

## STUDIES ON HETEROGENEITY OF FIBRINOLYTIC ACTIVITY IN ARTIFICIALLY INDUCED INTRAVASCULAR CLOTS

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### SUMMARY

This study was designed to investigate the effectiveness of enhancing local fibrinolytic activity in intravascular clots preventing side reactions resulting from fibrinolytic activation in the circulating blood. For this purpose, the following procedures were performed.

Artificial intravenous clots were induced in femoral and external jugular veins of dogs by three different methods. To dogs at various time intervals after clot formation, EACA or trans-AMCHA was intravenously injected following with simultaneous human serum, and then various units of streptokinase or urokinase were intravenously administered.

Fibrinolytic activity of these clots after treatment was assayed according to the fibrin plate method, at the same time fibrinolytic activity of a thrombosed vein was assayed.

The results of these experiments were as follows:

- 1) Artificially induced intravenous clots showed no fibrinolytic activity except thrombin-induced clots.
- 2) Activator activity of one hour to six day old clots treated with EACA or trans-AMCHA, was significantly enhanced by the simultaneous injection of streptokinase or urokinase.
- 3) Activator activity of intima of a thrombosed vein was also enhanced with streptokinase or urokinase.
- 4) The administration of EACA or trans-AMCHA did not sacrifice the fibrinolytic activation in an intravenous clot with streptokinase or urokinase.
- 5) A fibrinolytic mechanism in an intravascular clot showed different characteristics from that of the circulation.

### INTRODUCTION

In the normal human body, blood coagulation and fibrinolysis are always well balanced and intravascular blood coagulation is a continuous physiologic process<sup>1)2)</sup>. A continuous clotting and by necessity a continuous lysing process may be part of normal hemostasis, and hemofluidity the result of a dynamic balance between these processes<sup>3)</sup>.

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The endothelium of blood vessels under normal conditions are coated with a fibrin layer essential for the integrity of the capillary wall<sup>4)5)</sup>. Unwanted fibrin formed in the body is usually disposed by enzymatic digestion of plasmin which exists in the normal plasma, principally in the form of an inactive precursor, plasminogen<sup>6)7)8)</sup>. The precursor can be activated *in vivo* by shock, cyanosis, anxiety, severe exercise, pyrogens, injection of adrenaline and many other means<sup>9)</sup>. *In vitro*, plasminogen is activated by tissue activators<sup>10)11)</sup>, urinary activator (urokinase)<sup>12)13)14)</sup>, trypsin<sup>15)</sup>, chymotrypsin<sup>16)</sup>, plasmin<sup>17)18)</sup>, streptokinase<sup>19)</sup> and body fluids<sup>10)20)21)22)</sup>. Streptokinase (SK), extract of beta-hemolytic streptococci<sup>23)</sup>, does not react directly with plasminogen; rather, it interacts initially with a proactivator which is converted to activator which, in turn, catalyzes the plasminogen-plasmin reaction<sup>24)25)26)</sup>. Plasmin digests not only fibrin, but also fibrinogen, thrombin, prothrombin, blood clotting factors V, VII, VIII, IX and many other proteins<sup>8)27)</sup>, resulting in hemorrhagic diathesis<sup>28)</sup> or allergic reactions<sup>29)</sup>.

The destruction of plasma proteins by plasmin is limited by inactivation of the enzyme by inhibitors present in the plasma. A plasmin-inhibiting substance was found in the pseudoglobulin fraction of human serum by Milstone<sup>30)</sup>.

Norman<sup>31)</sup> reported slow and fast type inhibitors which are active against plasmin, activator and certain kinases. Plasmin inhibitors are found in tissues as well as in the circulating blood. Saline extracts of human liver, kidney, spleen, adrenal, thyroid, muscle, lung, heart and brain contain plasmin inhibitors<sup>32)</sup>. Although the etiology of thrombosis has been obscure, hypercoagulability and hypoplasminemia may be responsible for thromboembolic disorders.

Alkjaersig, *et al.*<sup>33)</sup> demonstrated that the chief and primary mechanism of plasma thrombolysis involves the activation of intrinsic clot plasminogen with resulting autodigestion of the thrombus and that the secondary mechanism of thrombolysis by exogenous plasmin action appears to be relatively unimportant. On the other hand, Ambrus<sup>3)</sup> reported that at concentrations of antiplasmin at which caseinolytic effect completely disappears, considerable fibrinolytic activity persists, and suggested that fibrin can compete for plasmin with antiplasmin while other proteins may be less effective in this respect. This may explain the specificity of plasmin for fibrin *in vivo*, in the presence of the inhibitor. Antiplasmin may play an important physical role in protecting normal plasma proteins while allowing lysis of fibrin clots by plasmin. So that a plasmin-antiplasmin complex may function as a circulating reservoir of plasmin, innocuous as a proteolytic enzyme for circulating proteins, but available whenever needed for clot lysis.

A number of reports on the ability of fibrinolytic enzymes in thromboembolic diseases have been presented<sup>34)35)36)</sup>. Intravenous administration of fibrinolytic agents has been often followed with undesirable side reactions, chill, hyper-

thermia, vomiting and oozing from the wounds<sup>37)(38)(39)(40)</sup>.

In the clinical application of fibrinolytic enzymes, it is desirable that these side effects are prevented by the administration of antiplasmin agents, such as EACA<sup>(41)</sup> and trans-AMCHA<sup>(42)</sup>, without inhibiting fibrinolytic activity in intravascular clots.

The purpose of the present study is to explore the possibility of producing an active fibrinolytic system in the intravenous clots of dogs by intravenous injection of EACA or trans-AMCHA, while inhibiting the circulatory fibrinolytic activity, and their effects on artificially induced intravascular clots were observed. Injection of EACA or trans-AMCHA was followed with simultaneous human serum and streptokinase or urokinase administration.

