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Physicochemical and Biological Properties of Human and Canine Plasmins

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Research Article

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Physicochemical and Biological Properties of Human and Canine Plasmins

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ABSTRACT Three kinds of plasmin were found to be generated when plasminogen or [^{125}I]plasminogen was incubated at 32°C for longer than 20 min in urokinase and 50% glycerol. Each plasmin was then separated by G-200 or G-75 Sephadex filtration, and its physicochemical properties were determined. The molecular weights of the three 'plasmins as determined by G-200 Sephadex filtration were 125,000±5,000(SD), 63,000±2,000(SD), and 31,500±1,000(SD), and those by sodium dodecyl sulfate(SDS)-polyacrylamide electrophoresis were 130,000±5,000(SD), 64,000±3,000(SD), and 32,000± 1,500(SD). It was also found that during the incubation of the smallest plasmin in SDS and beta-mercaptoethanol it was further split into two smaller pieces of about 16,000 mol wt and that polymer proteins of 95,000± 2,000(SD) and 48,000±1,500(SD) mol wt were formed. Despite these differences in the molecular size of the three plasmins, the specific activity of each plasmin was closely similar and in case of human plasmins averaged 29±0.9(SD) CTA units/mg plasmin and in case of canine plasmins 8.5±0.54(SD) CTA units/mg plasmin. Then, using human plasmin of the smallest size (mol wt 31,500), the total plasma antiplasmin capacity was determined in 20 normal human plasma, which averaged 7.8±2(SD) CTA units of plasmin per milliliter plasma. Studies were next made of the affinity of human [^{125}I]-plasmin of the smallest size with albumin, gamma globulin, α_2-macroglobulin, α_1-antitrypsin, fibrinogen, and fibrin. The results were 0%, 0%, 14.6±0.5(SD)%, 17.6±0.6(SD)%, 21±0.5(SD)%, and 20.5±0.6(SD)%, respectively, of [^{125}I]plasmin available and were unaltered when the amount of [^{125}I]plasmin was increased to twice and four times the original amount. Finally, the plasma disappearance half-life of canine [^{125}I]plasmin of the smallest size was studied in five healthy dogs, which averaged 14.2±0.63(SD) h. These results support the concept that the combination between plasmin and plasmin inhibitors is reversible and indicate that fibrinogen and fibrin have greater affinities than α_2-macroglobulin or α_1-antitrypsin.

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INTRODUCTION

A single kind of [^{125}I]plasmin is generated from [^{125}I]-plasminogen in vivo when urokinase is injected into dogs (1–3). This was also true in vitro when [^{125}I]plasminogen was incubated with urokinase at 38°C for 5 min (1–3). The [^{125}I]plasmin generated both in vivo and in vitro was detected and quantified by the method devised in this laboratory (1–3), using disc gel electrophoresis (4). Since then, our further studies showed some irregular results. Namely, when [^{125}I]plasminogen or plasminogen was incubated in vitro for a period longer than 20 min, three different plasmins with respect to their molecular size were found to be generated. The purpose of this communication is to describe details of the methods for preparation of each of these plasmins and of the physicochemical and biological properties of the three plasmins.

METHODS

Preparation of canine and human plasminogens. Canine plasminogen was prepared by the method previously reported (1), and human plasminogen was prepared by a minor modification of our previous method (1). About 1 liter of citrated human plasma obtained from the blood bank was proved to be free of Australia antigen and antibody by the hemagglutination assay methods (5). The modifications were in the use of centrifugation at 5,000 rpm for 5 min and 40% $(NH_4)_2SO_4$ saturation instead of what was described previously (1). The difference between canine and human plasminogens in DEAE cellulose chromatography was that the former appears in the effluent volumes of 200–300 ml (1), whereas the latter appears in the effluent volumes of about 600–800 ml.

Preparation of canine and human plasmins. About 50–100 mg of plasminogen were dissolved in 0.05 M sodium

phosphate buffer (pH 8.0) at plasminogen concentrations of about 4–12 mg/ml, and equal volumes of 100% glycerol and about 1,000–2,000 CTA units/mg plasminogen of NIH or Plough urokinases (Calbiochem, San Diego, Calif.) were added. The mixture was incubated at 32°C for 1 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 1 day, 2 days, 3 days, and 4 days and was diluted five times with cold distilled H_2O. The pH was then adjusted to 6.2 with 1 M KH_2PO_4 and 40% saturated with $(NH_4)_2SO_4$ to remove urokinase and to concentrate the plasmins generated. Our separate study showed that this procedure (6) does indeed remove the urokinase added. The mixture was then dissolved in appropriate volumes of 50% glycerol in 0.05 M sodium phosphate buffer (pH 8.0). It was then passed through a G-200 Sephadex column (Pharmacia, Uppsala, Sweden), 0.9 × 55 cm in size, equilibrated with 0.05 M sodium phosphate buffer (pH 8.0). The flow rate was about 10 ml/h, and the fraction volume was 1 ml. The chromatogram is shown in Fig. 1, in which three plasmin peaks are present. Then, 3-ml volumes around each peak were collected to obtain each plasmin. They were not 100% pure by disc electrophoresis (4), but this provided a rapid and convenient method for preparing about 90% pure peaks 1 and 2 and about 95% pure peak three plasmins. The three plasmins were preserved in 50% glycerol in 0.05 M sodium phosphate buffer (pH 7.0), which were then analyzed with respect to their extinction coefficients and specific activity as described previously (1). The plasmin activity was measured by the caseinolytic method (7) and by the fibrinolytic method of Moser and Frey (8).

