows antiplasmin activities relative to that of 6–aminocaproic

17) O.H. Lowry, N.J. Resebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

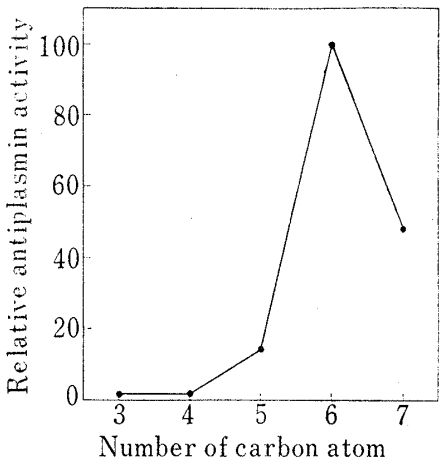

Fig. 7. Relative Antiplasmin Activities of Some ω–Aminoacids

ID_{50}s were determined for antiplasmin activities of β–alanine, 4–aminobutyric acid, 5–aminovaleric acid, 6–aminocaproic acid and 7–aminoheptanoic acid. They were represented in relative activities to antiplasmin activity of 6–aminocaproic acid (ε–ACA) by following equation: (ID_{50} of ε–ACA/ID_{50} of ω–amino-acid) × 100.

acid (ε–ACA) of other four ω–aminoacids: β–alanine, 4–aminobutyric acid, 5–aminovaleric acid and 7–aminoheptanoic acid. ε–ACA was found to be the most potent inhibitor in this series of compounds.

Comparison of Antiplasmin Activities of ε-ACA and the Two Stereoisomers of AMCHA

The two stereoisomers of 4–aminomethyl-cyclohexanecarboxylic acid, *cis*– and *trans*–AMCHA were assayed for their antiplasmin activities in comparison with that of ε–ACA. Fig. 8 shows graphical determination of their ID_{50}s. *trans*–AMCHA was found to be the most potent inhibitor and its inhibitory effect was 5—7 times more potent than that of ε–ACA and about 50 times more potent than that of *cis*–isomer (Table I).

Antiplasmin Activity of the Mixture of *cis*- and *trans*-AMCHA

Antiplasmin activities of the mixtures which contained *cis*– and *trans*–AMCHA in various proportions were assayed. As shown in Fig. 9, antiplasmin activity of the mixture was apparently proportional to the amount of *trans*–AMCHA contained, and *cis*–isomer did not affect the activity of *trans*–AMCHA.

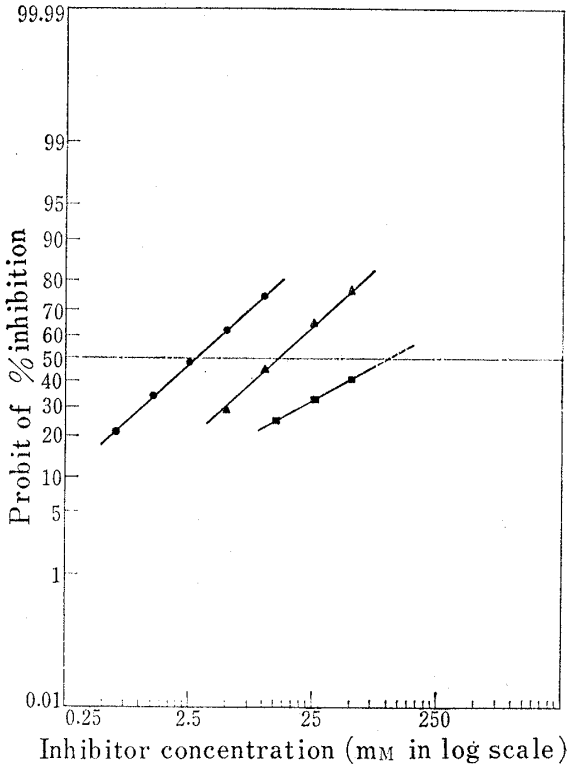

Fig. 8. A Graphical Determination of ID_{50}s of ε–ACA, *cis*– and *trans*–AMCHA

Experimental conditions were described in the text. (●) *trans*–AMCHA, (▲) ε–ACA, (■) *cis*–AMCHA.