Treated clots at various stages of organization were assayed for their fibrinolytic activity by the fibrin plate method.

#### MATERIALS AND METHODS

##### *Fibrinogen*

Bovine fibrinogen, Fraction I (Daiichi Kagaku, Japan) was used. Prior to use, real fibrinogen concentration was determined by the fibrin clot weighing method<sup>(43)</sup>, and fibrinogen solution was prepared with veronal buffer, pH 7.4, at each time of use.

##### *Thrombin*

A sample containing 500 units in each vial was supplied by Mochida Seiyaku (Japan), of bovine origin. It was dissolved in physiological saline to 100 units per ml.. This sample was found contaminated with a fibrinolytic active substance, revealed in this study.

Thrombin solution was kept in a refrigerator at  $-15^{\circ}\text{C}$  and used in need. The duration of storage after being dissolved was not more than three days.

##### *Streptokinase (SK)*

Streptokinase-streptodornase Mixture, Varidase (Lederle Laboratories Division), was used. Each vial contains 100,000 units of streptokinase and 25,000 units of streptodornase, and was used in a physiological saline solution. This streptokinase solution was prepared immediately before use.

##### *Urokinase (UK)*

A sample containing 900 units in each vial was supplied by Green Gross (Japan). This sample was dissolved in physiological saline just prior to use.

##### *Veronal buffer solution*

This was prepared as follows. 11.75 g of sodium diethyl barbiturate, 11.69

g of sodium chloride, and 430 ml of 1/10 N hydrochloric acid were added to 1,570 ml of distilled water. This solution was adjusted to pH 7.4.

*EACA (Epsilon-amino caproic acid)*

An antiplasmin agent which inhibits the activation of a fibrinolytic system was obtained from Daiichi Seiyaku (Japan).

*Trans-AMCHA (trans-4-aminomethyl cyclohexane-1-carboxylic acid)*

This is a more effective antiplasmin agent than EACA, also supplied by Daiichi Seiyaku (Japan).

*Human serum*

This was prepared from expired Blood Bank plasma and stored at  $-15^{\circ}\text{C}$  until use.

*Preparation of fibrin plate*

Standard fibrin plate was prepared by the method of Astrup and Müllertz<sup>44)</sup>. In each petri dish 9 cm in diameter, 9.0 ml of 0.1% fibrinogen solution was measured with a pipette and clotted by 0.1 ml (10 units) of thrombin solution. After clotting, the petri dish was kept at room temperature for 30 minutes, thereafter materials were placed on the surface of the fibrin layer. This procedure was always performed on a horizontal glass plate. After incubation for 18 hours at  $37^{\circ}\text{C}$ , the product of two perpendicular diameters was recorded in  $\text{mm}^2$  and the mean values of three determinations were calculated. These results were estimated as activator activity of a material.

Heated fibrin plate was prepared according to the method of Lassen<sup>45)</sup>, which consists of heating standard fibrin plate at  $85^{\circ}\text{C}$ , for 30 minutes in an oven. Heated plate was used for assay of plasmin activity of a material.

*Cold acetic acid solution*

This reagent was prepared as follows. 0.32 ml of 1.0% acetic acid was added to 19 ml of distilled water, and this mixed solution was adjusted to pH 5.2. This acetic acid solution was kept in a refrigerator at  $0^{\circ}\text{C}$  until use.

*Euglobulin fraction<sup>46)</sup>*

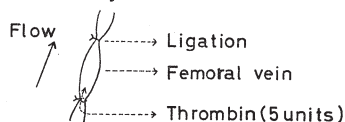
0.5 ml of citrated plasma or serum was added to 9.5 ml of cold acetic acid solution, there-after kept in an ice box for 30 minutes, then taken out, centrifuged for 30 minutes at 3,000 r.p.m., and immediately after centrifugation the supernatant was discarded and the precipitate was dissolved in 0.5 ml of veronal buffer solution.

*Artificial intravenous clot formation*

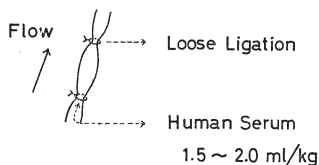
Dogs of both sexes weighing 8 to 12 kg were anesthetized with 30 mg of

intraperitoneal thiobarbiturate per kg of body weight. The neck and femoral regions were shaved and aseptic precautions were attempted. Both femoral and external jugular veins were exposed for three to five centimeters in length. All demonstrable tributaries of these veins were carefully ligated and then cut so that the vessels were entirely free from surrounding structures. Intravenous clot was induced by the following three different methods.

1) Thrombin Injection



2) Modified Wessler's Method



3) Pumping Method

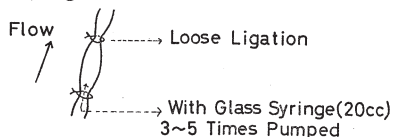


FIG. 1. Methods of intravenous clot formation.

1) Thrombin method (Fig. 1)

A proximal end of an exposed vein was ligated, then a distal end was loosely occluded. 5 units (0.05 ml) of thrombin solution was distally injected into the segment. The vein was allowed to refill with blood from the distal end, then ligated and left for one hour. Clot formation was verified by palpation.

2) Modified Wessler's method (Fig. 1)<sup>47)</sup>

Both proximal and distal ends were loosely ligated so that blood was able to flow through the half occluded channel, and then 1.5 ml per kg of human serum was injected into a segment from a distal site just below the ligation. Immediately after serum injection, only the proximal ligation was fastened. About one to two hours after

the operation, intravenous clot was noted.

