Purification of Human Plasminogen and Plasmin by Gel Filtration on Sephadex and Chromatography on Diethylaminoethyl-Sephadex*

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Highly purified human plasminogen has been prepared from a variety of plasma fractions and by different purification methods. The two major fractions used have been serum or plasma euglobulin and Cohn plasma Fraction III. The purification methods (1-14) have all yielded products of high purity. Plasminogen useful for further purification and isolation studies can be prepared by the Kline method (3). High purity plasmin can be prepared from Kline method plasminogen by activation with streptokinase (16).

The purpose of this study was to develop new methods for the preparation of pure plasminogen and plasmin. The Kline method was first modified to give a more reproducible procedure, with better yields. Plasminogen was precipitated, in the final step, at pH 6.0, with NaH₂PO₄. The use of plasma Fraction III₂,₃ instead of Fraction III as the starting material resulted in higher yields of proenzyme and gave preparations with higher specific activities. Gel filtration through Sephadex columns and chromatography on diethylaminoethyl-Sephadex columns resulted in preparations of highly purified proenzyme and enzyme. Ultracentrifugal and electrophoretic analysis, gel diffusion (Ouchterlony), and immunoelectrophoretic studies were carried out to characterize and to determine homogeneity of the proenzyme and enzyme.

EXPERIMENTAL PROCEDURE

Determination of Plasminogen and Plasmin Activity—The assay is a modification of the method described by Remmert and Cohen (1). The ice-cold plasminogen solution was pipetted into test tubes in a 4° water bath. The activator, 500 Christensen units of streptokinase (Varidase, Lederle) or 250 Ploug units of urokinase, in distilled water, was pipetted into the tubes. The volume was adjusted to 2.0 ml with 0.067 M phosphate buffer, pH 7.4, followed by 2.0 ml of ice-cold 4% casein solution. After mixing, the tubes were transferred to a 37° water bath. At the end of 30 minutes, the tubes were removed from the 37° water bath and placed into a 4° water bath; 6.0 ml of 15% trichloroacetic acid were added. After 18 hours at 4°, the tubes were allowed to warm up to room temperature. The precipitate was removed by filtration through Whatman No. 50 filter paper. The absorbancy of the solution was read at 280 mλ. The proenzyme blank contained less than 5% of the total activity calculated as plasmin. Duplicate determinations were carried on each sample. This method is valid and reproducible with enzyme concentrations which give absorbancy values between 0.10 and 0.40. The accuracy of the method is ±10%.

Plasmin activity was also measured by the same method except that activator was omitted. The plasminogen or plasmin proteolytic unit was calculated as the amount of enzyme which liberated 450 μg of trichloroacetic acid-soluble tyrosine in 1 hour. This unit is similar to that described by Remmert and Cohen (1). The Remmert and Cohen casein unit described by Kline and Fishman (16) is approximately 1.4 times this unit, whereas the Remmert and Cohen casein unit described by Sgouris et al. (7) is approximately 1.1 times this unit. The National Institutes of Health plasminogen and plasmin reference preparations, tentative, No. 1, when assayed by this method, gave values which were 10% less than labeled potency. The unit used in this study is approximately 1.1 times the N.I.H. unit. The calculation is made as follows

1 casein unit per ml = \( \frac{\text{absorbancy}}{\text{sample volume (ml)}} \times 2 \times 10 \times 150 \times \frac{1}{450} \)

or

1 casein unit per ml = \( \frac{\text{absorbancy}}{\text{sample volume (ml)}} \times 6.67 \)

Determination of Urokinase—Urokinase was determined by activation of plasminogen and measurement of the resulting plasmin in the casein assay described above. Plasmin preparations containing urokinase should be diluted to contain less than 0.06 casein units. In each assay 27 ± 3 casein units of plasminogen were used and the incubation was carried out for 15 minutes at 37°. The urokinase activity is read off a standard curve prepared from a urokinase preparation containing 3500 Ploug units per mg (Leo Pharmaceutical Company, Denmark). This method was used primarily for measuring urokinase in supernatant solutions after isoelectric precipitation of plasmin.

* This investigation was supported in part by a grant (H4366) from the National Heart Institute, United States Public Health Service.

