Effects of Heparin and ε-Aminocaproic Acid in Dogs on Plasmin-^{125}I Generation in Response to Urokinase Injections and Venous Injury

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ABSTRACT The isotopic method described previously for quantification of plasmin-^{125}I by disc gel electrophoresis was modified by inclusion of euglobulin precipitation to expand its applicability to plasmas containing low radioactivity of plasmin-^{125}I and plasminogen-^{125}I. It was found that the euglobulin precipitation method precipitates 72.4±2.1 (SD) % of both plasmin-^{125}I and plasminogen-^{125}I. Using this method and plasminogen-^{125}I as a tracer, studies were first made of the effects of heparin and ε-aminocaproic acid in dogs on plasmin-^{125}I generation in responese to a single injection of urokinase and to venous injury; second, of the effects of venous occlusion and thrombosis on plasmin-^{125}I generation; and third, in vitro studies of plasminogen-^{125}I affinity to fibrin and its activation in blood clots. The venous injury was produced by the damage of venous endothelium by an injection of 90% phenol and the thrombosis by a thrombin injection into an occluded vein. Heparin and ε-aminocaproic acid under the present experimental conditions inhibited about 78 and 100%, respectively of plasmin-^{125}I generation by the urokinase injection. Similar inhibitory effects of heparin and ε-aminocaproic acid were observed on plasmin-^{125}I generation in response to venous injury. The venous occlusion caused a small degree of plasmin-^{125}I generation, but thrombin thrombosis did not seem to stimulate the generation of plasmin-^{125}I. The in vitro studies showed that plasminogen-^{125}I does not have a specific affinity to fibrin and is incorporated into blood clots in approximately equal concentrations as those in serum during clotting processes, and that blood clots per se do not stimulate plasmin-^{125}I generation. These results suggest that injured veins release considerable amounts of vascular plasminogen activators into circulation and that these play an important role in thrombus dissolution in vivo.

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INTRODUCTION

Studies have been reported of the in vivo plasmin generation in response to vascular injury (1), but it was not entirely clear whether the plasmin generation was due to the vascular injury alone or to both the vascular injury and the accompanying thrombosis. A number of in vivo and in vitro experiments were therefore made in an attempt to solve this problem, using plasminogen-^{125}I as a tracer. This paper first describes studies of the effects of heparin and ε-aminocaproic acid on plasmin generation in dogs in response to a single injection of urokinase and to vascular injury, second, studies of the effects of venous occlusion and thrombin thrombosis on plasmin generation, and third, in vitro experiments to study the plasminogen affinity to fibrin and its activation in clots.

METHODS

Quantification of plasmin-^{125}I. Canine plasminogen-^{125}I was prepared as described previously (1). The separation and quantification of plasmin-^{125}I were achieved by the use of disc electrophoresis (2) as described elsewhere (1), but some modifications were occasionally employed, which are described below. When sufficient amounts of plasminogen-^{125}I and plasmin-^{125}I were present in plasma, volumes of plasma up to 0.05 ml were directly analyzed by the disc electrophoresis (2), but when the plasma radioactivity was too low, larger volumes of plasma had to be used in order to obtain accurate results. In this case, volumes of plasma up to about 0.8 ml were diluted 16 times in distilled H_2O and euglobulins were precipitated by exposing the diluted plasma to CO_2 gas for 4 min and centrifugation at 3000 rpm

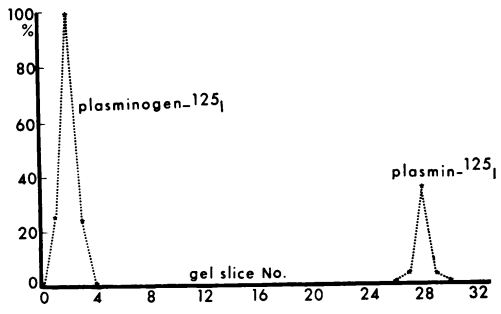

FIGURE 1 Disc electrophoretic analysis of plasma containing plasmin-^{125}I and plasminogen-^{125}I. The gel was cut into 2-mm slices and each was counted. Plasmin-^{125}I was then expressed as per cent of plasminogen-^{125}I.