Another method was used to prepare 100% pure peak three plasmin. After the activation of plasminogen for 2 days and the removal of urokinase as described above, the activated mixture was passed through a G-75 Sephadex column, 0.9 × 110 cm in size, equilibrated with 0.05 M sodium phosphate buffer (pH 8.0). The flow rate was about 8 ml/h, and the fraction volume was 1 ml. The chromatogram is shown in Fig. 2, in which three plasmin peaks are again found. Effluents from 32 to 44 ml (Fig. 2) were collected, 40% saturated with $(NH_4)_2SO_4$ and centrifuged at 5,000 rpm for 30 min. The precipitate was dissolved in appropriate volumes of 50% glycerol in 0.05 M sodium phosphate buffer (pH 7.0).

Preparation of human and canine [^{125}I]plasmins. [^{125}I]-plasminogen was first prepared by the use of the iodine monochloride method (9), activated and separated into peaks 1, 2, and 3 [^{125}I]plasmins as described above, using the Sephadex G-200 column (Fig. 1). Each plasmin was then electrophoresed by the use of disc electrophoresis (4) as described elsewhere (1). Fig. 3*A* shows the analysis of the activation products of [^{125}I]plasminogen, and it is seen that three plasmins are present. Figs. 3*B*, 3*C*, and 3D show the presence of peak 1, peak 2, and peak 3 plasmins, respectively. To prepare 100% pure peak 3 [^{125}I]-plasmin, Sephadex G-75 column was used as described for preparation of unlabeled 100% pure peak 3 plasmin.

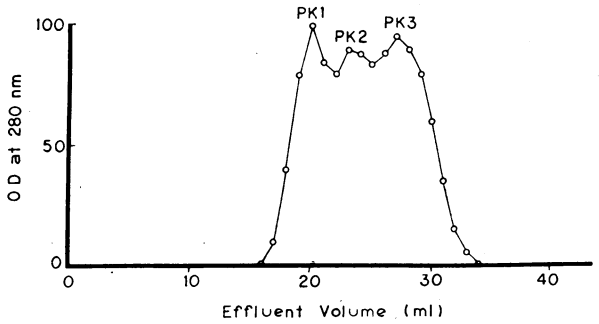

FIGURE 1 G-200 Sephadex filtration patterns of human plasminogen activated with 50% glycerol and urokinase for 2 days. Three plasmin peaks are seen. About 3-ml volumes around each peak were collected to obtain each plasmin.

Molecular weight determination of peaks 1, 2, and 3 plasmins. This was first done by the method of Andrews (10). However, in the present study, Sephadex G-200 column, 2.5 × 70 cm in size, equilibrated with 0.05 M sodium phosphate buffer (pH 8.0) was used. The flow rate was about 36 ml/h, and the fraction volume was 3 ml. For standards, blue dextran, aldolase, human serum albumin, ovalbumin, and chymotrypsinogen (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) were used. The molecular weight of each of these standards is 2 million, 158,000, 68,000, 43,000, and 25,000, respectively. About 5 mg of each standard and about 0.01 mg of each of peaks 1, 2, 3 [^{125}I]plasmins were dissolved in 1 ml of 0.05 M sodium phosphate buffer (pH 7.0) and were analyzed.

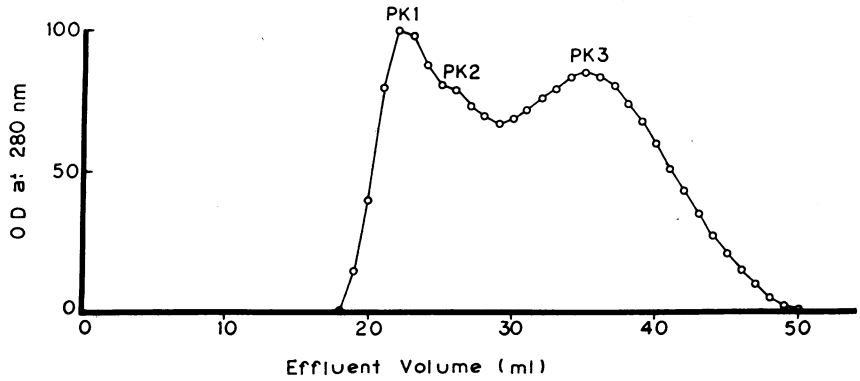

FIGURE 2 G-75 Sephadex filtration patterns of human plasminogen activated with 50% glycerol and urokinase for 2 days. Three plasmin peaks are again found, but peak 3 plasmin is much further away from peak 1 and 2 plasmins than shown in Fig. 1. Effluent volumes of from 32 to 44 ml were collected and further processed to obtain 100% pure peak 3 plasmin.