3) Pumping method (Fig. 1)

This procedure was designed to produce artificial intravascular clot without any other thrombotic agents, and by loose ligation, a 3 to 5 cm long venous segment was formed. Nonsiliconized needle was inserted into the lumen of a segment through a distal end, then the blood in the segment was gently aspirated with a nonsiliconized glass syringe of 20 cc. When 10 to 20 ml of blood was drawn into the syringe, the blood was reinjected into the segment, and this procedure was repeated 3 to 5 times until the piston of the syringe could no longer be pushed because of blood coagulation. The proximal end was tightly occluded immediately after completion of this procedure, the distal ligation was kept loose, so that the blood can flow into the segment. Fifteen minutes later,

this distal end was also occluded. Intravenous clot was formed within 30 minutes to 2 hours after operation, and this was ascertained by palpation and aspiration.

#### *Fibrinolytic activation of intravenous clots*

To clots one hour to 6 day old after formation, at first, EACA or trans-AMCHA, then human serum, and finally streptokinase or urokinase of varying units, were intravenously injected. Thirty minutes after administration, these clots were removed and fibrinolytic activity was assayed.

#### *Assay of fibrinolytic activity of a thrombosed and normal venous intima*

Six day old thrombosed venous intima and normal intima were assayed for their fibrinolytic activity by the fibrin plate method. These intima were dissected into slices, were placed on the fibrin layer, and activator activity was estimated by products of the perpendicular diameters of the lysed area.

#### *Determination of whole plasmin in serum and plasma*

Euglobulin fraction was obtained from human serum and plasma, 0.5 ml of euglobulin solution was activated by 500 units of streptokinase, and after 5 minutes incubation, whole plasmin activity was measured by the fibrin plate method.

#### *Extraction of activator in clot*

According to the method reported by Okamoto<sup>48</sup>, in a ratio of ten to one, 30% saccharose solution was added to a clot, thereafter this was crushed and mixed well, and kept in an ice box at 0°C for two hours. After these procedures, the mixed solution was centrifuged at 3,000 r.p.m. for 15 minutes, and the supernatant was used for assay of activator activity in a clot.

#### *Fibrinolytic activity of tissues adjacent to a clot*

The following study was designed to investigate the fibrinolytic activity of the tissues around a clot as they may be responsible for the fibrinolytic activity in clots.

The aspirated venous blood of a dog was injected into the peritoneal cavity and subcutaneous tissue of the dog, and clotted. An intravenous clot was induced with 1.0 unit of thrombin.

These clots and coagula were assayed for their fibrinolytic activity before and 30 minutes after 1,800 units of urokinase administration. Simultaneously, venous intima was assayed for fibrinolytic activity.

## RESULTS

### *A) Fibrinolytic activity of artificially induced clots (Fig. 2)*

Intravenous clots induced by three different methods were assayed for

activator activity 24 hours after clot formation.

Intravenous clots induced by Wessler's and pumping methods showed no activator activity or plasmin activity, but a thrombin induced clot revealed activator activity, which might be due to contamination with a fibrinolytic substance in the thrombin preparation, such as proactivator, activator, because of impurity of the sample.

It is postulated that in clot formation, adsorption of activator into a clot depends upon activator volume in the circulation and surrounding tissues. When a higher level of activator activity is present in the circulating blood, an activator rich clot will be formed, which is sensitive to clot-lysing agents.

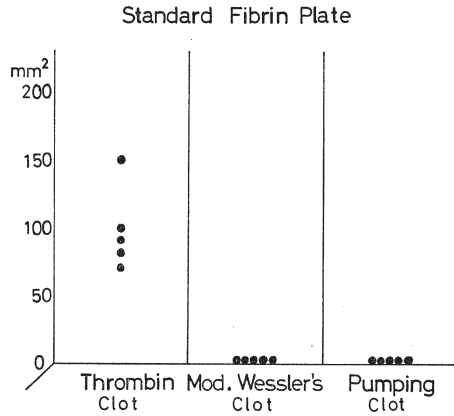


FIG. 2. 24 hours after clot formation, activator activity of clots induced by thrombin injection, modified Wessler's and pumping methods was assayed. No activator activity was observed except in thrombin induced clot.

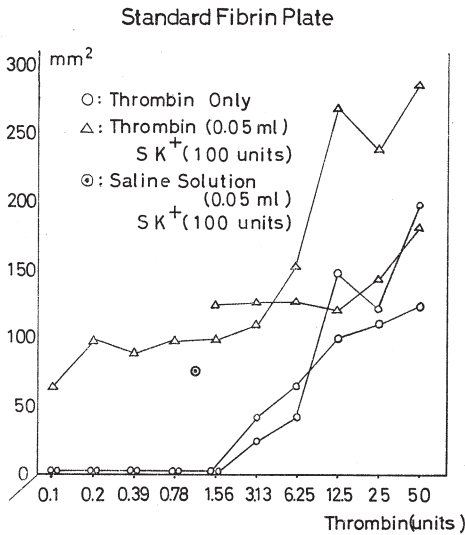


FIG. 3. Fibrinolytic activity of thrombin preparation was assayed. Thrombin solution showed a significant amount of activator activity, also proactivator contamination was observed.

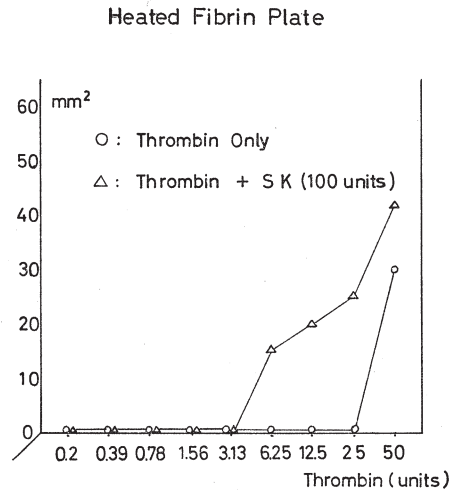


FIG. 4. Fibrinolytic activity of thrombin preparation was assayed by the fibrin heated plate. Thrombin solution of over ca. 6 units showed plasminogen contamination. No fibrinolytic activity was found with under ca. 3 units of thrombin solution.

