Studies on the Isolation of the Multiple Molecular Forms of Human Plasminogen and Plasmin by Isoelectric Focusing Methods*

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SUMMARY

Human plasminogen and diisopropyl phosphorofluoridate or L-1-chloro-3-tosylamido-7-amino-2-heptanone-treated plasmin, prepared from plasma Fraction III; or by ion exchange and gel filtration methods, and the equimolar human plasmin-streptokinase complex show multiple molecular forms in acrylamide gel electrophoresis at pH 8.4 in either 0.3 M L-aminocaproic acid or 8 M urea. Eight similar bands were found in the gel patterns of the zymogen and enzyme. The S-carboxymethyl heavy (A) chain derivative of plasmin did not show multiple molecular forms in acrylamide gel electrophoresis in either 0.3 M L-aminocaproic acid or 8 M urea. However, the S-carboxymethyl light (B) chain derivative showed multiple forms in acrylamide gel electrophoresis but in 8 M urea only.

Plasminogen prepared from either pooled plasma, or plasma Fractions III or III; or by affinity chromatography on lysine-substituted-Sepharose columns showed the same multiple molecular forms in acrylamide gel electrophoresis in 0.3 M L-aminocaproic acid as were found in our highly purified plasminogen preparations but with different quantitative distributions. Affinity chromatography of only plasma Fraction III; gave preparations with specific potential proteolytic activities which were equivalent in activity to our standard preparations.

The multiple molecular forms of plasminogen and plasmin prepared from plasma Fraction III; by ion exchange and gel filtration methods were isolated by isoelectric focusing methods. Eight plasminogen forms were obtained with isoelectric points ranging from pH 6.4 to pH 8.5. All of the forms had similar specific potential proteolytic activities which were equivalent in activity to our standard preparations.

The multiple molecular forms of plasminogen and plasmin prepared from plasma Fraction III; by ion exchange and gel filtration methods were isolated by isoelectric focusing methods. Eight plasminogen forms were obtained with isoelectric points ranging from pH 6.4 to pH 8.5. All of the forms had similar specific potential proteolytic activities. Each isoelectric form contained two closely related acrylamide gel electrophoretic components (doublet bands), both common to either adjacent isoelectric forms. Plasminogen isoelectric forms pH values 7.2 and 7.5 and diisopropylphosphoryl-plasmin isoelectric forms pH values 7.4 and 7.7 show the same acrylamide gel electrophoretic mobilities, respectively; the zymogen and enzyme forms differ in isoelectric point by approximately 0.2 pH unit. Plasminogen form pH 7.2 and its derived diisopropylphosphoryl-plasmin form show the same acrylamide gel electrophoretic mobilities.

The S-carboxymethyl heavy (A) and light (B) chain derivatives of plasmin were studied in isoelectric focusing. Three major light (B) chain fractions were isolated with isoelectric points of pH values 5.8, 5.9, and 6.0. The amount of each of the three major acrylamide gel electrophoretic forms in each isoelectric fraction was different; none of the fractions showed a single pure form. The heavy (A) chain derivative gave a single form with an isoelectric point of pH 4.9. The plasminogen isoelectric forms pH values 7.5 and 7.8, after conversion to plasmin, followed by reduction and alkylation, each gave light (B) chain preparations containing all of the major acrylamide gel electrophoretic forms. No changes in acrylamide gel electrophoretic mobility could be found in any of the multiple molecular forms of zymogen, enzyme, or light (B) chain derivative following isoelectric focusing or other preparative procedures.

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75,400 in which the chains are apparently connected by a single disulfide bond (3, 8). Homogeneous S-carboxymethyl heavy (A) and light (B) chain derivatives of plasmin have been isolated and characterized (7, 8). The heavy (A) and light (B) chains have molecular weights of 48,800 and 25,700, respectively. The active site serine and histidine residues are found in the light (B) chain (9, 10). The COOH-terminal amino acids of the heavy (A) and light (B) chains were determined to be arginine and asparagine, respectively, indicating that the light (B) chain of plasmin is derived from the COOH-terminal portion of the plasminogen molecule. Since the molecular weights of plasminogen and plasmin differ by approximately 6000, it is possible that either an activation peptide is released during activation of the zymogen (11), or a second bond is cleaved following the formation of the active site resulting in the release of a peptide. The position of this peptide in the plasminogen molecule has not as yet been definitely determined.

Human plasminogen has been shown to exist in multiple molecular forms (12-16). Slotta and Gonzales (12) showed that human plasminogen separated into multiple bands in starch gel electrophoretic systems containing either e-aminoacaproic acid or urea, at pH values between 7.4 and 10.0. Barg, Boggi, and DeRenza (13) obtained the same results using similar systems. These investigators studied highly purified plasminogen preparations and found that all of the excised bands contained plasminogen. We found multiple bands in plasminogen, plasmin, light (B) chain and the equimolar plasmin-streptokinase complex in acrylamide gel electrophoretic systems containing either e-aminoacaproic acid or urea at pH 8.4 (14). Wollén and Weimar (15) reported multiple bands with plasminogen in starch gel systems containing e-aminoacaproic acid at pH values 8.4 and 8.65 and determined enzymatic activity by a zymographic method using the intact gel. Deutsch and Mertz (16) found that plasminogen prepared from plasma by an affinity chromatographic method using L-lysine-substituted Sepahrose contained multiple bands in acrylamide gel electrophoretic systems at pH 8.3 in the absence of either e-aminoacaproic acid or urea. All of the bands were excised and found to contain plasminogen. They state that, in the presence of e-aminoacaproic acid at pH 8.3, a single band was obtained. Multiple molecular forms of canine plasminogen have also been reported (17). Although the existence of multiple electrophoretic forms of human plasminogen have been shown, the significance of those forms has not as yet been established. Multiple molecular forms of other zymogens of proteolytic enzymes, namely, chymotrypsinogen (18), pepsinogen (19), trypsinogen (20), and proelastase (21) have been isolated and characterized.