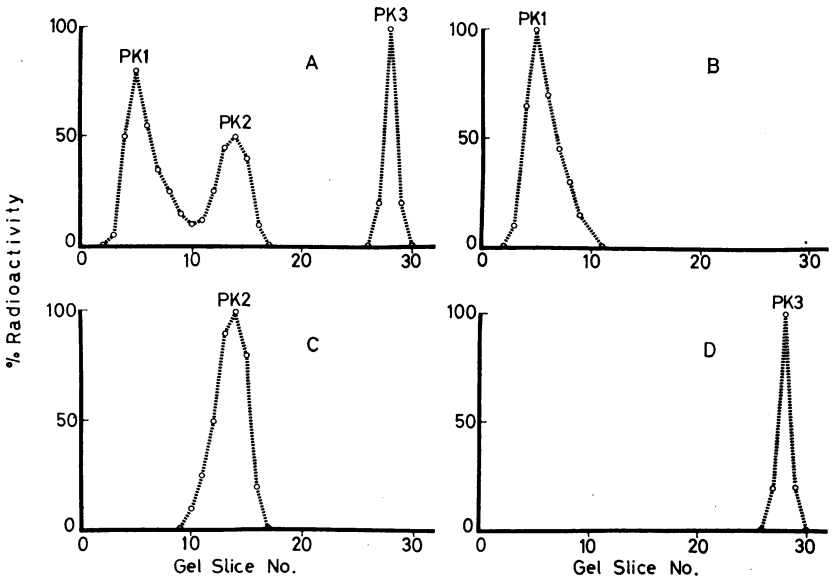

FIGURE 3 Disc gel electrophoresis of human [^{125}I]plasmins. Electrophoretic patterns of the activation products of [^{125}I]plasminogen with 50% glycerol and urokinase at 2 day incubation (*A*), peak 1 [^{125}I]plasmin (*B*), peak 2 [^{125}I]plasmin (*C*), and peak 3 [^{125}I]plasmin (*D*) are shown. About 1 μg of proteins was applied and electrophoresed at 5 mA per gel for 1 h, and the gels were cut into 2-mm slices and each was counted in a scintillation counter. The gels were of 7 g/100 ml polyacrylamide in 70.7 mg/100 ml ammonium persulfate, 0.726 g/100 ml Tris, 29 mg/100 ml TEMED, and 0.184 g/100 ml methylenebisacrylamide in H_2O at pH 8.9 (4). The buffer in the upper and lower chambers consisted of 0.63 g/100 ml Tris and 0.39 g/100 ml glycine in H_2O at pH 8.9.

Next the molecular weights of the three plasmins were determined by a minor modification of the method by Weber and Osborn (11). 7 g/100 ml polyacrylamide gel of 10 cm length was made in 1 g/100 ml sodium dodecyl sulfate (SDS), 70.7 mg/100 ml ammonium persulfate, 0.726 g/100 ml Tris, 29 mg/100 ml TEMED (*N,N,N′,N′*-tetramethylethylenediamine), and 0.184 g/100 ml methylenebisacrylamide in H_2O as described by Davis (4), and the pH of the gel was usually 8.9. The buffer in the upper and lower chambers consisted of 1 g/100 ml SDS, 0.63 g/100 ml Tris, and 0.39 g/100 ml glycine in H_2O at pH 8.9. For standards, some described above in addition to IgG (mol wt 150,000), pepsin (mol wt 35,000), and ribonuclease (mol wt 13,700) were used. About 50 μg of the standards and the three plasmins were separately incubated at 37°C for 30 min in the chamber buffer described above to which beta-mercaptoethanol was added in 1 g/100 ml concentration. The proteins were then separately applied onto the gels and electrophoresed at 5 mA per gel for about 4 h. The gels were then stained and analyzed exactly as described by Weber and Osborn (11).

In vivo testing of canine peak 3 [^{125}I] plasmin. In addition to the testing of both human and canine [^{125}I]plasmins with respect to their electrophoretic behavior and specific activities as described above, canine peak 3 [^{125}I]plasmin was further tested with respect to its in vivo metabolic behavior as described elsewhere (1). [^{125}I]plasmin was injected intravenously into a puppy and screened for 2 h to remove any denatured material. After 2 h, the puppy was bled by heart puncture, and citrated plasma containing essentially undenatured [^{125}I]plasmin was harvested. Then, about 30 μCi of screened [^{125}I]plasmin was injected intravenously into five recipient dogs, and its in vivo behavior was determined. This served as the standard. In a week, the same dogs were injected with unscreened [^{125}I]plasmin, and its in vivo behavior was compared with that of the standard. It is seen in Fig. 4 that the in vivo behavior of the two is very similar indicating that our [^{125}I]plasmin is not appreciably denatured.

Total plasma antiplasmin capacity. This was first determined by the fibrinolytic rate method of Moser and Frey (8). Human fibrinogen was prepared by the method described elsewhere (12), and small amounts of contaminating plasminogen were removed by adsorption with bentonite suspension as described previously (13). Serial dilutions of normal human plasmas in 0.9% saline were made, and 0.1 ml of each diluted plasma and 0.1 ml of our peak 3 human plasmin in 50% glycerol in 0.05 M sodium phosphate buffer (pH 7.0) (2.0 CTA units/ml) were mixed and incubated for 1 h at 38°C. Then, 0.1 ml of the above incubation mixtures was added to 1 ml of 4 mg/ml solution of human fibrinogen in tris-acetate-NaCl buffer as specified by Moser and Frey (8), and 0.1 ml of bovine thrombin (Parke, Davis & Co., Detroit, Mich.) in 0.9% saline (10 NIH units/ml) was added. The mixtures were all incubated for 15 min at 38°C, and the reaction was stopped as described elsewhere (8). The optical density of the supernate of each mixture after removal of residual fibrin by centrifugation at 3,000 rpm for 10 min at 3°C was then measured at 280 nm. For the controls, 0.1 ml of various dilutions of plasmas was mixed with 0.1 ml of 50% glycerol in 0.05 M sodium phosphate buffer (pH 7.0). The control mixtures were then subjected to the same procedures as the test mixtures, and the optical densities at 280

nm of the supernates of the control mixtures were subtracted from that of corresponding test mixtures. Analyses were also carried out by the caseinolytic method (7). The incubated mixtures of diluted plasmas and our peak 3 plasmin as above were directly analyzed with respect to the residual caseinolytic activity.