1 Casein, Hammersten quality, washed with water, 95% ethanol and ether, or devitaminized casein (Sheffield Chemical) was used. A 4% suspension in 0.067 M phosphate buffer, pH 7.4, was prepared and clarified by centrifugation. The substrate was stored at 4° and could be used for at least 1 week.

2 D. L. Kline, personal communication. Assays were also carried out by Dr. Kline on the preparations described in this paper.
RESULTS

Preparation of Plasminogen from Plasma Fraction III₂,₃—Partially purified human plasminogen was prepared by a modification of the method described by Kline (3). One kilogram of Fraction III₂,₃ paste was suspended in 20 liters of 0.05 N HCl. The suspension was stirred for 1 hour. The insoluble material was removed by centrifugation. The supernatant solution which had a specific activity of about 0.32 units per absorbancy unit was adjusted to pH 11.0 with N NaOH. After a few minutes, the pH was adjusted to 5.3 with N HCl. After storage at 4° for 18 hours, the suspension was adjusted to 2.0 with N HCl and the insoluble material removed by centrifugation. The supernatant solution was adjusted to pH 8.7 with N NaOH and then to pH 6.0 with 0.2 M NaH₂PO₄. The final concentration of NaH₂PO₄ was 0.003 M. After storage at 4° for 18 hours, the plasminogen precipitate was collected by centrifugation, dissolved in approximately 800 ml of 0.005 N HCl. The pH of the solution is approximately 3.7. These plasminogen preparations were stable for months in the liquid state when stored at 4°. Preparations stored at −25° were stable for longer periods of time.

This procedure is similar to the Kline method with the exception of the final step in which plasminogen was precipitated at pH 6.0 with NaH₂PO₄ and the precipitate was dissolved in HCl at pH 3.7. Higher specific activities can be obtained by increasing the phosphate concentration, at pH 6.0, to 0.03 M. Also, the use of Fraction III₂,₃ instead of Fraction III resulted in greater reproducibility in the method, and preparations with higher specific activities.

The data obtained in nine experiments are summarized in Table I. The average recovery of plasminogen from starting Fraction III₂,₃ was 37,000 units per kg with a yield of 42%. The preparations showed average specific activities of 4.7 units per mg, 7.7 units per mg of protein, and 3.3 units per absorbancy unit. The purification factor calculated from the acid extract of Fraction III₂,₃ was 10. The results obtained with this method with Fraction III are also summarized in Table I. The specific activities of the final products are much lower than those obtained with Fraction III₂,₃.

Further Purification of Plasminogen by Gel-Filtration—Plasminogen prepared by the modified Kline method could be further purified by gel filtration through Sephadex. The plasminogen solutions were filtered through either Sephadex G-50, medium, or G-75, medium, columns equilibrated with 0.0005 N HCl, pH 3.5, at 4°. The eluting and equilibrating solutions were the same. The result of a typical experiment is shown in Fig. 1. The plasminogen was separated into two groups of components. The first peak contained over 50% of the optical density but no proenzyme. The second peak contained 35% of the optical density and 60% of the proenzyme. The remainder of the optical density and some of the proenzyme could be removed from the column by a 0.05 M lysine-0.02 M borate buffer, pH 8.6. The specific activity of the plasminogen peak (Fractions 48 to 80) was 6.7 units per absorbancy unit. The specific activity of some of the fractions was as high as 10.0 units per absorbancy unit. The variations in the specific activity throughout the proenzyme peak may be due to the presence of different plasminogens in the preparation. The highest specific activities were always found in the tailing portion of the peak. Gel filtration of plasminogen preparations of different specific activities showed that the higher the purity of the starting material, the higher the purity of the final plasminogen preparation. There was always a loss of proenzyme activity, 25 to 50%, in the gel filtration experiments. The most active material prepared by
TABLE I
Preparation of plasminogen by modified Kline method

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Purification factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/kg</td>
<td>%</td>
<td>units/absorbancy unit</td>
</tr>
<tr>
<td>A. Fraction III₂₁†</td>
<td>28,800-53,500</td>
<td>34-58</td>
<td>2.7-4.7</td>
</tr>
<tr>
<td></td>
<td>37,000</td>
<td>42</td>
<td>3.3</td>
</tr>
<tr>
<td>B. Fraction III₂</td>
<td>12,300-48,400</td>
<td>35</td>
<td>1.7-2.6</td>
</tr>
<tr>
<td></td>
<td>25,800</td>
<td></td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Calculated from specific activity based on units per absorbancy unit.
† Data from 9 experiments with 1 kg of Fraction III₂₁ paste per experiment; the Fraction III₂₁ was obtained from Cutter Laboratories.
‡ Data from 26 experiments with 1 kg of Fraction III paste per experiment; the Fraction III was obtained from Cutter Laboratories.