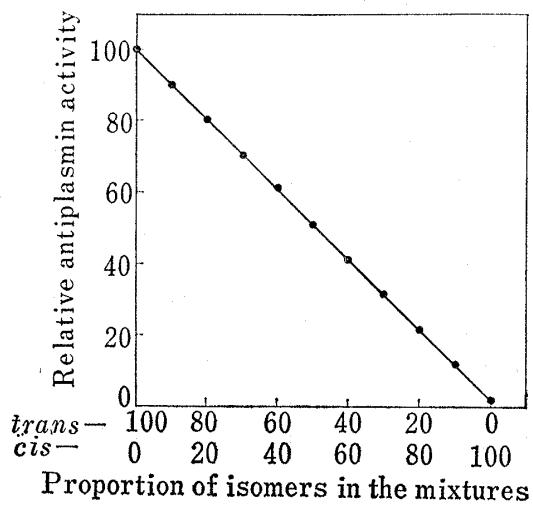

Fig. 9. Relative Antiplasmin Activity of the Mixtures of *trans*– and *cis*–AMCHA

trans–AMCHA was mixed with the *cis*–isomer at various proportions. Each mixture was assayed for antiplasmin activity and compared with *trans*–AMCHA in each ID_{50}.

Antiplasmin Activity of *trans*-AMCHA in Fibrinolysis

Inhibitory effect of *trans*–AMCHA on fibrinolysis was examined by the fibrin–clot lysis time assay and by the fibrin–plate assay. As shown in Table I, antifibrinolytic activity

Table I. Antiplasmin Activities of ε-ACA, *cis*- and *trans*-AMCHA

	Present method		Fibrin-clot lysis time method		Fibrin-plate method	
	ID_{50} (M)	R.A.[a]	ID_{50}[b] (M)	R.A.	ID_{50}[c] (M)	R.A.
ε-ACA	$1—1.3\times10^{-2}$	1	1.02×10^{-3}	1	1.12×10^{-3}	1
trans-AMCHA	$1.5—3\times10^{-3}$	5—7	1.26×10^{-4}	8.1	1.31×10^{-4}	8.5
cis-AMCHA	approx. 0.1	0.1	—		—	

Antiplasmin activities of ε-ACA, *cis*-AMCHA and *trans*-AMCHA were assayed by the present caseinolytic method, the fibrin-clot lysis time method and the fibrin-plate method.

a) Relative antiplasmin activity.

b) ID_{50} in the clot lysis time method was presented as the concentration of the inhibitor which increased lysis time by two times.

c) ID_{50} in the fibrin-plate method was presented as the concentration of the inhibitor which reduced lyzed area to 50% of the control.

of *trans*-AMCHA was about 8 times more potent than that of ε-ACA in both assays. *cis*-AMCHA was again little active also in both fibrinolytic assay systems.

Discussion

Several methods have been reported for screening antiplasmin drugs. They include fibrinolytic assay methods[18,19] and caseinolytic assay methods.[20,21] ε-ACA,[22,23] AMCHA[24,25] and *p*-aminomethylbenzoic acid[26] were reported to inhibit the activation process of plasminogen to plasmin competitively with the zymogen. For this reason, it seems to be important to make the amount of plasminogen constant in the assay system when the inhibitory effects of test compounds are examined in a system including the activation process. In fibrinolytic assay, it is difficult to control the amount of plasminogen in the reaction system because commercial preparations of fibrinogen (of human or bovine origin) contain a considerable amount of plasminogen.