B) *Fibrinolytic activity of thrombin preparation* (Fig. 3, 4)

0.05 ml of thrombin solution of various potencies ranging from 0.1 to 50 units were prepared as presented in Fig. 3, and activated with 100 units of streptokinase. After incubation for 5 minutes, fibrinolytic activity of these materials was assayed by the fibrin plate method. No activator activity was noted in samples of under 1.56 units, but in those of over 3.13 units some degree of activator activity was found.

By SK-activation, the activator activity of the thrombin preparation was much more enhanced than that of SK itself. From these results it may be suggested that a thrombin solution of over 3.13 units contains a fair amount of proactivator or activator. On the other hand, as shown in Fig. 2, an intravenous clot induced with 5 units of thrombin revealed more activator activity than 5 units of thrombin solution *in vitro*. It is probable that a contaminated fibrinolytic substance absorbed into a clot can be activated by an activator in the circulating blood, so that a clot *in vivo* is quite different from that *in vitro* in its fibrinolytic activity. Heated fibrin plate method showed that thrombin preparation was also contaminated with a trace of plasminogen (Fig. 4). 5 units of thrombin presented a little degree of fibrinolytic activity. It is probable that plasmin will be rapidly neutralized by antiplasmin in a clot.

C) *Fibrinolytic activation of intravenous clots* (Fig. 5, 6)

1) *One hour old clot*: To one hour old clot induced by a pumping method, 200 mg per kg of EACA, 1.5 ml per kg of human serum and 10,000 units per kg of streptokinase were intravenously injected as shown in Fig. 5. Thirty minutes after the final injection, the venous segment was removed and the clot was taken out. This clot which had been dissected into slices was placed on the fibrin standard plate. After 18 hours incubation, the lysis area was recorded. At the same time, the activator activity of arterial and venous blood was measured. Clot revealed various degrees of activator activity ranging from 62 to 380 mm<sup>2</sup> of lysis area. Arterial and venous blood also presented activator activity. On the other hand plasmin activity was measured with the fibrin heated plate (Fig. 6).

The result indicated that clot had less plasmin activity than the circulating blood, and the fibrinolytic activity of not only the circulating blood, but also an intravenous clot was not inhibited by the administration of EACA.

2) *3 day old clot induced with thrombin* (Fig. 6)

Three days after clot formation, streptokinase, 15,000 units per kg, was intravenously injected to a dog, and 30 minutes later a clot was taken out and the fibrinolytic activity was assayed by the fibrin plate method. No activator



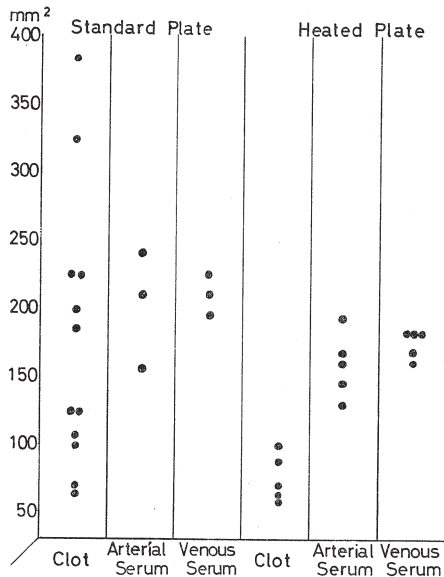


FIG. 5. One hour old clot induced by pumping method. EACA(200 mg/kg), human serum (1.5 ml/kg), and streptokinase (10,000 units/kg) were intravenously injected.

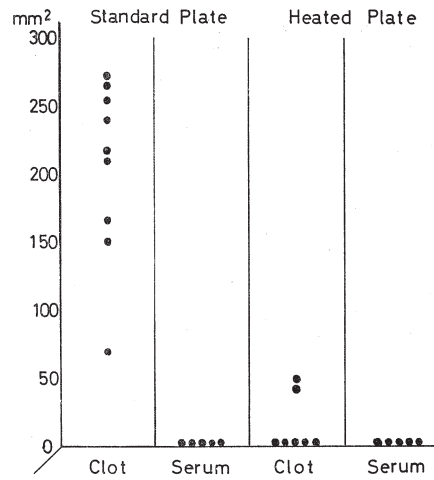


FIG. 6. 3 day old clot induced by thrombin solution. 30 minutes after clot formation, streptokinase (15,000 units/kg) was intravenously injected. Enhancement of activator activity was observed in clots.

and plasmin activity was found in the circulating venous blood, but a significant enhancement of activator activity in the clot was noted. Plasmin activity in the thrombus was less or absent. It was postulated that a proactivator rich clot such as that induced by thrombin might be activated by intravenously administered streptokinase.

3) 6 day old clot induced by modified Wessler's method (Fig. 7)

50 mg per kg of trans-AMCHA, 1.5 ml per kg of human serum, and 10,000 units per kg of streptokinase were intravenously injected to a dog with a 6 day old clot. 30 minutes later, the clot was removed and assayed for fibrinolytic activity by the fibrin plate method, while at the same time fibrinolytic activity of venous blood was measured. 6 day old clot revealed activator activity producing lysis areas of 94 to 128 mm<sup>2</sup> by the heated plate, and the clot showed plasmin activity, but serum not. Fibrinolytic activity of a thrombosed venous intima which was measured by the fibrin plate method, showed fibrinolytic activity much higher than that of clot. It is suggested that venous intima may be significantly activated by a fibrinolytic agent, such as streptokinase, and fibrinolytically activated intima will influence the activation of fibrinolytic system in a clot.