for 10 min. The euglobulins were then dissolved in 0.2 ml 20% sucrose in 0.06 M tris buffer at pH 8.9, and were electrophoresed at 2 ma/gel for about 3 hr. The gels were then cut into 2-mm slices and each was counted in a scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with the efficiency of about 34%. The plasminogen-^{125}I peak appears at gel slice number 2 and that of plasmin-^{125}I at gel slice number 28 as shown in Fig. 1. The ratio of plasmin-^{125}I counts to those of plasminogen-^{125}I was then calculated. It became necessary to test if the euglobulin precipitation alters the ratio of plasmin-^{125}I to plasminogen-^{125}I compared with that in plasma. First, studies were made of the recovery rate of plasminogen-^{125}I and plasmin-^{125}I by the euglobulin precipitation method. In 10 analyses of the same plasma sample containing plasminogen-^{125}I and plasmin-^{125}I, the combined recovery in per cent of the radioactivity was 75.5, 72.6, 70.6, 73.7, 74.0, 68.0, 72.4, 70.6, and 72.5 with a mean of 72.4±2.1 (SD) %. Then, to determine if the euglobulin precipitation alters the ratio of plasmin-^{125}I to plasminogen-^{125}I in plasma, 13 different plasma samples containing varying proportions of plasmin-^{125}I and plasminogen-^{125}I were electrophoresed with and without the euglobulin precipitation. The analytical results are given in Table I. Statistical analysis showed that the results were not significantly different ($0.6 > P > 0.5$). This indicates that the euglobulin precipitation does not alter the ratio of plasmin-^{125}I to plasminogen-^{125}I and that both plasmin-^{125}I and plasminogen-^{125}I are precipitated similarly at the rate of about 72.4%.

The sensitivity of the isotopic method for plasmin-^{125}I measurement compared with the standard caseinolytic method. The isotopic method described previously (1) was compared with the caseinolytic method of Johnson, Kline, and Alkjaersig (3). In all the experiments described here, the total volume of the incubation mixture was 2.5 ml and the reagents used were exactly the same as described by Johnson et al. (3). To 0.1 ml of citrated canine plasma were added 25 Plough U of human urokinase (Calbiochem, San Diego, Calif.) or equivalent amounts of NIH human urokinase proven to be free of thromboplastic materials by the method of Alkjaersig, Fletcher, and Sherry (4), 0.005 ml of plasminogen-I^{125}, 1.15 ml of pH 7.5 tris-NaCl buffer, and 1.25 ml of α-casein solution. For the reagent controls, every reagent except the plasma was added. About 20,000 CTA U of NIH human urokinase was kindly given to us by Dr. Edward Genton. The mixture was incubated for 30 min at 38°C and was analyzed by the isotopic and caseinolytic methods. In six measurements, 17±2(SD) % of plasminogen-^{125}I were converted to plasmin-^{125}I by the isotopic method (1), but no measurable amounts of plasmin were generated by the caseinolytic method (3). In the reagent controls, no detectable amounts of plasmin were found. Next, 250 USP U of heparin (Riker Laboratories, Northridge, Calif.) was added to the incubation mixture, and after 30 min incubation, the mixture was analyzed by the two methods. The isotopic method showed that there was no measurable degree of plasmin-^{125}I generation and the caseinolytic method also showed no measurable degree of plasmin generation both in the test mixture and reagent controls. Then, 10 mg of ε-aminocaproic acid (Lederle Laboratories, division of American Cyanamid Co., Pearl River, N. Y., 250 mg/ml) was added to the incubation mixtures, and after 30 min incubation, measurements were again made by the two methods. There was no measurable degree of plasmin-^{125}I or plasmin generation both in the test mixture and reagent controls. These studies seem to indicate that the isotopic method (1) is more sensitive than the caseinolytic method (3) and that heparin and ε-aminocaproic acid have inhibitory effects on plasmin generation by urokinase. Next, the incubation period was extended to 12 hr and all the experiments described above were repeated six times. Under this condition, 18±3(SD) % of plasminogen-^{125}I were converted to plasmin-^{125}I, but in the presence of heparin or ε-aminocaproic acid as described above, no measurable amounts of plasmin-^{125}I were generated. By the caseinolytic method (3), measurable amounts of plasmin were generated without heparin or ε-aminocaproic acid in the incubation mixture, but in the presence of heparin or ε-aminocaproic acid, the plasmin generation appeared to be less. The optical density at 275 mμ was 0.143±0.1(SD) without heparin or ε-aminocaproic acid, 0.121±0.05(SD) with heparin and 0.08±0.005(SD) with ε-aminocaproic acid, but no measurable amounts of plasmin were generated in the reagent controls. Thus, the inhibitory effects of heparin and ε-aminocaproic acid on plasmin gen-

TABLE I

Quantification of Plasma Plasmin-^{125}I by Disc Electrophoresis with and without Euglobulin Precipitation