An understanding of the significance of the multiple electrophoretic forms of plasminogen and plasmin requires their isolation, and the determination of the differences between the various molecules. In a preliminary report (14), we described the use of isoelectric focusing methods for separating these multiple molecular forms. This report describes in detail the use of isoelectric focusing methods for separating and isolating the multiple molecular forms of human plasminogen and plasmin. The heavy (A) and light (B) chains were also studied by these techniques. Plasminogen was prepared for this study both by our ion exchange and gel filtration methods from plasma Fractions III1,2,3 (1, 2) and by the affinity chromatographic method (16) from pooled plasma and from different human plasma protein fractions. The isolated multiple molecular forms were characterized by chemical, physical, and immunochromatographic methods.

**MATERIALS AND METHODS**

**Human Plasminogen and Plasmin**—Human plasminogen was prepared from plasma Fraction III1,2 by ion exchange and gel filtration methods previously described (1, 2). Plasmin was prepared from the zymogen by activation with urokinase in 25% glycerol (1, 2, 6). Proteolytic activity was determined on a casein substrate after activation of the zymogen with streptokinase and directly with the enzyme (1, 2). The specific activities of the zymogen and enzyme preparations used in these studies were approximately 22 to 26 casein units per mg of protein. In order to establish a relationship between the casein unit used in our laboratories and the CTA casein unit (22), a CTA standard preparation of human plasmin was assayed in our assay system. One casein unit was found to be equivalent to 1.2 CTA units.

Human plasminogen was also prepared from pooled plasma, serum, plasma euglobulin and plasma Fractions III and III1,2 by a modification of the method described by Deutsch and Mertz (16) using affinity chromatography with L-lysine-substituted Sepharose. The chromatographic experiments were carried out at 2° instead of room temperature which required the replacement of the 0.3 M phosphate buffer wash by a 0.1 M phosphate buffer wash. The column was eluted with 0.2 M e-aminoacaproic acid in 0.1 M phosphate buffer, pH 7.4. The eluted plasminogen was precipitated by adding 3.4 g of ammonium sulfate per ml of solution. After 18 hours at 2°, the suspension was centrifuged, at 2°, at 4000 rpm (International Centrifuge PR11, Head No. 845). The plasminogen was dissolved in 0.05 M Tris-0.02 M L-lysine buffer, pH 9.0, at a concentration of approximately 25 mg per ml and frozen.

**Derivatives of Human Plasminogen and Plasmin**—Human plasminogen and plasmin prepared from plasma Fraction III1,2 by ion exchange and gel filtration methods were treated with both TLCK and DFP by methods previously described (9, 10). Completely reduced and S-carboxymethylated heavy (A) and light (B) chain derivatives of plasmin were prepared and isolated by methods previously described (7, 8). The equimolar plasmin-streptokinase complex was prepared and isolated by methods previously described (9, 23).

Certain preparations were radio labeled with [3H]TLCK and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3003, in Insta Gel (Packard Instrument Co., Danvers, Massachusetts) (10).

**Acrylamide Gel Electrophoresis**—Acrylamide gel electrophoresis was carried out on a gel slab in the Beckman Microzone acrylamide gel system at pH values 8.4 and 3.1. The acrylamide preparation used contained 5% bisacrylamide. Gels (5.0%) were prepared in either 0.037 M Tris-0.29 M glycine buffer, pH 3.1.

1 The nomenclature subcommittee of The International Committee on Thrombosis and Haemostasis has suggested that the heavy and light plasmin chains be called A and B plasmin chains, respectively.

2 The abbreviations used are: TLCK, L-1-chloro-3-tosylamido-7-amino-2-heptanone; DFP, diisopropyl phosphorofluoridate; DFP, diisopropylphosphate.
HCl, ITrere placed in each slot; sucrose crystals were added to aminocaproic acid but not urea. The pl-I 3.1 cell buffer was in the p1-I 3.1 gel only, ascorbic acid (0.7 ml per ml) was added to aid in polymerization. The pH 3.1 cell buffer contained ε-aminocaproic acid but not urea. The pH 3.1 cell buffer was prepared by titrating 0.1 M formic acid with 0.05 M NaOH to pH 3.1 and did not contain urea.