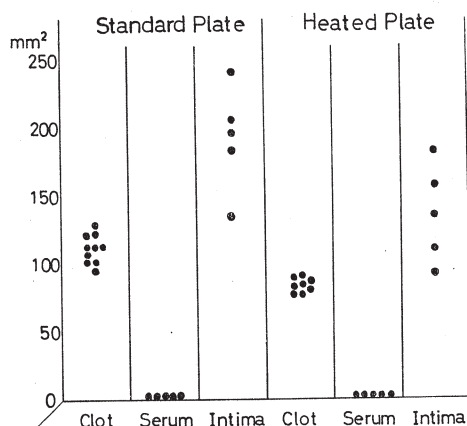


FIG. 7. 6 day old clot induced by modified Wessler's method. Trans-AMCHA (50 mg/kg), human serum (1.5 ml/kg), and streptokinase (10,000 units/kg) were intravenously injected.

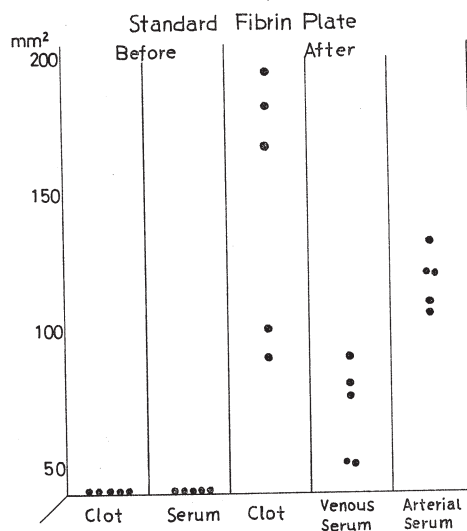


FIG. 8. 6 day old clot induced by pumping method. Trans-AMCHA (50 mg/kg), human serum (1.5 ml/kg), and streptokinase (20,000 units/kg) were intravenously administered. Remarkable enhancement of activator activity was observed in clots.

#### 4) 6 day old clot induced by pumping method (Fig. 8)

This stage of organized clot was treated with 50 mg per kg of trans-AMCHA, 1.5 ml per kg of human serum and 20,000 units per kg of streptokinase. Activator activity of the clot prior to treatment was not observed, but after the administration of these agents, activator activity of the clot was significantly enhanced, while venous and arterial serum also presented activator activity less than that of clot.

No plasmin activity was found in a thrombus and the circulatory blood before and after the treatment (Fig. 9)

From these results it was concluded that activator activity in clots would be enhanced by a fibrinolytic agent, such as SK, free from the effect of an antiplasmin agent.

#### 5) 6 day old clot induced by pumping method (Fig. 10)

This organized clot was treated with 50 mg per kg of trans-AMCHA, 2.0 ml per kg of human serum and 270 units per kg of urokinase. 30 minutes after treatment, the removed clot was assayed for fibrinolytic activity by the fibrin plate method. A significant enhancement of activator activity in clots was

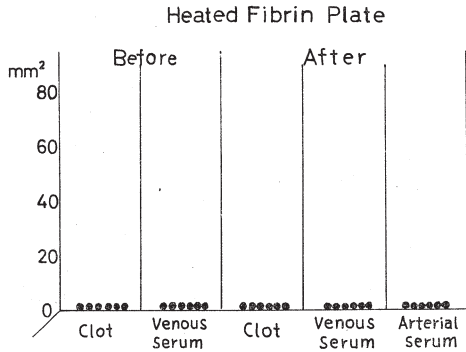


FIG. 9. 6 day old clot induced by pumping method. Trans-AMCHA (50 mg/kg), human serum (1.5 ml/kg), and streptokinase (20,000 units/kg) were intravenously administered.

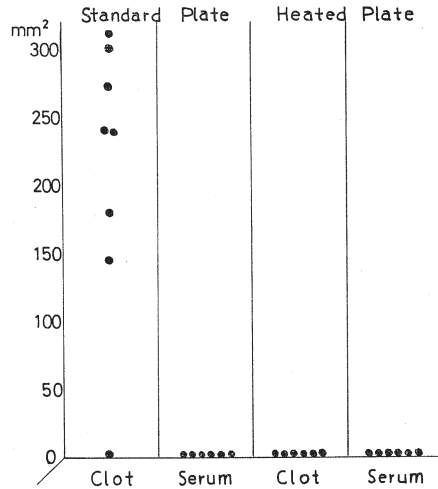


FIG. 10. 6 day old clot induced by pumping method. Trans-AMCHA (50 mg/kg), human serum (2.0 ml/kg), and urokinase (270 units/kg) were intravenously administered. Significant activator activity in clots was observed.

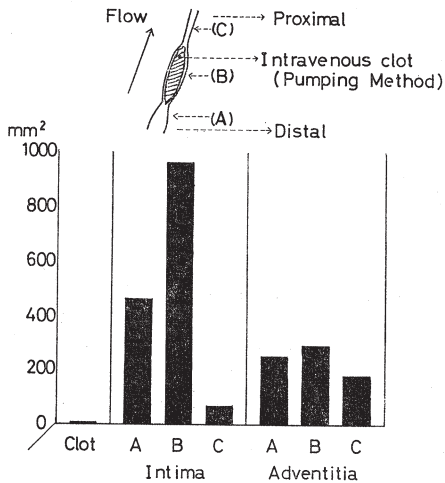


FIG. 11. Fibrinolytic activity of intravenous clot and venous wall was assayed by the fibrin standard plate. Venous intima of thrombosed site showed highest activator activity, and less in a proximal site. Clot showed no activator activity.

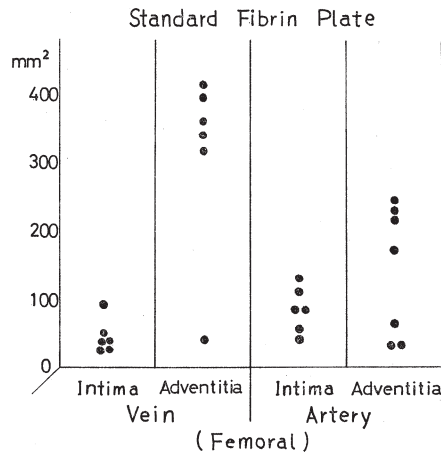


FIG. 12. Activator activity of intima and adventitia of blood vessel was assayed. Venous and arterial adventitia showed slightly higher activator activity than intima. There was no significant difference in activator activity of intima of vein and artery.

observed, but not in serum. No plasmin activity was noted in both clots and serum.