Affinity of peak 3 [^{125}I]plasmin to albumin, 7s gamma globulin, α_2-macroglobulin, and α_1-antitrypsin. It has been reported that several plasma proteins act as plasmin inhibitors (14, 15). Studies were therefore made of the affinity of human peak 3 [^{125}I]plasmin with several plasma proteins. Sephadex G-75 column, 0.9 × 55 cm in size, equilibrated with 0.05 M sodium phosphate buffer (pH 8.0), was used. The flow rate was about 12 ml/h, and the fraction volume was 1 ml. About 10 mg of all the proteins were dissolved in 1 ml of 0.05 M sodium phosphate buffer (pH 8.0), mixed with 0.1 ml of 0.05 mg of [^{125}I]plasmin in 50% glycerol in 0.05 M sodium phosphate buffer (pH 7.0) and incubated for 30 min or 60 min at 38°C before applying onto the Sephadex column.

Affinity of peak 3 [^{125}I]plasmin to fibrinogen and fibrin. Next, studies were made of the affinity of [^{125}I]plasmin with human fibrinogen. The plasminogen-free fibrinogen (12, 13) was dissolved in 0.05 M sodium phosphate buffer (pH 8.0), and 3 mg, 12 mg, and 24 mg in 3 ml volume were mixed with 0.1 ml of 0.05 mg [^{125}I]plasmin and incubated for 30 min or 60 min at 38°C. The control consisted of 3 ml of 0.05 M sodium phosphate buffer (pH 8.0) mixed with 0.1 ml of 0.05 mg or 12 mg of [^{125}I]-plasmin. Exactly 1 ml of each mixture including the control was then counted as the standards. Almost all fibrinogen is precipitated at 25% saturation with $(NH_4)_2SO_4$ (12). Therefore, the remainder of the above mixtures including the control were 25% saturated with $(NH_4)_2SO_4$ and spun at 5,000 rpm for 10 min. The precipitates were counted and compared with the control and respective standard. Then, the affinity of [^{125}I]plasmin with fibrin was studied. The plasminogen-free fibrinogen (12, 13) was dissolved in 0.05 M sodium phosphate buffer (pH 8.0), and 3 mg, 12 mg, and 24 mg fibrinogen in 3 ml volume were mixed with 0.1 ml of 0.05 mg [^{125}I]plasmin and incubated for 30 min or 60 min at 38°C. Exactly 1 ml of each mixture was then counted as the standards. Then, about 5 NIH units of bovine thrombin (Parke, Davis & Co.) were added to the remainder of each, which was then coagulated. The generated fibrin was collected on a glass rod and washed in distilled H_2O and counted. Also, exactly 1 ml serum of each mixture was counted, and the results were compared with the standards.

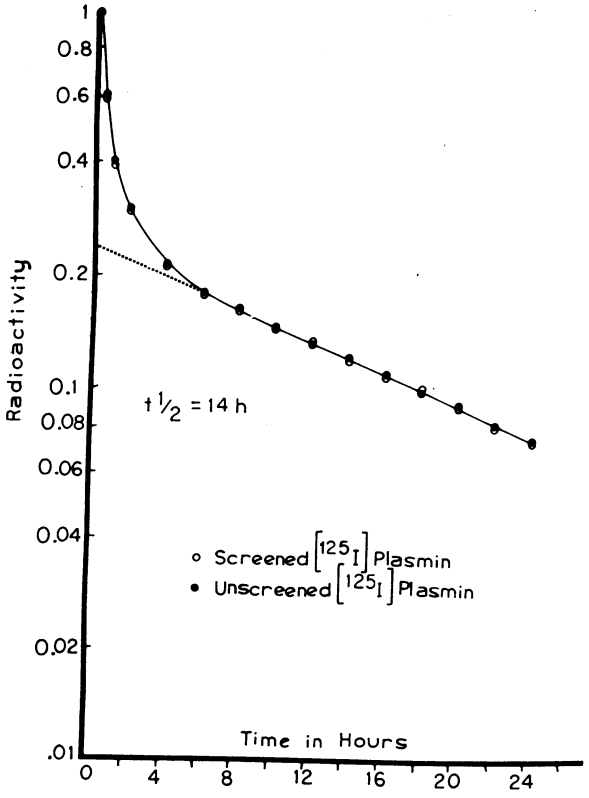

FIGURE 4 In vivo behavior of screened and unscreened canine peak 3 [^{125}I]plasmins. It is seen that the plasma behavior of the two are very closely similar.

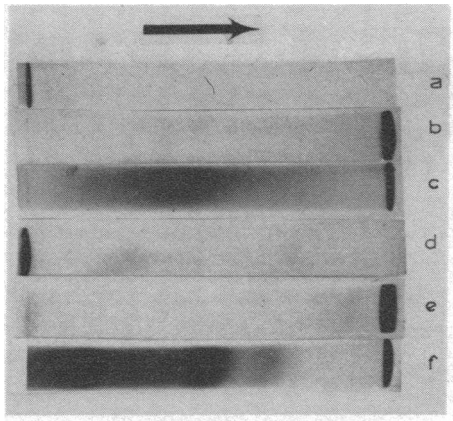

FIGURE 5 Disc gel electrophoresis of plasminogens and plasmins. The electrophoretic patterns of human plasminogen (*a*) and peak 3 plasmin (*b*), human plasmin provided by Dr. A.J. Johnson (*c*), canine plasminogen (*d*) and peak 3 plasmin (*e*), and the mixture of human peaks 1, 2, and 3 plasmins at 2-day incubation (*f*) are shown. Proteins of about 20–50 μg were applied and electrophoresed at 5 mA per gel for 1 h. It is seen that plasmin can readily be separated from plasminogen and that the electrophoretic mobility of our human and canine plasmins are very similar to that of plasmin provided by Dr. A. J. Johnson. The electrophoretic conditions are the same as in Fig. 3.