Plasma samples	A	B
	%	%
1	8.4	10.2
2	12.7	14.7
3	18.5	15.9
4	15.8	18.3
5	8.7	10.3
6	22.3	20.6
7	7.2	8.7
8	36.0	35.4
9	23.5	26.1
10	30.0	26.4
11	19.5	17.5
12	18.0	21.1
13	8.2	9.2
Means	17.6	18.0

A is plasmin-^{125}I expressed in per cent of plasminogen-^{125}I present in plasma and B is that after euglobulin precipitation of plasma.

eration under the present experimental conditions were 100% by the isotopic method (1) but by the caseinolytic method (3) they were 14% ($0.7 > P > 0.6$) and 44% ($0.2 > P > 0.1$), respectively. Thus, the results by the caseinolytic method (3) tended to support the results by the isotopic method (1) but not conclusively. The reasons for these discrepancies by the two methods are not clear, but one of the reasons seems to be the difference in the sensitivity of the two methods (1, 3).

Effects of heparin and ε-aminocaproic acid on plasmin generation in response to a single injection of urokinase. For the studies of heparin effects, five healthy dogs, 10–20 kg in weight, were used, and were fed on regular dry dog food mixed with horse meat and given by mouth about 0.5 g of KI daily in two divided doses to block the thyroid uptake of ^{125}I. At the start of the experiments, 50–100 μCi of plasminogen-^{125}I was intravenously injected into each dog. After a control period of 6 hr, 2000 Ploug U of human urokinase (Calbiochem) or equivalent amounts of NIH urokinase and 20,000 USP U of heparin (Riker Laboratories) were simultaneously injected intravenously. Thereafter, 10,000 USP U of heparin was injected subcutaneously every 3 hr for the 6 hr period. Blood samples of about 4 ml were obtained in tubes containing 143 USP U of dry heparin at the following times in days after the initial injection of plasminogen-^{125}I: 0, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{3}{8}$, $\frac{1}{2}$, $\frac{5}{8}$, $\frac{3}{4}$, 1, $1\frac{1}{4}$, and $1\frac{1}{2}$. Then, on the 3rd day, 2000 Ploug U of human urokinase was again injected intravenously. Blood samples were obtained at the following times in days and heparinized: 3, $3\frac{1}{8}$, $3\frac{1}{4}$, $3\frac{1}{2}$, 4, $4\frac{1}{2}$, 5, $5\frac{1}{2}$, and 6. Plasmas from every blood sample were analyzed by the disc electrophoresis as described above, and the ratio of plasmin-^{125}I to plasminogen-^{125}I was calculated. The Lee-White clotting time was greater than 4 hr during the period of $\frac{1}{4}$ to $\frac{1}{2}$ days, but it returned to normal values on 1 day.

For the studies of the effects of ε-aminocaproic acid, four healthy dogs, 10–21 kg in weight, were used. The general experimental procedures were the same as described above. Immediately after the intravenous injection of 50–100 μCi of plasminogen-^{125}I, 7 ml of ε-aminocaproic acid (Lederle Laboratories, 250 mg/ml) and 2000 Ploug U of human urokinase (Calbiochem) or equivalent amounts of NIH urokinase were simultaneously injected. Thereafter, 2 ml of ε-aminocaproic acid was intravenously injected every hour for a 9 hr period. Blood samples were obtained at the following times in days after the initial injection of plasminogen-^{125}I and heparinized: 0, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{3}{8}$, $\frac{1}{2}$, $\frac{2}{3}$, 1, $1\frac{1}{4}$, $1\frac{1}{2}$, and 2. On the 3rd day, 2000 Ploug U of human urokinase was again injected intravenously and blood samples were obtained at the following times in days and heparinized: 3, $3\frac{1}{8}$, $3\frac{1}{4}$, $3\frac{1}{2}$, 4, $4\frac{1}{2}$, 5, $5\frac{1}{2}$, and 6. Plasmas from every blood sample were electrophoresed and the ratios of plasmin-^{125}I to plasminogen-^{125}I were calculated as described above.

Plasmin generation in response to venous injury. The venous injury was produced as described previously (1) by a single injection of 1 ml 90% phenol into occluded veins which was removed after 1 min. Four healthy dogs, 10–19 kg in weight, were used for this study. After the venous injury was produced, 50–100 μCi of plasminogen-^{125}I was injected intravenously, and blood samples were obtained and heparinized at the following times in days: 0, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$, 3, $3\frac{1}{2}$, 4, $4\frac{1}{2}$, and 5. Plasma samples were analyzed as described above. Venous biopsies obtained at 1 day after the venous injury showed the presence of thrombosis in all the dogs used.