Samples of 0.025 to 0.05 mg in 2.5 ul of buffer, or 0.001 x HCl, were placed in each slot; sucrose crystals were added to stabilize the samples. Electrophoresis of the pH 8.4 gel was carried out at a constant voltage of 450 volts (35 to 40 ma) for 2 hours at 20°C; electrophoresis of the pH 3.1 gel was carried out at a constant amperage of 45 ma (200 to 220 volts) for 2 hours at 4°C. The gels were stained for 90 min with 0.075% Smido black 1013 in 7.5% acetic acid. Electrolytic destaining of the gels was carried out in 2% acetic acid. The gels were washed in distilled water and photographed, or dried and photographed.

**Isoelectric Focusing**—Separations by isoelectric focusing methods were carried out as described by Vesterberg and Svensson (24), with the LKB model 8100 jacketed columns (110 ml and 440 ml capacity). A stabilizing linear sucrose gradient (from 45% to 1%) was formed continuously by the use of a two chamber gradient mixer (LKB 8121). Carrier ampholytes (Ampholines) were obtained as water solutions with a solids content of 40% w/w, with various pH ranges. The final average concentration of carrier ampholytes during a separation was either 1% or 2%. A temperature controlled 10-liter water bath with a circulating pump was used to maintain the desired temperature within ±1°C. In all separations, the voltage was increased stepwise to avoid electrical loads greater than 1 watt. Voltages between 700 and 1000 volts were used.

The bottom electrode solution in all separations contained 60% w/v sucrose and 1% phosphoric acid, and the top electrode solution contained 2% ethylene diamine. The carrier ampholyte solution contained either 0.3 M ε-aminocaproic acid or 7 M urea. The isoelectric gradient was established by applying a potential of 300 volts for 24 hours. The sample was either dissolved in, or dialyzed against, either 0.3 M ε-aminocaproic acid or 7 M urea. The sucrose concentration of the sample was adjusted to 30% by adding sucrose crystals and the sample was introduced into the column by layering it at its isodense position. Fractions of 2.0 ml were collected by gravity flow at a regulated flow rate of 1 ml per mm. The protein concentration of each fraction was determined by absorbance measurements at 230 nm; the pH of each fraction was measured at room temperature (Radiometer TTTla pH meter). The proteolytic activity was determined on fractions exhaustively dialyzed against 0.001 M NaCl. The fractions were then lyophilized. Radioactivity measurements were made on the lyophilized fractions dissolved in 0.001 M HCl.

**Electrophoresis, Immunoelectrophoresis, and Immunodiffusion**—Electrophoresis, immunoelectrophoresis, and immunodiffusion were carried out on cellulose acetate. The electrophoresis method was previously described (25). For immunoelctrophoresis, the electrophoresis was carried out at 150 volts at room temperature for 1 hour. Protein samples at a concentration of 3 to 6 mg per ml were subjected to electrophoresis in 0.02 M phosphate-0.01 M lysine buffer, pH 8.0. After electrophoresis, a specific rabbit anti-plasminogen γ-globulin preparation (4) was added, and diffusion was allowed to proceed under mineral oil for 24 hours. The membranes were then washed with petroleum ether and saline before staining with 0.2% Ponceau S-fixative dye solution. The stained membranes were then rinsed in 10% acetic acid and dried between blotters at room temperature.

In the immunoafifaxis experiments, protein samples were dissolved in 0.02 M phosphate-0.01 M lysine buffer at pH 8.0, at a concentration of 1.0 mg per ml and the specific rabbit anti-plasminogen antibody was used. Diffusion was allowed to proceed under mineral oil for 24 hours. The membranes were then washed with petroleum ether and 0.9% NaCl solution before staining with the 0.2% Ponceau S-fixative dye solution. The stained membranes were then rinsed in 10% acetic acid and dried between blotters at room temperature.

**Sedimentation Equilibrium Analyses**—The sedimentation equilibrium runs were carried out with the Spincoro model E analytical ultracentrifuge equipped with a split beam photoelectric scanning system as previously described (3). The samples were run in 0.1 M NaCl-0.001 M HCl, pH 3.2.

**RESULTS**

**Preparation of Plasminogen by Affinity Chromatography**—Plasminogen was prepared by affinity chromatography from plasma, serum, plasma euglobulin, plasma Fraction III and plasma Fraction III+II using a lysine-substituted-Sepharose column (2.5 x 26 cm). The results are tabulated in Table I. Plasminogen was precipitated from fresh plasma by diluting 50 ml of plasma with 700 ml of distilled water at 2°C and adjusting the pH to 5.1 with 1 M acetic acid. The precipitate was dissolved in 0.16 M HCl for 20 min at 25°C and then neutralized.

<table>
<thead>
<tr>
<th>Source</th>
<th>Volume</th>
<th>Activity a</th>
<th>Plasmin activity b</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Plasmin c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>8.4</td>
<td>100</td>
<td>2.3</td>
</tr>
<tr>
<td>Plasma</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>18.5</td>
<td>100</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Plasma</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>15.6</td>
<td>100</td>
<td>7.1</td>
</tr>
<tr>
<td>Plasma euglobulin</td>
<td>45</td>
<td>140</td>
<td>70</td>
<td>18.5</td>
<td>79</td>
<td>3.4</td>
</tr>
<tr>
<td>Fraction III</td>
<td>35</td>
<td>100</td>
<td>31</td>
<td>21.8</td>
<td>91</td>
<td>3.8</td>
</tr>
<tr>
<td>Fraction III+II</td>
<td>4</td>
<td>157</td>
<td>15</td>
<td>25.0</td>
<td>96</td>
<td>3.8</td>
</tr>
</tbody>
</table>

a Activity in serum and plasma determined after plasma was incubated with an equal volume of 0.16 M HCl for 20 min at 25°C and then neutralized.