RESULTS

Purity, extinction coefficient, and specific activity of plasminogens and plasmins. In Fig. 5 it is seen that our human and canine plasminogens and peak 3 plasmins are electrophoretically homogenous. The extinction coefficient of human plasminogen determined as described previously (1) averaged 16.0±0.8(SD) for 10 mg/ml at pH 7.0 and 280 nm, using 1 cm quartz cuvette, and the specific activity was 29±1.0(SD) CTA units/mg plasminogen. The extinction coefficients of peaks 1, 2, and 3 plasmins (Figs. 1–3) were very similar and in

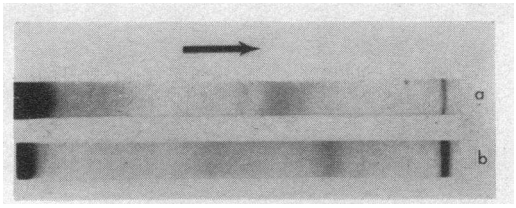

FIGURE 6 Disc gel electrophoresis of activation mixtures of human plasminogen at 5 and 10 min incubation. The electrophoretic conditions were the same as described in Fig. 3. Fig. 6*a* (5-min incubation) and Fig. 6*b* (10-min incubation) both show the presence of plasminogen at the left end of the gel and peak 3 plasmin at the right end. Also, there seem to be small amounts of homogeneous materials between the two.

case of canine plasmins averaged 14.0±0.60(SD), and the specific activities were also very similar and averaged 8.5±0.54(SD) CTA units/mg plasmin. In case of human plasmins, the extinction coefficients and specific activities averaged 15.9±0.7(SD) and 29±0.9(SD) CTA units/mg plasmin, respectively. The specific activities of the three [^{125}I]plasmins were unaltered compared with unlabeled plasmins, and the radioactivity ranged from 10 to 20 μCi/mg plasmin.

Plasminogen activation. To determine the sequence of the three plasmin evolution (Figs. 1–3), the activation mixture was electrophoresed at various incubation times. Fig 5 shows that plasminogen was converted to three plasmins after 2-day incubations. This always occurred after 20-min or longer incubations, and there did not seem to be any appreciable difference in the amount of each of the three plasmins with respect to time. However, at 1-min incubation no detectable amount of plasmin was found by the caseinolytic assay (7) or by the use of disc electrophoresis (4). At 5-min incubation, some plasmin activities were found by the caseinolytic assay, and as shown in Fig. 6*a* there is plasmin at the right end of the gel and plasminogen at the left end of the gel. At 10-min incubation, the finding was essentially the same (Fig. 6*b*).

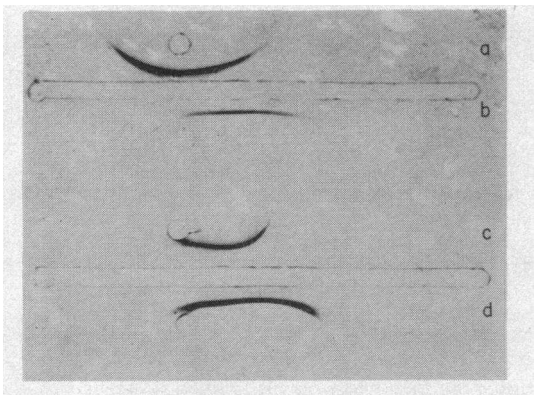

FIGURE 7 Immunoelectrophoresis of plasminogens and plasmins. The electrophoretic patterns of human plasminogen (*a*) and plasmin (*b*), and of canine plasminogen (*c*) and plasmin (*d*) are shown. About 10 μg of proteins were applied and electrophoresed at 3 mA per slide for 90 min. The upper trough was filled with anti-human plasminogen and the lower trough with anti-canine plasminogen. It is seen that plasmin moves somewhat faster than its respective plasminogen. The electrophoretic conditions were as described elsewhere (1).

Comparative behavior on immunoelectrophoresis of both human and canine plasminogens and peak 3 plasmins. Anti-canine plasminogen was prepared as described elsewhere (1) and anti-human plasminogen (batch no. 2104D) was obtained from Behring Diagnostics, Somerville, N. J. In Fig. 7 it is seen that canine plasmin has a somewhat greater mobility than that of canine plasminogen, and this was also true with human plasmin and plasminogen. However, canine plasminogen appears to have a greater mobility than human plasminogen, and canine plasmin seems to have a somewhat greater mobility than human plasmin. These results were also found to be true by cellulose acetate electrophoresis (1). Namely, canine plasminogen migrates as a protein with a mobility between β_1- and β_2-proteins, whereas human plasminogen migrates as a β_2-protein. On the other hand, canine plasmin moves as a β_1-protein,