Effects of heparin and ε-aminocaproic acid on plasmin generation in response to venous injury. For the studies of the effects of heparin, four healthy dogs, 11–20 kg in weight, were used. At the start of the experiment, the venous injury was produced (1), and 50–100 μCi of plasminogen-^{125}I and 20,000 USP U of heparin were intravenously injected immediately thereafter. Heparin injection was continued thereafter at a rate of 10,000 USP U every 6 hr for the period of 5 days. The Lee-White clotting time was almost always greater than 4 hr. Blood samples were obtained at the following times in days: 0, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$, 3, $3\frac{1}{2}$, and 4. Plasmas were then electrophoresed as described above. For the studies of the effects of ε-aminocaproic acid, four healthy dogs, 10–22 kg in weight, were used. Immediately after the venous injury, 50–100 μCi of plasminogen-^{125}I and 7 ml of ε-aminocaproic acid were intravenously injected. Thereafter, 2 ml of ε-aminocaproic acid was intravenously injected every hour for the period of 9 hr. Blood samples were obtained at the following times in days and heparinized: 0, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{3}{8}$, $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$, 3, $3\frac{1}{2}$, 4, $4\frac{1}{2}$, and 5. Plasmas were then electrophoresed as described above.

Effects of venous occlusion on plasmin generation. Four healthy dogs, 11–22 kg in weight, were used. The venous occlusion was produced as follows: Leg veins were tightly occluded by a piece of cotton gauze at about 5 cm above the knee. After about 20 min, the cotton tourniquet was removed and 50–100 μCi of plasminogen-^{125}I was injected intravenously. Blood samples were obtained at the following times in days and heparinized: 0, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1, $1\frac{1}{2}$, $1\frac{1}{2}$, and 2. Plasmas from every blood sample were electrophoresed.

Effects of thrombin thrombosis on plasmin generation. Four healthy dogs, 10–21 kg in weight, were used. Thrombin thrombosis was produced as follows: Leg veins were tightly occluded by a piece of cotton gauze at about 5 cm above the knee, and 400 NIH U of bovine thrombin (Parke, Davis & Company, Detroit, Mich.) was injected into the occluded vein. After about 20 min, the cotton tourniquet was released and 50–100 μCi of plasminogen-^{125}I was injected into the vein on the other leg. Blood samples were obtained as described in the venous occlusion experiment and analyzed as described above. A few minutes after the thrombin injection, the vein became hardened and occluded as evidenced by the impossibility of obtaining blood samples from the vein, but in about 4 hr the vein became recanalized, indicating the difficulty of producing a sustained thrombosis by this means. Analytical results of the plasma samples showed a small degree of plasmin-^{125}I generation, which was closely similar to that found in the venous occlusion experiment.

RESULTS

Effects of heparin and ε-aminocaproic acid on plasmin generation in response to a single injection of urokinase. Five healthy dogs were used for the studies of heparin effects and the results are given in Fig. 2. The results by both Plough and NIH urokinases were closely similar and therefore only the former are shown. The first peak from the left represents the plasmin-^{125}I expressed in per cent of plasma plasminogen-^{125}I, which was generated in response to a single injection of urokinase during heparinization. The second peak is of plasmin-^{125}I generated in response to a single injection of urokinase without heparinization. The maximum values in the first peak were