b Calculated from casein units found in each fraction relative to casein units in original plasma pools. Assumed recovery of 25 kg of Fraction III and 5 kg of Fraction III+II per 1000 liters of pooled plasma.

c Affinity chromatography at 23°C (individual plasma).

d Affinity chromatography at 25°C (individual plasma).

e Euglobulin was precipitated from fresh plasma by dialyzing 50 ml of plasma with 700 ml of distilled water at 2°C and adjusting the pH to 5.1 with 1 M acetic acid. The precipitate was dissolved up to 17 ml with 0.05 M Tris-0.02 M lysine-0.1 M NaCl buffer, pH 9.

f Fractions III and III+II were obtained from Cutter Laboratories, Berkeley, California as frozen pastes. Sixty grams of each fraction was dissolved in 500 ml of 0.05 M Tris-0.02 lysine-0.1 M NaCl buffer, pH 9, and clarified.

g Standard plasminogen preparation.
Preparations were obtained with specific activities of 17 (plasma) to 22 (Fraction III1,3) casein units per mg of protein. The purity varied with the starting material used. The highest specific activities were obtained with the more highly enriched plasminogen plasma Fractions III and III2,3. This method gives varying recoveries, again depending on the starting material used. The starting materials also contain different amounts of original plasma plasminogen; Fractions III and III2,3 contain 78% and 31% of original plasma plasminogen, respectively. Highly purified plasminogen prepared from plasma Fraction III2,3 by our ion exchange and gel filtration methods (1, 2) was chromatographed on the lysine-Sepharose column and recovered quantitatively, with no increase or decrease in specific activity.

Plasmin activity was found in the plasminogen preparations prepared from plasma chromatographed at room temperature instead of 2°, serum, plasma euglobulin, and Fraction III1,3. Plasminogen prepared from plasma at 2° and Fraction III did not contain plasmin activity. It appears that the various starting materials have trace amounts of a plasminogen activator which apparently activates small amounts of the zymogen under certain conditions.

Plasminogen, Fraction III and Fraction III1,3 extracts were chromatographed on a larger lysine-substituted-Sepharose column (4.2 x 23 cm) and the eluted plasminogen peaks were divided into fractions. The plasminogen specific activities throughout the plasma, Fraction III and Fraction III1,3 peaks are compared in Table II. Plasminogen was found throughout each peak. The specific activities were lower at either end of the peaks. The highest specific activities, between 25 and 30 casein units per mg of protein, were obtained with the most highly enriched plasminogen starting material, Fraction III2,3.

**Acrylamide Gel Electrophoresis**—The acrylamide gel electrophoretic patterns of plasminogen and plasmin prepared from plasma Fraction III1,3 by our ion exchange and gel filtration methods, and the S-carboxymethyl heavy (A)- and light (B)-chain derivatives of plasmin in 8 M urea at pH 3.1 are shown in Fig. 1A. All preparations appear to be essentially single components in this system. A minor, slower moving, component was seen in the heavy (A) chain preparation. The acrylamide gel electrophoretic patterns obtained with the same preparations in 8 M urea at pH 8.4 are shown in Fig. 1R. Multiple bands can be seen in the plasminogen, plasmin, and S-carboxymethyl plasmin light (B) chain preparations but not in the S-carboxymethyl plasmin heavy (A) chain preparation. Multiple bands were found in plasmin preparations prepared by activating the zymogen with either streptokinase, urokinase, or trypsin. Greater resolution of the bands was obtained when acrylamide gel electrophoresis was carried out in 0.3 M e-aminocaproic acid at pH 8.4 (Fig. 1C). The e-aminocaproic acid gel is more discriminating than the urea gel. Eight major components were seen in the plasminogen and plasmin preparations. No multiplicity of bands was observed with the S-carboxymethyl plasmin heavy (A) chain derivative; the S-carboxymethyl plasmin light (B) chain derivative did not migrate in this system since it is insoluble in the running buffer. Multiple bands were also seen in an equimolar plasmin-streptokinase complex preparation. Multiple bands were also seen in gels not containing e-aminocaproic acid but 0.3 M concentrations of this compound gave the optimal resolution of bands.

The acrylamide gel electrophoretic patterns of plasminogen fractions prepared by affinity chromatography of plasma Fractions III and III1,3 (Table II) in 0.3 M e-aminocaproic acid at pH 8.4 are shown in Fig. 1D. The electrophoretic mobilities of every band in each sample were identical. All of the plasminogen fractions obtained from Fraction III contained several faster moving bands not seen in the Fraction III1,3 plasminogen fractions. However, the slower moving major bands were the same in the Fraction III and III1,3 plasminogen fractions. The fractions with the highest specific activities prepared from plasma, Fraction III and Fraction III1,3 by affinity chromatography (Table II) were compared to our highly purified plasminogen prepared from plasma Fraction III1,3 by ion exchange and gel filtration methods and with plasminogen prepared from an individual plasma by affinity chromatography (16 casein units per mg of protein). All of the highly purified plasminogen preparations showed the same slow band distribution. The plasminogen prepared from an individual plasma by affinity chromatography showed a different quantitative distribution of bands; it contained primarily faster moving bands which corresponded to the faster moving bands in the Fraction III preparation. Plasma, and plasma Fractions III and III1,3, which contain different amounts of original plasma plasminogen, vary in their distribution of plasminogen bands.