It was concluded that free plasmin in the circulation would be rapidly neutralized by antiplasmin, although activator activity in the clot may not be interfered by an antiplasmin agent.

*D) Fibrinolytic activity of a thrombosed vein (Fig. 11)*

The activator activity of a thrombosed venous wall was assayed by the fibrin standard plate method. The results shown in Fig. 12 indicated that a venous clot revealed no activator activity, although the venous intima of the thrombosed vein showed notable activator activity.

The activator activity of venous intima was highest in the affected thrombosed site, next in the distal vein and less in the proximal portion which had no blood flow. Plasmin activity was negative in the clot, intima and adventitia.

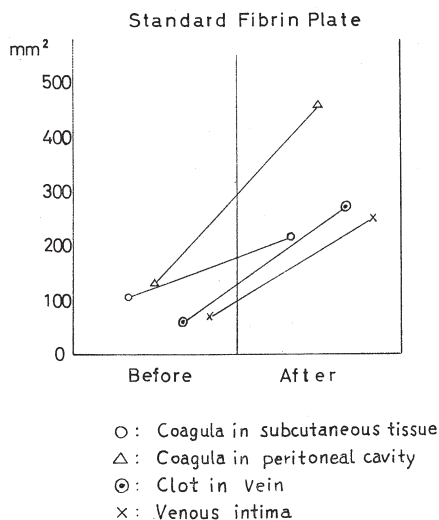


FIG. 13. This procedure was designed to investigate that in blood coagulation, fibrinolytic activity of clot will be correlated with fibrinolytic constituents of surrounding tissues. 30 minutes after administration of 1,800 units of UK, activator activity of intraperitoneal coagula became highest, and also thrombin-induced intravenous clot was significantly activated. These results indicate that fibrinolytically potent tissues adjacent to clot will produce an activator rich clot.

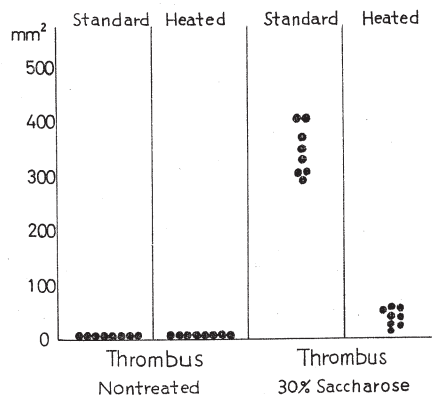


FIG. 14. Thrombi obtained from a patient suffering from acute thrombosis of abdominal aorta, 3 days after onset, was assayed for its fibrinolytic activity by the fibrin plate method. 30% saccharose treated clot revealed remarkable activator activity and slight plasmin activity, but nontreated clot showed no activator and plasmin activity.

*E) Fibrinolytic activity of blood vessel (Fig. 12)*

The normal arterial and venous wall of a dog was assayed for its fibrinolytic activity by the method of the fibrin standard plate.

The activator activity of the intima was much lower in both artery and vein than the adventitia, while the venous adventitia showed a little higher activator activity than that of the artery, but there was no significant difference in activator activity between venous and arterial intima.

*F) Fibrinolytic activity of clot and adjacent tissues (Fig. 13)*

As shown in Fig. 13, after urokinase injection, activator activity of coagula and clot was significantly enhanced. Before treatment the coagula in a peritoneal cavity showed highest activator activity and lowest in an intravenous clot, but after treatment an intravenous clot presented a little higher activator activity than that of the venous intima.

From this study, it may be suggested that when blood coagulation occurs, activator activity of the surrounding tissues will influence the fibrinolytic activity of a clot, so that if a venous intima has high activator activity, the clot formed in it will show high activator activity, and it may be concluded that activator activity of an intravenous clot is dependent upon that of the venous intima.

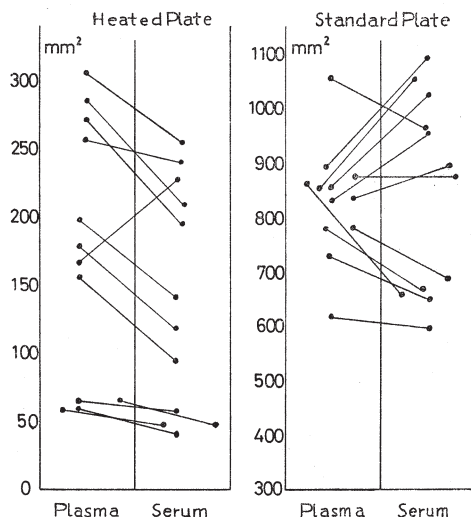


FIG. 15. 0.1 ml of euglobulin fraction of serum and plasma was activated by 100 units of SK, and after 5 minutes of incubation, fibrinolytic activity was assayed by the fibrin plate method.

G) *Fibrinolytic activity of clinical thrombus* (Fig. 14)

Thrombus obtained from a patient suffering from acute thrombosis of the abdominal aorta, 3 days after onset, was assayed for its fibrinolytic activity. Thrombus itself, dissected into slices, showed no activator activity or plasmin activity, but after treatment with 30% saccharose solution, thrombus showed significant activator activity or slight plasmin activity. It may be postulated that acute thrombus has a significant activator activity available for clotlysis, but the fibrin plate method is not usefull for assay of such activator activity.