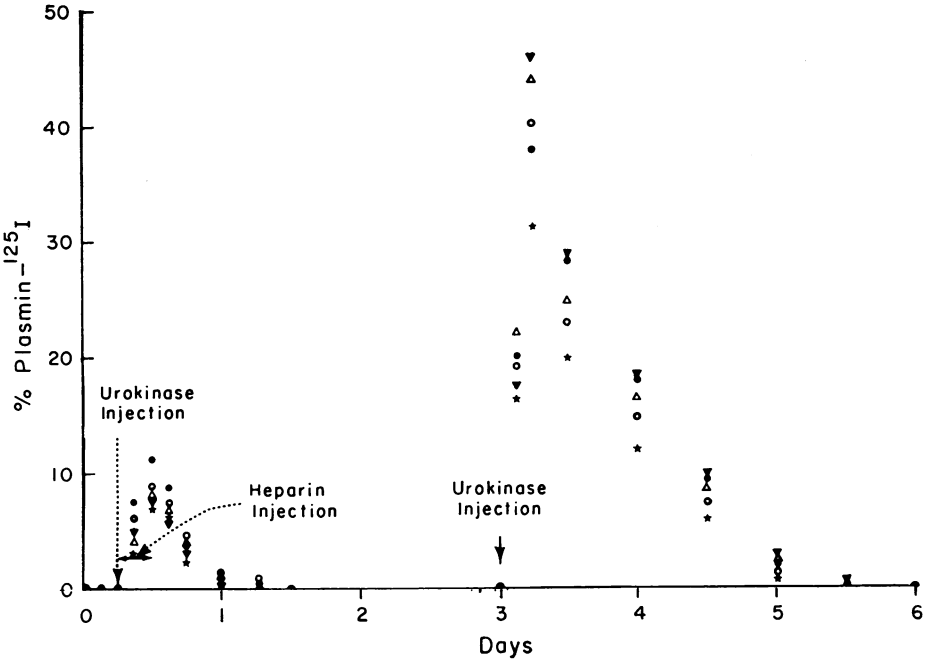

FIGURE 2 Effects of heparin on plasmin-^{125}I generation in response to urokinase injections. The first peak is of plasmin-^{125}I generated in response to a single injection of urokinase during heparinization, and the second is that in response to a single injection of urokinase without heparinization. The second peak is much greater than the first, and the inhibitory effects of heparin on urokinase are obvious.

reached at the 6th hr after the injection of urokinase and averaged 8.7±1.5 (SD) %, but the plasmin-^{125}I completely disappeared from plasma within 24 hr after the urokinase injection. The maximum values in the second peak were also reached at the 6th hr after the second urokinase injection, but were much higher than those in the first peak and averaged 39.8±5.8 (SD) %. The plasmin-^{125}I in the second peak persisted in plasma for about 2½ days. These results clearly indicate the inhibitory effects of heparin on urokinase.

Four healthy dogs were used for the studies of the effects of ε-aminocaproic acid and the results are shown in Fig. 3. It is seen that plasmin-^{125}I generation was completely inhibited during the 9 hr of ε-aminocaproic acid administration, but that upon its discontinuation plasmin-^{125}I was generated and reached its peak values within 6 hr thereafter, which averaged 6.5±0.9 (SD) %. The second peak from the left is of plasmin-^{125}I generated in the same dogs in response to a single injection of urokinase without the injections of ε-aminocaproic acid. These results indicate that ε-aminocaproic acid in the dose given is a complete inhibitor of urokinase, but that it is rapidly removed from circulation.

Plasmin generation in response to venous injury. Four healthy dogs were used for this study and the results are given in Fig. 4. It is seen that plasmin-^{125}I was generated within 3 hr after venous injury, and reached its peak values at the 6th hr, which averaged 28.4±2.7 (SD) %. Thereafter, the plasma levels of plasmin-^{125}I stayed relatively constant during the remainder of the study.

Effects of heparin and ε-aminocaproic acid on plasmin generation in response to venous injury. Four healthy dogs were used for the studies of heparin effects. The results are shown in Fig. 5. It is seen that plasmin-^{125}I was generated despite the heparinization within 3 hr after venous injury and reached its maximum values on the 6th hr, which averaged 32.1±3.8 (SD) %. However, it declined rapidly thereafter and completely disappeared from circulation within 3 days. For the studies of the effects of ε-aminocaproic acid, four healthy dogs were also used. The results are given in Fig. 6. It shows that during the 9 hr of ε-aminocaproic acid administration the generation of plasmin-^{125}I was completely inhibited, but that upon its discontinuation plasmin-^{125}I was rapidly generated in the manner similar to that shown in Fig. 4, and reached its peak values on the 6th hr, which averaged 31.9±2.4 (SD) %. Thereafter, the plasma levels of plasmin-^{125}I stayed relatively constant during the remainder of the study.

Effects of venous occlusion on plasmin generation. Four healthy dogs were used. Within 3 hr after the venous occlusion plasmin-^{125}I was generated and reached its maximum values on the 6th hr, which averaged