We established a new caseinolytic assay method for screening antiplasmin drugs after detailed examination on assay conditions: concentrations of plasminogen, casein and streptokinase and reaction time. This assay method is an application of Norman's casein assay for plasminogen,[12] and can give informations about the effects of test compounds on the activation process of plasminogen to plasmin, as well as on the action of active plasmin. A similar assay system was employed in studies on antiplasmin activities of some lysine and arginine derivatives by Nagamatsu, *et al.*[20]

In the present assay method, the reaction time is another important factor for evaluating the antiplasmin activity of a test compound, because the inhibitory effect varies dependently on incubation time (Fig. 6). In a longer period of incubation may be missed the inhibitory effect of a test compound. The reaction curve of this system with human plasminogen is sigmoidal and the effect of an inhibitor should be examined in an early phase of the reaction (ΔE_{280}=0.1—0.4).

Introduction of ID_{50} to our assay method could quantify the effect of inhibitors and made it possible easily to compare the inhibitory effects among many compounds. The

18) M. Yokoi, *J. Physiol. Soc. Japan* (in Japanese), **22**, 1098 (1960).
19) A.F. Bickford, F.B. Taylor, and R. Sheena, *Biochim. Biophys. Acta*, **92**, 328 (1964).
20) A. Nagamatsu, T. Okuma, M. Watanabe, and Y. Yamamura, *J. Biochem.*, **54**, 491 (1963).
21) T. Onishi, M. Muramatsu, T. Sato, and S. Fujii, *J. Biochem.*, **60**, 87 (1966).
22) F.B. Ablondi, J.J. Hagan, M. Philips, and E.C. De Renzo, *Arch. Biochem.*, **82**, 153 (1959).
23) N. Alkjaersig, A.P. Fletcher, and S. Sherry, *J. Biol. Chem.*, **234**, 832 (1959).
24) A.H.C. Dubber, G.P. McNicol, and A.S. Douglas, *Brit. J. Haematol.*, **11**, 237 (1965).
25) M. Maki and F.K. Beller, *Thrombos. Diathes. Haemorrh.*, **16**, 668 (1966).
26) F. Markwardt, H. Landmann, and A. Foffmann, *Hoppe-Zeiler's Physiol. Chem.*, **340**, 174 (1965).

results from experiments on relative antiplasmin activities of ω-aminoacids (Fig. 7) agreed with that reported by other investigators[27-29] using fibrin-clot lysis time assay, and relative antiplasmin activities of ε-ACA, *trans*- and *cis*-AMCHA in the casein assay were parallel with that in the two fibrin assays: the fibrin-clot lysis time assay and the fibrin-plate assay. These facts indicate that the present assay method is reliable in screening antiplasmin drugs.

Okamoto, *et al.*,[7,8] Lohman, *et al.*,[30] and Mangyo[29] demonstrated that antiplasmin activity of AMCHA was more potent than that of ε-ACA when examined by the fibrin-clot lysis assay. Recently, Okamoto, *et al.*[10] and Shimizu, *et al.*[11] reported that *trans*-form isomer of AMCHA was an active compound and that *cis*-AMCHA had little activity. Dubber, *et al.*[31] also reported the existence of an active stereoisomer and an inactive one of AMCHA. Studies on the separation and chemical nature of the two stereoisomers of AMCHA will be reported in detail in the following paper.[9] *trans*-AMCHA was about 50 times more potent than *cis*-isomer and 5—7 times more potent than ε-ACA (Table I). *cis*-AMCHA did not only exert the effect on plasmin system but also did not affect the inhibitory action of *trans*-AMCHA (Fig. 9). This make it possible to determine enzymatically the content of *trans*-AMCHA in the mixture. It is very interesting that *trans*-AMCHA is a far more potent inhibitor in plasmin system than *cis*-AMCHA.

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27) M. Yokoi, *J. Physiol. Soc. Japan* (in Japanese), **22**, 1103 (1960).
28) M. Miyamoto and K. Lee, *J. Pharm. Sci.*, **51**, 912 (1962).
29) M. Mangyo, *J. Jap. Biochem. Soc.* (in Japanese), **36**, 735 (1964).
30) K. Lhoman, F. Markwardt, and H. Landmann, *Naturwiss.*, **50**, 502 (1963).
31) A.H.C. Dubber, G.P. McNicol, A.S. Douglas, and B. Melander, *The Lancet*, **2**, 1317 (1964).