Further Purification of Plasminogen by Chromatography on DEAE-Sephadex Columns—Plasminogen prepared by the modified Kline method could be further purified by chromatography on DEAE-Sephadex columns. The experiments were carried out with DEAE-Sephadex, type A-50, medium, capacity of 3.0 to 3.9 meq per g, in columns at 4°C. Plasminogen could be adsorbed at pH 8.0 to 9.0, in a Tris-lysine buffer, ionic strength of 0.07, and eluted by increasing the ionic strength of the buffer to 0.17, with NaCl. It was found that chromatography of the proenzyme with starting agent development conditions was preferable to adsorption and elution methods. The best conditions for starting agent development were pH 8.0 or 9.0 in a buffer containing 0.05 M Tris, 0.02 M lysine, and 0.10 M NaCl. The lysine is essential. The result of a typical experiment, with starting agent development conditions at pH 9.0, is shown in Fig. 2. The plasminogen preparation was dialyzed against 10 volumes of buffer for 1 hour at 4°C before chromatography. The peak represents the proenzyme passing through the column under equilibrium conditions. The RF value of the peak was close to 1.0. The highest specific activity obtained in individual fractions was 11.0 units per absorbancy unit. The specific activity curve showed a number of peaks. The total proenzyme recovery was 78%. The specific activity of the pool of Fractions 40 to 56 was 10.0 units per absorbancy unit; the recovery of this preparation had a specific activity of 8.7 units per absorbancy unit with a purification factor of 2.6. The purification factor is 26-fold relative to the acid extract of Fraction III₂₁ (see Table II). The salt-free gel-filtered plasminogen preparations were dried from the frozen state. Gel-filtered plasminogen on refiltration through gel under the same conditions gave a single peak with quantitative recovery of protein and proenzyme. The proenzyme and optical density peaks were in the same position.

Gel filtration of plasminogen solutions through Sephadex G-25, medium, columns, at pH 3.5, gave a single peak, the proenzyme appearing in the tailing portion of the peak. Gel filtration through Sephadex G-50 and G-75, at pH 3.0, also gave a single peak but with a broad tailing edge with the proenzyme appearing in the tailing edge. The specific activities were similar to those obtained at pH 3.5 but the separation of the proenzyme from the impurities was more difficult. At pH 2.0, gel filtration of plasminogen through G-50 and G-75 gave a single peak with the proenzyme appearing throughout the optical density peak.

TABLE II
Preparation of plasminogen and plasmin from modified Kline method fractions by gel filtration on Sephadex and chromatography on DEAE-Sephadex

<table>
<thead>
<tr>
<th>Preparation and method</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Purification factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>units/absorbancy unit</td>
<td>-fold</td>
</tr>
<tr>
<td>Plasminogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel filtration (Sephadex)</td>
<td>50-75</td>
<td>8.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Chromatography (DEAE-Sephadex)</td>
<td>50-57</td>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Plasmin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel filtration (Sephadex)</td>
<td>32-34</td>
<td>4.3</td>
<td>1.3†</td>
</tr>
<tr>
<td>Chromatography (DEAE-Sephadex)</td>
<td>34-41</td>
<td>11.0</td>
<td>3.3†</td>
</tr>
</tbody>
</table>

* Calculated from specific activity based on units per absorbancy unit.
† Relative to Fraction III₂₁.
‡ Based on starting plasminogen preparation.
pool was 57%. The pooled material was adjusted to pH 3.7
with 1 N HCl and dried from the frozen state. The dried enzyme
preparation was dissolved in a small volume of 50% glycerol.
This preparation had a specific activity of 9.0 units per absorb-
ancy unit. It contained 170 units and 10.0 mg of protein per
ml. Rechromatography under the same conditions, after
dialysis of the preparation against 10 volumes of buffer at 4°C,
gave a single peak. The specific activity of each fraction was
the same. The recovery of proenzyme and optical density
material was 81%. The specific activity increased by about
10%.