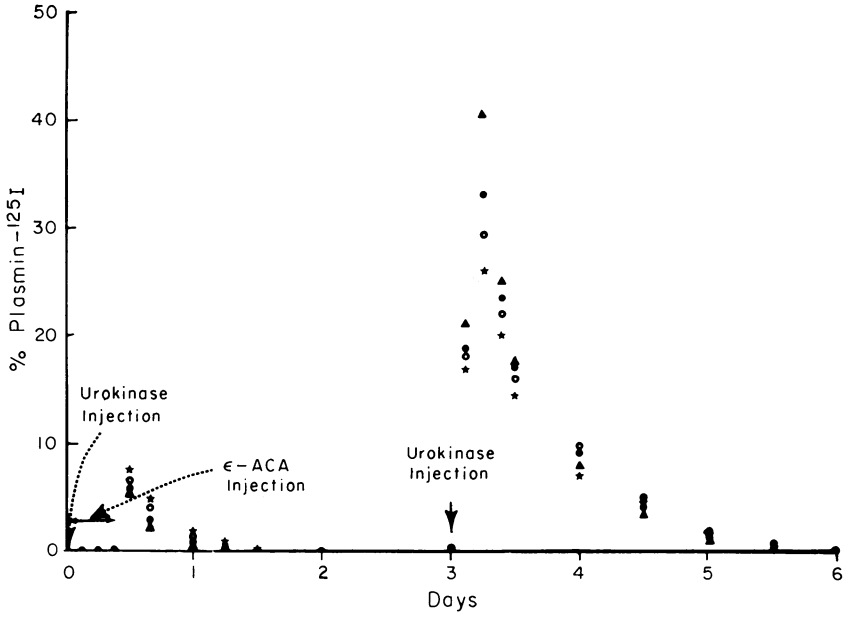

FIGURE 3 Effects of ε-ACA on plasmin-^{125}I generation in response to urokinase injections. The first peak is of plasmin-^{125}I generated in response to a single injection of urokinase during ε-ACA administration, and the second is that in response to a single injection of urokinase without ε-ACA administration. It is seen that during the 9 hr of ε-ACA administration the generation of plasmin-^{125}I was completely inhibited. ε-ACA is ε-aminocaproic acid.

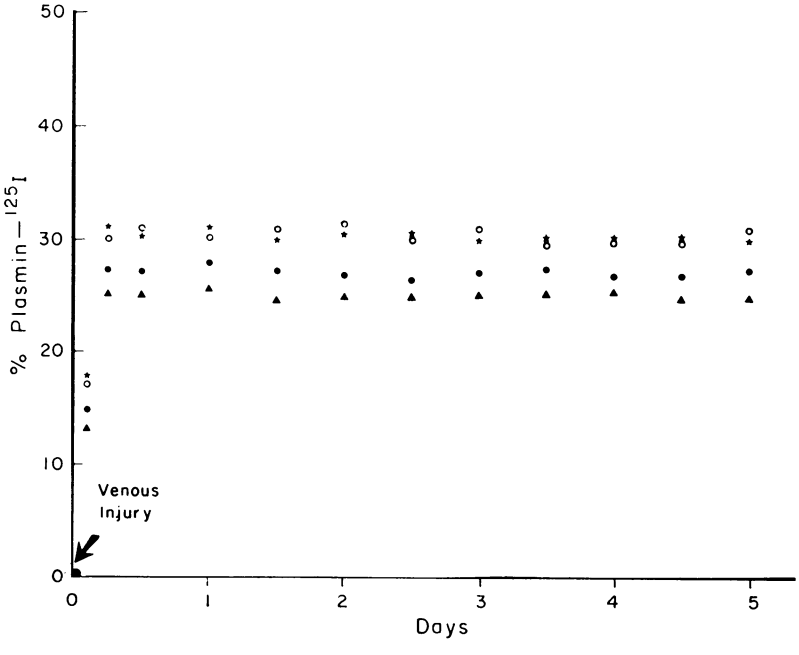

FIGURE 4 Plasmin-^{125}I generation in response to venous injury. It is seen that within 3 hr after the venous injury plasmin-^{125}I was generated and reached its maximum value on the 6th hr, and thereafter remained relatively constant.

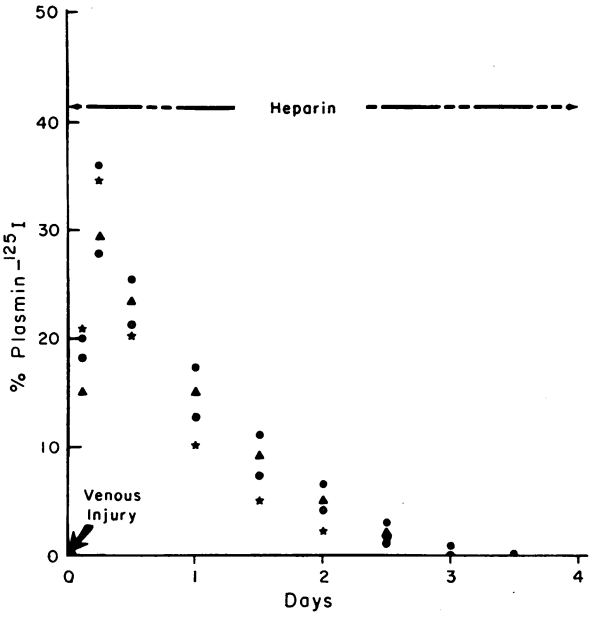

FIGURE 5 Effects of heparin on plasmin-^{125}I generation in response to venous injury. It is seen that despite the heparinization plasmin-^{125}I was generated but disappeared from circulation within 3 days, and it is obvious that heparin partially inhibited the generation of plasmin-^{125}I (compare with Fig. 4).