**Isoelectric Focusing of Human Plasminogen**—Preliminary experiments with the isoelectric focusing method with Ampholine, pH range of 3 to 10, containing either 0.3 M e-aminocaproic acid or 7 M urea showed that plasminogen prepared from plasma Fraction III1,3 by ion exchange and gel filtration methods was composed of a group of isoelectric forms with isoelectric points between pH values 6 and 9. Resolution and stability were greater in e-aminocaproic acid than in urea. A TLCK-treated plasminogen preparation (100 mg), with a specific activity of 22 casein units per mg of protein, was electrofocused in the 440-ml column in 2% Ampholine, pH range of 5 to 10, containing 0.3 M e-aminocaproic acid at 1000 volts for 72 hours at 2°. The absorbance-pH patterns are shown in Fig. 2A. Eight protein peaks were obtained with isoelectric points ranging from pH 6.4 to 8.5. The protein distribution is shown in Table III. Each of the 2.0-ml fractions obtained in all of the eight peaks was exhaustively dialyzed against 0.001 M HCl and lyophilized. All of the fractions contained plasminogen. The specific activity of the peak fraction in each protein peak was found to be between 18 and

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**TABLE II**

_Preparation of human plasminogen from plasma, and Fractions III and III1,3 by affinity chromatography using lysine-Sepharose columns (4.2 x 23 cm): elution profiles_

<table>
<thead>
<tr>
<th>Eluent volumes</th>
<th>Plasma</th>
<th>Fraction III</th>
<th>Fraction III1,3</th>
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<tbody>
<tr>
<td>ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>320-350</td>
<td>8.8</td>
<td>14.1</td>
<td>17.4</td>
</tr>
<tr>
<td>351-375</td>
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<td>376-400</td>
<td>18.9</td>
<td>20.7</td>
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<td>401-425</td>
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<td>451-475</td>
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<td>476-500</td>
<td>17.4</td>
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<tr>
<td>501-520</td>
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</tr>
<tr>
<td>521-560</td>
<td>5.4</td>
<td>19.4</td>
<td>22.2</td>
</tr>
<tr>
<td>561-600</td>
<td>11.4</td>
<td></td>
<td>16.1</td>
</tr>
</tbody>
</table>

* Peak fraction.
FIG. IA. Acrylamide gel electrophoretogram, pH 3.1, in 8 M urea, of: 1, plasminogen; 2, TLCK-treated plasmin; 3, Cm-heavy (A) chain derivative; 4, Cm-light (B) chain derivative. B, acrylamide gel electrophoretogram, pH 8.4, in 8 M urea, of: 1, serum; 2, Cm-light (B) chain derivative; 3, Cm-heavy (A) chain derivative; 4, TLCK-treated plasmin; 5, DFP-treated plasminogen; 6, plasminogen. C, acrylamide gel electrophoretogram, pH 8.4, in 0.3 M e-aminocaproic acid, of: 1, plasminogen; 2, TLCK-treated plasmin; 3, Cm-heavy (A) chain derivative; 4, Cm-light (B) chain derivative; 5, equimolar human plasmin-streptokinase complex.

D, acrylamide gel electrophoretogram, pH 8.4, in 0.3 M e-aminocaproic acid, of affinity chromatography fractions (Table II), 1, Fraction III, fraction volume 360 to 400 ml; 2, Fraction III, fraction volume 401 to 424 ml; 3, Fraction III, fraction volume 425 to 448 ml; 4, Fraction III, fraction volume 504 to 520 ml; 5, Fraction III, fraction volume 360 to 376 ml; 6, Fraction III, fraction volume 408 to 424 ml; 7, Fraction III, fraction volume 425 to 441 ml; 8, Fraction III, fraction volume 504 to 520 ml.

22 casein units per mg of protein (Table III). Six isoelectric forms (88% of the protein) had specific activities between 21 and 22 casein units per mg of protein. The molecular weights of isoelectric forms pH values 6.7 and 7.8 were determined to be approximately 81,000. The amino acid compositions of isoelectric forms pH values 7.2 and 8.1 were identical and the same as the amino acid composition of plasminogen previously reported (1). These preparations did not contain e-aminocaproic acid. The peak fractions obtained from isoelectric forms pH values 6.4 to 8.5 were analyzed by acrylamide gel electrophoresis in 0.3 M e-aminocaproic acid at pH 8.4 (Fig. 2B). Each of these isoelectric forms contained two electrophoretic components, both common to the adjacent isoelectric forms. Isoelectric focusing separated the eight electrophoretic acrylamide components into eight isoelectric forms with isoelectric points between pH values 6.4 and 8.5. The electrophoretic mobilities in acrylamide gel of the two components in each isoelectric form corresponded with the electrophoretic mobilities of the corresponding component, or band, in the original plasminogen preparation. No change in acrylamide gel electrophoretic mobility was seen after isoelectric focusing, dialysis, or lyophilization.