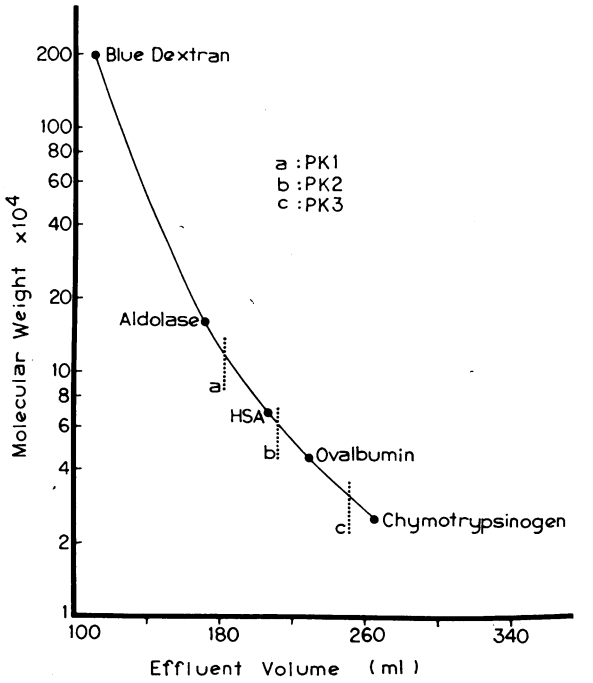

FIGURE 8 Molecular weight determination of human [^{125}I]-plasmins by G-200 Sephadex gel filtration. HSA represents human serum albumin. It is seen that the molecular weight of peak 1 [^{125}I]plasmin (*a*) is about 125,000; that of peak 2 [^{125}I]plasmin (*b*) is about 63,000; and that of peak 3 [^{125}I]plasmin (*c*) is about 32,000.

and human plasmin migrates as a protein with a mobility between β_1- and β_2-proteins.

Molecular weight determination of peaks 1, 2, and 3 [^{125}I]plasmins by the method of Andrews. This was repeated three times and the results were essentially the same. The elution patterns of the standard proteins and human peaks 1, 2, and 3 [^{125}I]plasmins are shown in Fig. 8. It can be seen that the molecular weight of peak 1 plasmin is about 125,000±5,000(SD), that of peak 2 plasmin is about 63,000±2,000(SD), and that of peak 3 plasmin is about 31,500±1,000(SD). The same analysis was also applied to canine peaks 1, 2, and 3 [^{125}I]plasmins and the results were identical with those of human [^{125}I]plasmins. The molecular weight of the plasmin provided by Dr. A. J. Johnson (American National Red Cross, Bethesda, Md.) was also determined by the same method, and was found to be identical with that of our peak 3 plasmin. Thus, these studies indicate that our peak 3 plasmin is the smallest of the three plasmins. The molecular weight of human plasminogen was additionally determined, and found to be about 130,000.

Molecular weight determination of peaks 1, 2, and 3 plasmins by SDS electrophoresis. The relationships between the mobility of each protein (11) and their molecular weights is shown in Fig. 9. The analysis was repeated six times, and the molecular weights of the three plasmins were 130,000±5,000(SD), 64,000± 3,000(SD), and 32,000±1,500(SD), respectively, and were closely similar to those obtained by the method of

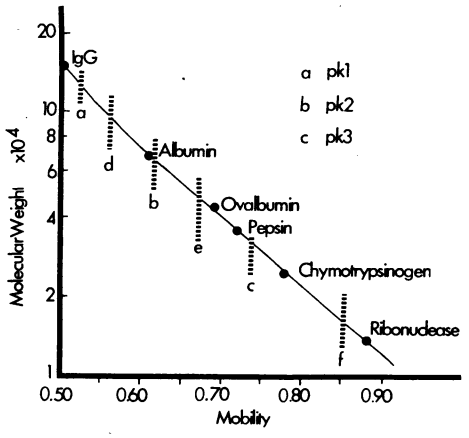

FIGURE 9 Molecular weight determination of human plasmins by SDS-polyacrylamide electrophoresis. About 50 μg of the standard proteins and peaks 1, 2, and 3 human plasmins were separately incubated for 30 min in 1 g/100 ml SDS and beta-mercaptoethanol and separately electrophoresed at 5 mA per gel for about 4 h. It is seen that the molecular weight of peak 1 plasmin (*a*) is about 130,000; that of peak 2 plasmin (*b*) is about 64,000; and that of peak 3 plasmin (*c*) is about 32,000. *d*, *e*, and *f* are explained in Fig. 10. See text for details of the electrophoretic conditions.

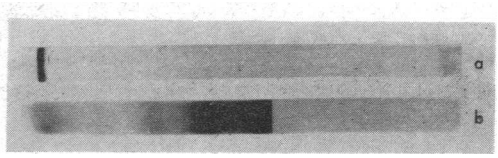

FIGURE 10 Polymer plasmin formation from and further separation into smaller protein pieces of peak 3 plasmin. Fig. 10*a* shows the electropherogram of human peak 3 plasmin obtained by the usual polyacrylamide electrophoresis as was in Fig. 6. Fig. 10*b* shows that peak 3 plasmin shown in Fig. 10*a* was converted to three additional proteins when incubated for 30 min in 1 g/100 ml SDS and beta-mercaptoethanol and electrophoresed by SDS-polyacrylamide electrophoresis as was in Fig. 9. The molecular weights of the three additional bands (*d*, *e*, and *f*) were obtained from the standard curve given in Fig. 9 and were 95,000, 48,000, and 16,000.

Andrews (10). However, during the incubation of peak 3 plasmin in SDS and beta-mercaptoethanol, three additional protein bands were found to be generated as shown in Fig. 10. It is seen that peak 3 plasmin (Fig. 10*a*) was converted to three additional bands (Fig. 10*b*). The mobility of each band was calculated as described by Weber and Osborn (11), and their molecular weights were obtained from the standard curve (Fig. 9). They were found to be 95,000±2,000(SD), 48,000± 1,500(SD), 32,000±1,500(SD), and 16,000±1,000(SD) in six experiments.

Total plasma antiplasmin capacity. The fibrinolytic rate method of Moser and Frey (8) was applied to 20 normal plasmas, and the total plasma antiplasmin capacity averaged 7.8±2(SD) CTA units of plasmin per milliliter plasma. 10 additional normal human plasmas were analyzed by the caseinolytic method (7). The results were 9.0±3(SD) CTA units of plasmin per milliliter plasma, showing no significant difference between the two ($0.2 > P > 0.1$).