H) *Whole plasmin in serum and plasma* (Fig. 15)

Euglobulin fraction was obtained from serum and plasma, and after SK activation, whole plasmin activity was assayed. As shown in Fig. 15, serum showed less whole plasmin activity than plasma, but proactivator and activator activity in plasma and serum showed indefinite results. It may be suggested that when a clot is formed, plasminogen will be adsorbed onto fibrin, but proactivator or activator is not always constant.

#### DISCUSSION

It appears from these studies that in thrombolytic treatment with streptokinase and urokinase, simultaneous administration of antiplasmin agents, such as EACA and trans-AMCHA will not inhibit the enhancement of activator activity in intravascular clots. Even 6 day old clots were fibrinolytically activated with the injection of EACA and trans-AMCHA following simultaneous administration of SK or UK, whereas the fibrinolytic activity of circulating blood was completely or to some extent inhibited with antiplasmin agents. It is suggested from this study that the fibrinolytic system in the clot is different in characteristics from that of the blood stream. Norman<sup>31)</sup> has shown that the plasmin inhibiting capacity of blood is very much greater than potential plasmin and that blood contains two type of inhibitor, immediate and slow, which can inactivate thirty times the volume of plasmin in circulating blood, so that free plasmin does not exist in the normal blood stream. The question arises whether under such sufficient amounts of antiplasmin thrombolysis may occur. Fibrin has more affinity for plasmin than antiplasmin and fibrin protects plasmin from inhibitor, so fibrinolytically activated plasmin in clot continued to lyse clot so far as clot lysis will be accomplished<sup>3)</sup>.

Celander and Guest<sup>49)</sup> have reported the greater susceptibility of fibrin to plasmin when compared with fibrinogen. In addition, they suggested that when a clot is formed, plasmin becomes incorporated and physically protected from the circulating inhibitor, so fibrinolytically activated plasmin in clot may cause complete lysis of the clot.

Ambrus and Markus<sup>3)</sup> have reported that caseinolytic effect is abolished by antiplasmin while considerable amounts of fibrinolytic activity still exist, and a plasmin-antiplasmin complex may function as a circulatory reservoir of plasmin, innocuous as a proteolytic enzyme for circulating proteins, but available whenever needed for clot lysis.

The therapeutic effectivity of plasmin infusions in presence of high levels of circulating antiplasmin, and without obvious proteolytic effect, is thus understandable.

Ambrus *et al.*<sup>53)</sup> studied the mechanism of thrombolysis by plasmin, producing I<sup>131</sup>-labeled purified human fibrin clots in veins of dogs, followed by infusions of human plasmin, and showed that there was no significant free plasmin activity demonstrable in the blood. Nevertheless, the clots removed from these animals exhibited *in vitro* lysis upon incubation. The activator (streptokinase) alone, used to activate plasminogen to plasmin, was significantly less effective than streptokinase activated-plasmin, alone or in complex form with antiplasmin, and fibrin may compete effectively with antiplasmin for plasmin. Thus antiplasmin may serve as a transportable reservoir of plasmin, releasing it when fibrin clots are available, but protecting other plasma proteins from its proteolytic effect.

Back *et al.*<sup>51)</sup> using immunodiffusion technics revealed the heterogeneity of the various enzyme preparations used and showed that the active fibrinolytic enzyme preparations were found to localize onto and diffuse into the matrix and core of the clot, and a high degree of localization has been seen with streptokinase-, urokinase-, and spontaneously activated human plasmin, as well as human plasminogen. They also stated that chloroform-activated bovine plasmin is localized to a lesser extent and no difference was observed in the results, whether the fibrin clots were of human, canine or bovine origin. It may be postulated from these investigations and the present studies that the administration of EACA and trans-AMCHA before an infusion of streptokinase or urokinase, would inhibit the activation of plasminogen by fibrinolytic agents in the circulating blood, while at the site of the clot these antiplasmin agents, such as EACA or trans-AMCHA may be less effective in inhibiting plasminogen activation, and this antiplasmin agent could not penetrate the matrix of the clot, probably due to these agents being combined with activator or plasminogen at the surface of the clot and be made inactive, whereas streptokinase or urokinase may infuse into a clot when the incorporated plasminogen will be activated to plasmin.

In this study various ages of intravenous clots were assayed for their activator and plasmin activity, and clots treated with EACA or trans-AMCHA following simultaneous treatment with streptokinase or urokinase, revealed demonstrable enhancement of activator activity, while no noticeable plasmin activity

was observed.

Müllertz<sup>52)</sup> demonstrated, with the external scintillation counting method following intravenous administration of I<sup>131</sup>-labeled plasminogen to dogs with femoral venous thrombosis, the greatest uptake of radioactivity occurring in clots 48 hours old or less, but all clots of up to seven days duration demonstrated some uptake of the tracer. In the present study, an elevation of activator activity in clots was observed to up to 6 day old clots after treatment with anti-plasmin agents and streptokinase or uokinase.

Back *et al.*<sup>53)</sup> studied the capacity of plasmin to adsorb selectively preformed venous and arterial clots and showed that the highest degree of adsorption to fibrin clots were exhibited by human plasminogen activated with streptokinase or urokinase, while spontaneously activated human plasmin or chloroform-activated bovine plasmin exhibited less localizing capacity and the inactive precursor plasminogen was also localized in clots to some extent.

Alkjaersig *et al.*<sup>53)</sup> prepared I<sup>131</sup>-labeled plasminogen deficient, plasminogen normal and plasminogen rich clots, incubated in 0.1 M phosphate buffer solution at pH 9.6 with SK, UK, and spontaneously activated human plasmin and determined the rate of supernatant radioactivity release. It was concluded that the rate of clot lysis is not only related to the surrounding activator concentration, but also to at any given activator concentration related to the clot plasminogen concentration.