Since each isoelectric form contained two components, one of

Unpublished experiments. The amino acid analyses were carried out as previously described (7) except that the hydrolysis mixture contained approximately 1 to 2 mg of protein in 2 ml of 6 N HCl and 1 ml of 2-mercaptoethanol.

### TABLE III

<table>
<thead>
<tr>
<th>Isoelectric focusing of plasminogen</th>
<th>Protein distribution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effluent volume of peak fraction</td>
<td>Specific activity</td>
</tr>
<tr>
<td>ml</td>
<td>%</td>
</tr>
<tr>
<td>68</td>
<td>18.4</td>
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<td>92</td>
<td>20.7</td>
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<td>168</td>
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<td>363</td>
<td>21.4</td>
</tr>
<tr>
<td>390</td>
<td>20.8</td>
</tr>
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</table>

* Determined from absorbance measurements using an extinction coefficient, E280, of 17.0.

The isoelectric forms (pH 7.8) was rerun by isoelectric focusing in the 440-ml column containing 0.3 M e-aminocaproic acid at 1000 volts for 72 hours at 20°. The absorbance pattern showed a single peak with an isoelectric point of pH 7.8 (Fig. 2C). Each 2.0-ml fraction in the isoelectric peak still showed the same two original electrophoretic components in acrylamide gel electrophoresis (Fig. 2D). These doublet
components could represent two plasminogens with the same isoelectric points but with different primary sequence, or perhaps, carbohydrate content.

Each isoelectric form prepared by isoelectric focusing (Fig. 2A and Table III) was analyzed for plasminogen in immunodiffusion and immunoelectrophoretic systems. All of the isoelectric forms reacted with the specific antibody; six of these forms are shown in Fig. 3A. A comparison was made of the immunoelectrophoretic mobilities of six of the isoelectric forms (pH values 7.2 to 8.5) (Fig. 3B). The various isoelectric forms showed different electrophoretic mobilities in the immunoelectrophoretic system in the absence of e-aminocaproic acid. Also, six of the
FIG. 3A, B, AND C.
(See p. 4098 for legend.)
isoelectric forms differ in electrophoretic mobility on cellulose acetate (Fig. 3C), again in the absence of e-aminocaproic acid.

**Isoelectric Focusing of Human Plasmin**—A preparation of DFP-treated urokinase-activated plasmin (95 mg), prepared from plasminogen obtained from plasma Fraction III2 by ion exchange and gel filtration methods, was electrofocused in the 440-ml column in 2% Ampholine, pH range of 5 to 10, containing 0.3 M e-aminocaproic acid at 1000 volts for 72 hours at 2°C. The absorbance-pH patterns are shown in Fig. 4. Eight apparent protein peaks were obtained with isoelectric points ranging from pH 7.0 to pH 8.5. The protein distribution is shown in Table IV. The isoelectric points of the multiple forms of DIP-plasmin were very similar to those found for plasminogen but not identical. The relative concentrations of protein in the zymogen and enzymatic isoforms were different (compare Tables III and IV). Each of the 2.0 ml fractions obtained in all of the 110-ml column was not as good as was obtained above with the 440-ml column. The major isoelectric forms in the 110-ml column was not as good as was obtained above with the 440-ml column. The major isoelectric forms in this experiment had isoelectric points of pH 7.3, 7.9, 8.2, and 8.4 which are similar to the isoelectric points obtained in the experiment with DIP-plasmin (Fig. 4) and plasminogen (Fig. 2A). The resolution with the 110-ml column was not as good as was obtained above with the 440-ml column. The major isoelectric forms in this experiment had isoelectric points of pH 7.3, 7.9, 8.2, and 8.4. The specific radioactivity per mg of protein was nearly identical in each isoelectric form. Each of these isoelectric forms reacted with specific antibody. Measurable differences in electrophoretic mobilities were obtained in cellulose acetate electrophoresis in the absence of e-aminocaproic acid.

**Isoelectric Focusing of the S-Carboxymethyl Heavy (A) Chain Derivative of Human Plasmin**—The S-carboxymethyl plasmin heavy (A) chain derivative (50 mg), prepared from plasminogen isoelectric form of pH 7.7. The plasminogen isoelectric form of pH 7.2 was activated with low molar ratios of urokinase in 25% glycerol and the resulting plasmin was treated with DFP. This pH 7.2 urokinase-activated DIP-plasmin was compared in the acrylamide gel electrophoretic system to the DIP-plasmin isoelectric forms and was found to be similar to the DIP-plasmin isoelectric form of pH 7.4 (Fig. 5B). The plasminogen form pH 7.2 and the DIP-plasmin form derived from this plasminogen form show the same acrylamide gel electrophoretic mobilities. But this derived plasmin form has the same electrophoretic mobility as the DIP-plasmin isoelectric form pH 7.4. The faster moving bands seen in Fig. 5B, No. 5 may represent some autolysis of plasmin during the activation process.