Affinity of peak 3 [^{125}I]plasmin to albumin, 7s gamma globulin, α_2-macroglobulin, and α_1-antitrypsin. These studies were carried out using both 30 and 60 min incubation times. We did not find any appreciable difference in the results depending on the incubation times. Therefore, the results at 30 min incubation only are described below. The results of the study with albumin (Calbiochem) is shown in Fig. 11. It is seen that [^{125}I]-plasmin has no appreciable degree of affinity with albumin. The results were the same with 7s gamma globulin (Calbiochem), but [^{125}I]plasmin showed considerable degree of affinity to human α_2-macroglobulin (Behring Diagnostics) as shown in Fig. 12. The calculation of the areas under the peaks of bound and unbound [^{125}I]-plasmin showed that 14.6±0.5(SD)% of the total [^{125}I]-plasmin was bound to α_2-macroglobulin in five experiments, and these results were unchanged when 0.1 mg and 0.2 mg of [^{125}I]plasmin were used. Also, studies with

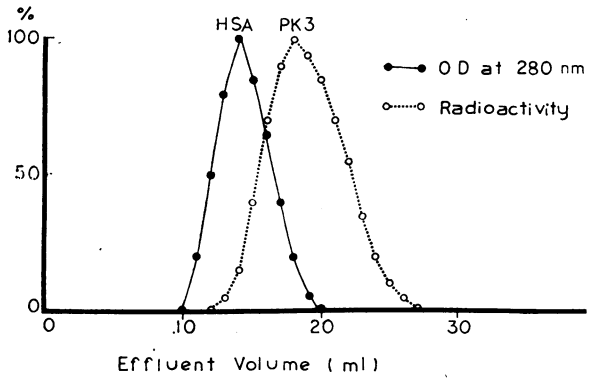

FIGURE 11 G-75 Sephadex gel filtration patterns of human peak 3 [^{125}I]plasmin and albumin. About 10 mg of albumin and 0.05 mg of [^{125}I]plasmin were mixed and incubated for 30 min at 38°C, and were passed through the column. It is seen that there is no appreciable degree of affinity between the two.

human α_1-antitrypsin (Behring Diagnostics) showed similar results. The calculation showed that 17.6 ± 0.6(SD)% of the total [^{125}I]plasmin was bound to α_1-antitrypsin in three experiments, and these results were unchanged when 0.1 mg and 0.2 mg of [^{125}I]plasmin were used.

Affinity of peak 3 [^{125}I]plasmin to fibrinogen and fibrin. The studies were carried out using both 30 and 60 min incubation. However, the results did not vary appreciably depending on the incubation times. Therefore, the results at 30 min incubation are given below. The results showed that in the control the precipitated [^{125}I]plasmin was 6±0.5(SD)% in three analyses and that when fibrinogen was present in concentrations of 3, 12, and 24 mg/3 ml the precipitated [^{125}I]plasmin was 27±0.5(SD)%, 26±0.4(SD)%, and 27±0.3(SD)%, respectively, each in four experiments. Thus, the net percent bound was 21±0.5(SD)%, 20±0.5(SD)%, 21±0.4(SD)%, respectively. Next, the amount of [^{125}I]-plasmin was increased to 0.1 and 0.2 mg, and the same procedure was carried out, but the results were essentially unchanged. With respect to the affinity of peak 3 [^{125}I]plasmin to fibrin, the results were as follows: When the original amount of fibrinogen was 3 mg/3 ml, the percent [^{125}I]plasmin bound to fibrin was 20.5± 0.5(SD)% in five experiments, and the results were 20.2±0.6(SD)% and 20.6±0.7(SD)% in five analyses, when the original amount of fibrinogen was 12 mg and 24 mg/3 ml, respectively. Next, the amount of [^{125}I]-plasmin added to fibrinogen was increased to 0.1 and 0.2 mg, but the results were not appreciably different.

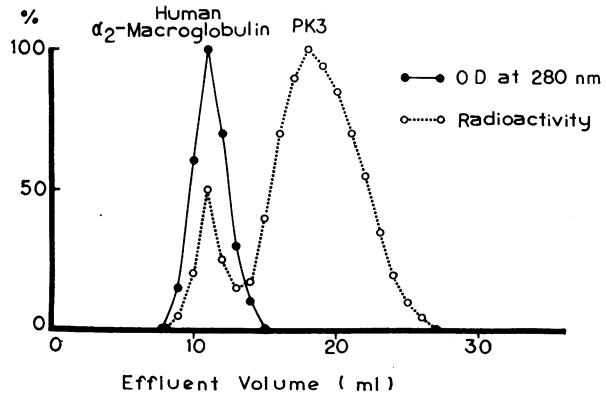

FIGURE 12 G-75 Sephadex gel filtration patterns of human peak 3 [^{125}I]plasmin and α_2-macroglobulin. About 10 mg of α_2-macroglobulin and 0.05 mg of [^{125}I]plasmin were mixed and incubated for 30 min at 38°C, and were passed through the column. It is seen that [^{125}I]plasmin has a considerable degree of affinity with α_2-macroglobulin.