5.2±1.4 (SD) %. Thereafter, it declined rapidly and completely disappeared from the circulation within 24 hr.

In vitro studies of plasmin generation in clots. About 50 ml of venous blood was withdrawn into a plastic tube, 2.6 × 10 cm in size and sufficient amounts of plasminogen-^{125}I were added immediately, and were spontaneously clotted after mixing at room temperature. In a few minutes the coagulated blood was broken down into several pieces by the use of glass rod, and serum samples were obtained periodically for a 7 day period. The serum samples were electrophoresed as described in the Methods section but no detectable amounts of plasmin-^{125}I were found to be generated in any of the samples, and the original volume of clots did not appear to have decreased in the end of the observation period. Next, studies were made to determine if plasminogen-^{125}I has a specific affinity to fibrin. First, about 30 ml of citrated, platelet-poor plasma was obtained from a healthy dog and was divided into 10-ml portions. Appropriate amounts of plasminogen-^{125}I were added to each and mixed. Then, about 50 NIH U of bovine thrombin was added to each, and formed fibrin was collected on a glass rod by squeezing out the serum was as much as possible. The three mixtures were spun at 3000 rpm for 10 min to remove the remainder of fibrin. The serum samples were then counted and compared with the radioactivity of

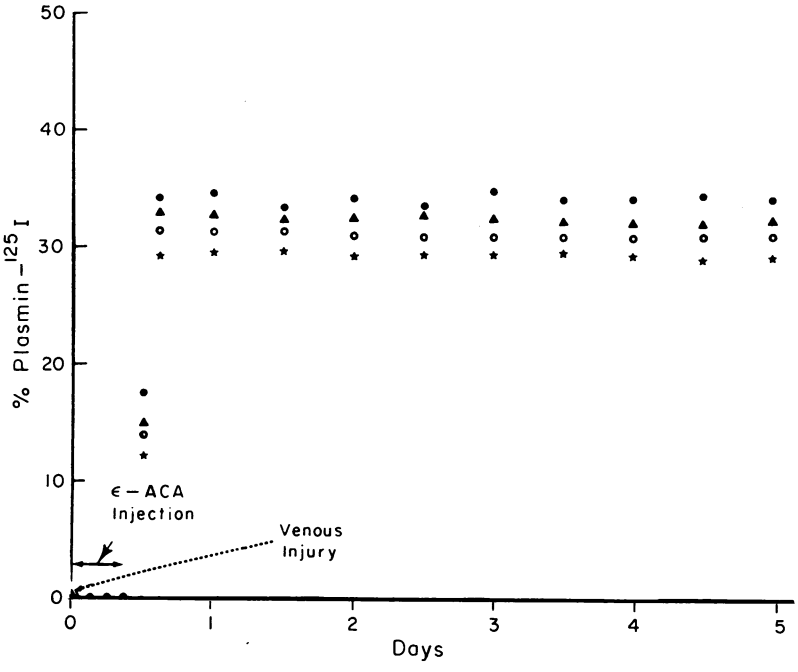

FIGURE 6 Effects of ε-ACA on plasmin-^{125}I generation in response to venous injury. It is seen that plasmin-^{125}I generation was completely inhibited during the 9 hr of ε-ACA administration, but that upon its discontinuation plasmin-^{125}I was generated within 3 hr and thereafter behaved similarly to that shown in Fig. 4.

original plasmas. There was no significant difference ($P > 0.9$) between the counts/minute per milliliter of plasma and serum samples after correction for the dilution due to the thrombin addition. Then, the fibrin collected on glass rods from the three samples were counted, but no appreciable amounts of radioactivity were found to be present. Next, about 100 ml of citrated blood was obtained from a healthy dog and appropriate amounts of plasminogen-^{125}I were added and mixed, which were then divided into 10-ml portions. 1-ml portions of the five plasma samples were counted as controls, and then the blood in five separate plastic tubes was recalcified by adding 1 ml of 5% $CaCl_2$ to each. After about 20 min, serum samples were obtained and their radioactivity was compared with the control plasma counts. There was no significant difference ($P > 0.9$) between the counts/minute per milliliter plasma and that of serum after correction for the dilution due to the addition of $CaCl_2$. However, the recovery of the volume of serum from the clotted blood was less than that of plasma volume from the citrated blood by centrifugation at 3000 rpm for 10 min. This indicates that the difference in the recovery of plasma and serum volumes from originally equal volumes of blood was contained in the clots. Thus, these in vitro studies showed that plasminogen has no specific affinity to fibrin and that clots contain plasminogen in concentrations approximately equal to those in serum.