Plasminogen activation occurs as a two stage process and requires a pro-activator as an essential component for its completion; clots containing plasminogen also contain a sufficient amount of proactivator to allow their lysis by streptokinase alone.<sup>54)</sup>

Alkjaersig *et al.*<sup>53)</sup> concluded that the chief and primary mechanism of plasma thrombolysis involves the activation of intrinsic clot plasminogen with resulting autodigestion of the thrombus and that the secondary mechanism of thrombolysis by exogenous plasmin action appears to be relatively unimportant.

Sherry *et al.*<sup>55)</sup> reported that plasmin in the circulation has very little effect on a preformed thrombus, since it can not activate the intrinsic plasminogen, and the ability of plasma to dissolve thrombin is dependent upon the presence of an activator which can diffuse into the thrombus and activate the fibrinolytic enzyme from within. They have employed streptokinase intravenous administration to 50 patients with thrombo-embolic disease, resulting in oozing and ecchymoses at the site of needle puncture, or in a few instances, fresh bleeding at the site of previous operative wounds. To prevent these side reactions the simultaneous administration of hydrocortisone with the streptokinase infusion have been used and the development of antithrombin activity has been blocked but without sacrificing any fibrin-lysing activity.

It was concluded that intravenous streptokinase should prove to be a success-



full, systemic fibrinolytic therapy that may then be applied to any number of diseases with undesirable accumulations of fibrin clot.

It was found that the ability of plasmin at a dose level of 30 plasmin units (Loomis) per kg to dissolve a clot is closely correlated with the degree of organization of the clot.<sup>56)</sup> It was observed that even by the third day, most of the free edges of the clot were covered with a layer of endothelial cells, so daily treatments with 30 plasmin units (Loomis) per kg of human plasmin did not affect clots older than three days.

Mole<sup>57)</sup> suggested that the vascular endothelium is a source of the fibrinolytic activity in cadaver blood. Kwaan *et al.*<sup>58)59)</sup> applied stimuli to the vein wall in man and in rabbits and observed the release of fibrinolytic activity through a local cholinergic mechanism.

Astrup *et al.*<sup>60)</sup> demonstrated plasminogen activator in the adventia of arteries and veins, and in the intima of veins. Todd<sup>61)</sup> found the plasminogen activator in tissues localized to the small veins and the venous endothelium. The recent demonstration that plasminogen activator is brought into an area of tissue repair by the newly formed capillaries suggested that such a situation could exist in an organizing thrombus<sup>62)</sup>. Presumably, in a venous thrombus, capillaries originating from the venous endothelium would invade the thrombus, bringing with them fibrinolytic active endothelial cells, thus assisting in the resolution of fibrin<sup>63)</sup>. After venous thrombosis in rats thrombolysis occurs rapidly when there has been no previous injury to the endothelial lining, and recanalization eventually occurs beginning from the open distal end of the venous segment and with these endothelial cells fibrinolytic activity is brought into the thrombosed area<sup>63)</sup>.

In the present study, the intima of a 6 day old thrombosed vein of a dog revealed significant activator activity, while a clot showed no activator activity. From these results, it may be concluded that a thrombosed venous intima is fibrinolytically active and such an activator rich intima will influence the fibrinolytic activation of a thrombus resulting in recanalization or clotlysis.

Whole plasmin activity in human plasma and serum assayed by the fibrin plate method revealed a significant difference and showed that whole plasmin activity in plasma is higher than serum, but proactivator or activator activity in plasma and serum is indefinite and there was no significant difference in proactivator or activator between plasma and serum. These results indicate that in blood coagulation, plasminogen will be adsorbed onto fibrin clot but proactivator or activator not always.

From this point of view, it may be suggested that natural intravascular clot will be formed with varying fibrinolytic activities as follows:

- 1) a plasminogen rich and proactivator or activator rich clot which is easily lysed spontaneously.

2) a plasminogen rich and proactivator or activator deficient clot to which thrombolytic agents may be effective by endogenous activation.

3) a plasminogen and proactivator or activator deficient clot which may be resistant to thrombolytic agents.

4) a plasminogen deficient and proactivator or activator rich clot not sensitive to thrombolytic agents.

In view of these considerations, it may be concluded that the effectiveness of thrombolytic agents in thromboembolic disorders is correlated with the fibrinolytic property in the thrombus, fibrinolytic activity of endothelial cells and the age of organization.

Hitherto, as thrombolytic agent, trypsin<sup>15)</sup>, chymotrypsin<sup>16)</sup>, streptokinase<sup>19)</sup>, urokinase<sup>14)</sup>, and plasmin<sup>18)</sup> have been clinically used by many investigators, but more or less side reactions after parenteral administration of these agents have been reported, such as chills, hyperthermia, nausea, oozing from wound, hemorrhagic diathesis resulting from fibrinolytic activation in the circulating blood. It is desirable for clotlysis that local fibrinolysis in clots should be enhanced without any side reactions, protecting general circulating fibrinolysis.

For this purpose, a new attempt was performed, and it was found that the simultaneous administration of EACA or trans-AMCHA with SK or UK was effective in enhancing activator activity in intravenous clots, and inhibiting the general fibrinolytic activity.

#### CONCLUSION

Artificially induced intravascular clots showed no fibrinolytic activity except thrombin induced clots.

Six day old clot which showed no activator activity was fibrinolytically activated by the intravenous administration of EACA or trans-AMCHA with simultaneous administration of streptokinase or urokinase, while fibrinolytic activity of the circulating blood was sacrificed.

A fibrinolytic activation system in an intravascular clot was different in characteristics from that of the circulating blood.

A fibrinolytic activation of a clot may depend upon fibrinolytic property of the clot, fibrinolytic activity of endothelial cells and the age of organization.

In the fibrinolytic treatment of thromboembolic disorders, the administration of EACA or trans-AMCHA with thrombolytic agents may be effective for activation of intravascular clots, preventing side reactions following fibrinolytic activation in the blood.

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