In vivo fate of plasmin. Canine peak 3 [^{125}I]plasmin was screened as described in Methods, and its in vivo behavior was studied in five healthy dogs. The graphical analysis (16) of the tracer data (Fig. 4) showed that the plasma behavior of [^{125}I]plasmin (x) can be closely described by a two-exponential function of the form, $x = C_1e^{-at} + C_2e^{-bt}$, where $C_1 + C_2 = 1.0$. The x values in the five dogs were $x = 0.24e^{-1.19t} + 0.76e^{-4.17t}$, $x = 0.26e^{-1.10t} + 0.74e^{-4.50t}$, $x = 0.30e^{1.25t} + 0.70e^{-4.35t}$, $x = 0.27e^{-1.19t} + 0.73e^{-4.61t}$, and $x = 0.32e^{-1.10t} + 0.68e^{-4.50t}$, and averaged $x = 0.28e^{-1.16t} + 0.72e^{-4.42t}$. The plasma disappearance half-lives of [^{125}I]plasmin in the five dogs were 12, 15, 13, 14, and 15 h with the average value of 14.2± 0.63(SD)h. These data were further analyzed by the methods described elsewhere (1), and the fractional transcapillary transfer rate of plasma [^{125}I]plasmin (j_1), the fractional lymphatic return rate of extravascular [^{125}I]plasmin (j_2) and the fractional catabolic rate of plasma [^{125}I]plasmin (j_3) were obtained. j_1 in the five dogs ranged from 0.82 to 1.15 day^{-1} with the average value of 1.02±0.14(SD) day^{-1}, and j_2 values were 1.91, 1.98, 2.18, 2.11, and 2.19 with the mean value of 2.07± 0.12(SD) day^{-1}. The j_3 values were 2.63, 2.50, 2.60, and 2.26 day^{-1} and averaged 2.50±0.14(SD) day^{-1}.

DISCUSSION

The first question was whether our peak 3 plasmin is the very initial product of plasminogen activation and our peaks 1 and 2 plasmins (Figs. 1–3) are the polymers of the former, or our peak 3 plasmin is the end-product of the degradation of our peak 1 and 2 plasmins. In this connection, the analysis of samples at 5 min or less incubation is very important. Therefore, the samples at 1 and 5 min incubation in addition to samples at longer incubation were analyzed by the caseinolytic method (7) and disc electrophoresis (4). At 1-min incubation we did not find detectable amounts of plasmin, but at

5-min incubation some plasmin activities were generated. The electrophoretic analyses of the sample (Fig. 6*a*) showed in essential agreement with our previous results (1–3) that the predominant plasmin found at 5 min was one that has the electrophoretic mobility identical to that of our peak 3 plasmin (Figs. 5 and 6). However, there were minute amounts of homogeneous materials between plasminogen and peak 3 plasmin (Fig. 6*a*). These might be plasmins which we call peak 1 and 2 plasmins (Figs. 1–3). Also, the analysis of the 10 min sample gave essentially the same results (Fig. 6*b*). Therefore, these results were unable to provide definitive answers to the above question. However, we found that our peak 3 plasmin was further split into two pieces of about 16,000 mol wt and that polymers of 95,000 and 48,000 mol wt were formed during the incubation in SDS and beta-mercaptoethanol (Figs. 9 and 10). These findings seem to support the idea that our peak 1 and 2 plasmins (Figs. 1–3, 5) are formed by polymerization of peak 3 plasmin. However, these results must again be regarded as inconclusive, because what takes place in denaturing conditions such as in SDS and beta-mercaptoethanol does not necessarily reflect what occurs under normal incubation conditions of plasminogen activation.

Next, the biological properties of both human and canine peak 3 plasmins were studied using [^{125}I]plasmins. First, the total plasma antiplasmin capacity was measured which averaged 7.8±2(SD) CTA units plasmin/ml of normal human plasma. Then a question arose as to what fractions in plasma are responsible for the observed antiplasmin capacity and to what extent. Ratnoff, Lepow, and Pillemer (17) reported the presence of at least three plasmin inhibitors, and Schwick, Heimburger, and Haupt (18) purified five plasmin inhibitors. Thus, we studied the affinity of our [^{125}I]plasmin with albumin, gamma globulin, α_2-macroglobulin, α_1-antitrypsin, fibrinogen, and fibrin as described in Methods. Under the experimental conditions, our peak 3 human [^{125}I]plasmin did not show appreciable degree of affinity with human albumin (Fig. 11) or gamma globulin. However, considerable degrees of affinity were observed with α_2-macroglobulin (Fig. 12), α_1-antitrypsin, fibrinogen, and fibrin. The results indicated that the amount of plasmin bound to the above proteins will increase with the amount of plasmin available but that the percent of available plasmin bound remains constant. These results would be expected if the combination between the inhibitor proteins and plasmin were reversible. Also, the comparison of the affinity of α_2-macroglobulin, α_1-antitrypsin, fibrinogen, and fibrin with [^{125}I]plasmin showed that fibrinogen and fibrin do have greater affinities ($P < 0.001$) than the other two, but unfortunately we did not study what would happen to the affinity of each of these proteins in the presence of all of these proteins. However, it has been reported that the dissociation of plasmin from antiplasmin increased in the presence of fibrin substrate (19) and this lends a support to our findings described above. The next problem was the in vivo fate of plasmin. In our previous studies (1), this was indirectly determined by measuring the levels of [^{125}I]-plasmin generated from [^{125}I]plasminogen in dogs when a single injection of urokinase was given, using the electrophoretic method for detection and quantification of [^{125}I]plasmin (1, 2), and the plasma half-life of generated [^{125}I]plasmin was found to be 0.58±0.05(SD) days. In the present studies, the plasma half-life of [^{125}I]plasmin was directly determined by the use of purified peak 3 [^{125}I]plasmin (Fig. 3) and averaged 14.2±0.63(SD) h (Fig. 4), agreeing very closely with our previous results. The very fast plasma disappearance of [^{125}I]plasmin applies only to the plasma disappearance of circulating plasmin and does not apply to plasmin generated and entrapped within thrombi.

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