A preparation of [II(IV)CLK-treated urokinase-activated plasmin (26 mg), also prepared from plasminogen obtained from plasma Fraction III2, by ion exchange and gel filtration methods, was electrofocused in the 110-ml column in 1% Ampholine, pH range of 6 to 9, containing 0.3 M e-aminocaproic acid at 1000 volts for 48 hours at 2°C. Six distinct protein peaks were obtained with isoelectric points of pH 6.7, 7.3, 7.7, 7.9, 8.2, and 8.4 which are similar to the isoelectric points obtained in the experiment with DIP-plasmin (Fig. 4) and plasminogen (Fig. 2A). The resolution with the 110-ml column was not as good as was obtained above with the 440-ml column. The major isoelectric forms in this experiment had isoelectric points of pH 7.3, 7.9, 8.2, and 8.4. The specific radioactivity per mg of protein was nearly identical in each isoelectric form. Each of these isoelectric forms reacted with specific antibody. Measurable differences in electrophoretic mobilities were obtained in cellulose acetate electrophoresis in the absence of e-aminocaproic acid.

**Table IV**

<table>
<thead>
<tr>
<th>DIP plasmin</th>
<th>Protein distributiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoelectric point</td>
<td>Different volume</td>
</tr>
<tr>
<td>ml</td>
<td>pII</td>
</tr>
<tr>
<td>80</td>
<td>7.0</td>
</tr>
<tr>
<td>124</td>
<td>7.2</td>
</tr>
<tr>
<td>204</td>
<td>7.4</td>
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<td>292</td>
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<tr>
<td>308</td>
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<td>432</td>
<td>8.2</td>
</tr>
<tr>
<td>489</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*a Determined from absorbance measurements using an extinction coefficient, E1%cm, of 17.0.
Fig. 5A. Acrylamide gel electrophoretogram, pH 8.4, in 0.3 M  
ε-aminocaproic acid, of: 1, plasminogen isoelectric form pH 7.2;  
2, DIP-plasmin isoelectric form pH 7.5; 3, plasminogen isoelectric  
form pH 7.2; 4, DIP-plasmin isoelectric form pH 7.4; 5, plasmin-  
gen isoelectric form pH 7.8; 6, DIP-plasmin isoelectric form pH  
7.7.  

Fig. 5B. Acrylamide gel electrophoretogram, pH 8.4, in 0.3 M  
ε-aminocaproic acid, of: 1, plasminogen; 2, plasminogen isoelectric  
form pH 7.2; 3, DIP-plasmin derived from plasminogen isoelectric  
form pH 7.2; 4, DIP-plasmin isoelectric form pH 7.4; 5, DIP-  
plasmin isoelectric form pH 7.2; 6, DIP-plasmin isoelectric form pH  
7.5; 7, DIP-plasmin isoelectric form pH 7.8; 8, DIP-plasmin isoelectric form pH 7.4.  

Fig. 6. Isoelectric focusing of S-carboxymethyl heavy (A)  
chain derivative of plasmin.  

obtained from plasma Fraction III2 by ion exchange and gel  
filtration methods, was electrofocused in the 440-ml column in  
2% Ampholine, pH range of 3 to 7, containing 7 M urea at 700  
vols for 72 hours at 24°. The absorbance-pH pattern is shown  
in Fig. 6. One single peak was obtained with an isoelectric  
point of pH 4.9. When this heavy (A) chain derivative was elec-  
троfocused in the same system containing 0.3 M  ε-aminocaproic  
acid instead of 7 M urea at 1000 volts for 46 hours at 4°, a single  
protein peak was also obtained with the same isoelectric point  
(pH 4.9). The heavy chain derivative did not show multiple  
forms in acrylamide gel electrophoresis in either 8 M urea or  
0.3 M  ε-aminocaproic acid (Fig. 1, B and C).  

Isoelectric Focusing of the S-Carboxymethyl Light (B) Chain Der-  
ivative of Human Plasmin—The S-carboxymethyl plasmin light  
(B) chain derivative (20 mg) prepared from plasminogen obtained  
from plasma Fraction III2 by ion exchange and gel filtration  
methods, was electrofocused in the 110-ml column in 2% Am-  
pholine, pH range of 4 to 7, containing 7 M urea at 750 volts for  
72 hours at 24°. The absorbance-pH pattern is seen in Fig. 7A.  
Three major protein peaks were obtained with isoelectric points  
of pH 5.8, 5.9, and 6.0 containing approximately 75% of the total  
preparation. Each of the fractions obtained was dialyzed ex- 
tensively against 1 M acetic acid and lyophilized. The major  
peak fractions at pH values 5.8, 5.9, and 6.0, and the minor peak  
fractions at pH values 5.3 and 6.2, were analyzed by acrylamide  
gel electrophoresis in 8 M urea at pH 8.4 (Fig. 7B). Partial  
resolution of the isoelectric forms was obtained; the amount of  
each of the three major acrylamide gel electrophoretic forms in  
each fraction was different. The amino acid compositions of  
isolectric forms pH values 5.8, 5.9, an 6.0 were identical and  
the same as the amino acid composition previously reported (10).  
Since the isoelectric points of the different forms are practically  
the same, it may be difficult to prepare pure light chain forms.  
The possibility existed that each isoelectric plasminogen, or  
plasmin form would give a single light chain form. To test this  
hypothesis, two major plasminogen isoelectric forms, pH values  
7.5 and 7.8, were converted to plasm with urokinase, com-
pletely reduced and carboxymethylated, and the light chain was isolated by our dialysis procedure. Each light chain preparation showed the same three major isoelectric forms. These isoelectric forms may differ in either their carbohydrate composition or amide distribution. There appears to be some very labile components in the light chains.