DISCUSSION

In our previous studies (1), one of the findings was that venous injury causes plasmin-^{125}I generation, but the venous injury was always accompanied by thrombosis. Therefore, whether the plasmin-^{125}I generation was due to the venous injury alone or to the combined effects of venous injury and thrombosis was not entirely clear. Also, in our previous report (1), the effects of heparin on plasmin generation in response to venous injury were studied, but because of our ignorance of the effects of heparin on plasminogen activators, the results could not be interpreted fully. These difficulties prompted the present investigation. First, the effects of heparin and ϵ-aminocaproic acid on plasmin generation in response to a single injection of urokinase were studied. A number of studies have been reported of the effects of heparin on urokinase, streptokinase, and other plasminogen activators (5–20), but the results have been contradictory. Thus, many investigators found that heparin enhances or stimulates fibrinolytic activities (5–12), while others reported inhibitory effects of heparin (13–17), or both inhibitory and stimulative effects of heparin depending on the dose (18–20), or no detectable effects of heparin (21). In most of the previous studies, the euglobulin lysis time and its modifications have been used. The results by these methods depend on the amount of fibrinogen, plasmin, antiplasmin, plasminogen activators and antiactivators, etc., and are difficult to interpret properly except under unusual conditions. A recent development of a method for separation and quantification of plasmin-^{125}I by the disc gel electrophoresis (1) has offered unique opportunities to study these problems. In the present studies, the heparin and ϵ-aminocaproic acid were given in relatively large doses compared with the amount of urokinase administered. Under this condition, heparin produced a marked inhibition of plasmin generation by urokinase as shown in Fig. 2. The peak plasma values of plasmin-^{125}I averaged 8.7± 1.5 (SD) % during heparinization and 39.8±5.8 (SD) % without heparinization. This indicates that the inhibitory effects of heparin were not 100 but about 78%. On the other hand, ϵ-aminocaproic acid completely inhibited plasmin-^{125}I generation as shown in Fig. 3.

Next, studies were made of the effects of heparin and ϵ-aminocaproic acid on plasmin generation in response to venous injury (Figs. 4, 5, and 6). In the control studies of venous injury (Fig. 4), plasmin-^{125}I was generated within 3 hr and reached its peak values on the 6th hr, and thereafter stayed relatively constant for the remainder of the period, but the heparinization partially inhibited the generation of plasmin-^{125}I in response to venous injury (Fig. 5), and ϵ-aminocaproic acid completely inhibited the generation of plasmin-^{125}I as long as it was administered (Fig. 6). ϵ-Aminocaproic acid is a known inhibitor of plasminogen activation (22, 23). Because of the inhibitory effects of heparin on urokinase as demonstrated in the present study (Fig. 2), similar effects of heparin on other plasminogen activators might be assumed. Thus, the demonstrated inhibitory effects of heparin and ϵ-aminocaproic acid on plasmin-^{125}I generation (Figs. 2, 3, 4, 5, and 6) strongly suggest that vascular plasminogen activators are released into circulation from the injured veins. Studies of the effects of venous occlusion on plasmin-^{125}I generation also demonstrated a small degree of plasmin-^{125}I generation, which too can be explained by the same mechanism, namely, a release of vascular plasminogen activators from the veins mildly injured by their temporary occlusion, but future studies are required for more direct and absolute proof of this mechanism.

These studies, however, did not provide an answer to a question whether or not blood clots per se generate plasmin-^{125}I. Studies were therefore made of the effects of thrombin thrombosis as described in the Methods section. Plasmin-^{125}I was generated, but not in amounts greater than those in the venous occlusion experiments, suggesting that thrombus per se has little stimulative effects for plasmin generation. In order to further substantiate this thesis, several in vitro studies were carried out as described in the Results section. These studies showed

that plasminogen-^{125}I has no specific affinity to fibrin and that plasminogen-^{125}I is incorporated into clots in approximately equal concentrations as those in serum, but that plasminogen-^{125}I both in serum and clots was not appreciably activated to plasmin-^{125}I during the observation period of 7 days. Thus, the present studies strongly support the concept that vascular plasminogen activators are released from injured veins and that these play an important role in thrombolysis.

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