DISCUSSION

In all of our previous studies on the isolation and characterization of human plasminogen, plasmin, and the S-carboxymethyl heavy (A) and light (B) chain derivatives of plasmin, we have shown that these proteins were apparently homogeneous and monodispersed (1–4, 7, 8). The zymogen was always prepared from plasma Fraction III, by our ion exchange and gel filtration methods (1, 2, 26). The zymogen and enzyme were found to be monomers, and after reduction and alkylation of the enzyme, the resulting isolated heavy (A) and light (B) chains were also shown to be monomers (7, 8). Our previous electrophoretic studies using moving boundary electrophoresis (26), cellulose acetate electrophoresis (25), and starch gel electrophoresis (5) showed that the zymogen and enzyme were apparently homogeneous. All of our plasminogen preparations contained up to 3 to 4% active plasmin. Although plasmin activity is found in our zymogen preparations, there is no evidence that the enzyme causes proteolytic degradation of the zymogen. Our physical and chemical studies (1–3) do not show heterogeneity. Our NH₂- and COOH-terminal amino acid studies (5–8) show that lysine is the NH₂ terminus of plasminogen and the heavy (A) chain, valine is the NH₂ terminus of the light (B) chain, asparagine is the COOH terminus of plasminogen and the light (B) chain, and arginine is the COOH terminus of the heavy (A) chain. In a recent study, Wallén and Wiman (15) reported that plasminogen isolated from a plasma Fraction III extract containing a plasmin inhibitor (Trasylol) showed NH₂-terminal glutamic acid, but if the plasmin inhibitor was not added to the plasminogen extract, preparations were obtained which had NH₂-terminal glutamic acid, valine, and lysine. These authors infer that plasmin degrades plasminogen during the isolation procedures. An analysis of their results indicated that their plasminogen preparation could be a heterogeneous group of components. The specific activity calculated in CTA units is about 75%; the specific activity of our plasminogen preparations and no physical studies were reported to show homogeneity. The possibility exists that the inhibitor used may also bind to the zymogen. Dlouha and Keil had reported that this inhibitor binds specifically to trypsinogen (27). The preparations isolated by Wallén and Wiman could contain plasminogen-plasmin inhibitor and plasmin-plasmin inhibitor complexes. We have recently found that all of the plasminogen preparations used in our studies which were obtained from Fraction III, by our ion exchange and gel filtration methods, or from pooled plasma, and plasma Fractions III and III, by the lysine-substituted-Sepharose affinity chromatography method (Tables I and II) had NH₂-terminal lysine and valine and the same NH₂-terminal sequence using the Edman method. ⁶ We had previously reported (3) using the Stark and Smyth method that our plasminogen preparations had NH₂-terminal lysine and valine. We have never found a preparation with NH₂-terminal glutamic acid. The Fraction III plasminogen preparation used for sequence analysis had no plasmin activity. We have shown on the basis of all of our previous studies that plasminogen, plasmin, and the heavy (A) and light (B) chain derivatives are monomers with a high degree of homogeneity. Also, plasminogen prepared by our ion exchange and gel chroma-
Multiple molecular forms of human plasminogen have been reported by several investigators (12-16). The separation and isolation of these forms are discussed in this paper. Multiple molecular forms have been demonstrated in plasminogen preparations prepared by a single step from pooled fresh human plasma (four normal subjects) by the lysine-substituted-Sepharose affinity chromatographic method and from the sera of normal individual subjects by our ion exchange and gel chromatographic methods (28). Although the specific activity of the plasminogen preparations were about 70% of purity, the same bands were observed in the acrylamide gel electrophoretic systems containing ε-aminocaproic acid as were observed with the plasminogen prepared from Fractions III and III-2. In this report, we have shown that the multiple molecular forms of plasminogen can be isolated from our apparently homogeneous preparations of the zymogen by isoelectric focusing methods. The zymogen was always treated with either DFP or TLCK to inhibit any active plasmin before electrofocusing. Multiple molecular forms of plasminogen with isoelectric points at pH values 6.4, 6.7, 7.2, 7.5, 7.8, 8.1, 8.3, and 8.5 were isolated. The potential specific proteolytic activities of each isoelectric form were similar. Each isolated form gave two bands in acrylamide gel electrophoresis. The bands always corresponded with two of the eight bands isolated from our apparently homogeneous preparations of the plasminogen. The parameters responsible for the differences between the zymogen isoelectric forms are probably different from the parameters responsible for the differences between the light (B) chain isoelectric forms. Similar multiple forms have also been found in dog and bovine isolated light (H) chains.

The multiple molecular forms of plasminogen observed both in acrylamide gel electrophoresis and in isoelectric focusing did not appear to be naturally occurring isoenzymes since they are present in normal individual plasmas and sera (15, 16, 28). Since they can be isolated by isoelectric focusing methods in relatively large quantities, it should be possible to determine the biochemical differences between these different isoelectric forms of plasminogen.

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