Plasminogen preparations of different specific activities behaved differently on DEAE-Sephadex. Every partially purified preparation of plasminogen, regardless of specific activity, could be further purified by chromatography on DEAE-Sephadex, under the conditions described above, to give preparations with specific activities between 10.0 and 11.0 units per absorbancy unit, the maximal purity obtained by this method. The best chromatographed preparations have been purified approximately 30-fold relative to the acid extract of Fraction III_{2,3} (see Table II). These preparations, in 50% glycerol, are stable for over 1 year when stored at -25°C. They contain between 4.0 and 4.9% "active" proteinase.

Gel-filtered plasminogen preparations can be further purified by chromatography on DEAE-Sephadex. Also, preparations chromatographed on DEAE-Sephadex can be gel-filtered on Sephadex G-50 or G-75, at pH 3.7, with no loss in activity. This method can be used to desalt the preparations. DEAE-cellulose columns can also be used to further purify crude plas-
minogen preparations, under the same conditions as described for DEAE-Sephadex, but the highest specific activity obtained with DEAE-cellulose was 8.0 units per absorbancy unit.

**TABLE III**

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole pool</td>
<td>Selected pool</td>
</tr>
<tr>
<td>units/kg</td>
<td>%</td>
</tr>
<tr>
<td>Range</td>
<td>228,000-276,000</td>
</tr>
<tr>
<td>Average</td>
<td>243,000</td>
</tr>
</tbody>
</table>

* Data from 5 experiments.
† Calculated to represent highest purification factor from selected pools from specific activity based on units per absorbancy unit.
from the material prepared by chromatography of the modified Kline method material. The preparations are readily soluble at neutral pH values and are more soluble between pH 4 and 7. Rechromatography of these plasminogen preparations on diethylaminoethyl-Sephadex, under the same chromatographic conditions as described above, will not increase the specific activity of those preparations with initial specific activities of 9.0 to 9.7 units per absorbancy unit, but will increase the specific activity of preparations of lower purity. The maximal specific activity obtained on rechromatography is approximately 10 units per absorbancy unit. When a preparation with a specific activity of 9.7 units per absorbancy unit is rechromatographed, a single peak with nearly constant specific activity in all the fractions is obtained.

Gel filtration of these preparations on Sephadex G-25, G-50, or G-75, medium, columns at pH 9.0 did not increase the specific activity of the material. At pH 3.5, gel filtration on a Sephadex G-50, medium, column gave only one peak, with no loss in activity and with no increase in purity. Gel filtration of the modified Kline method material always resulted in a 50% loss in activity and always gave two peaks (Fig. 1).

Preparation of Plasmin—(a) Activation of plasminogen by urokinase. It had been previously reported that plasminogen could be activated in 50% glycerol, either spontaneously (21) or with small amounts of urokinase (6). In these studies, complete conversion of plasminogen to plasmin can be effected in 24 hours, at 25°, with trace quantities of urokinase. A system containing 25% glycerol (synthetic), 0.01 M phosphate, and 0.034 M glycine or 0.005 M lysine, at pH 7.4, gave the best results. Without these trace quantities of urokinase, it was not possible to completely activate plasminogen, in 25% glycerol, in a reasonable time. The concentration of modified Kline method plasminogen used was 17 to 33 units per ml and the urokinase to plasminogen ratio (unit basis) was approximately 1. The urokinase preparation contained 16,000 Ploug units per mg (Leo Pharmaceutical Company, Denmark). After activation, the plasmin was precipitated at pH 6.2 ± 0.2 with 0.2 M NaH₂PO₄ and dissolved at pH 3.7. The recovery of enzyme was about 75%. Two- to 3-fold dilution of the activation mixture before isoelectric precipitation gave somewhat better recoveries. Dialysis at pH 3.7 followed by drying from the frozen state is an alternate method. The bulk of the urokinase was found in the supernatant solution after isoelectric precipitation of the plasmin.

(b) Activation of plasminogen by trypsin. Modified Kline method plasminogen was activated with trace quantities of trypsin in 25% glycerol, under the same conditions as described for the preparation of urokinase-activated plasmin. For complete activation in 24 hours at 25°, 2.5 μg of crystalline trypsin (Wilson) were used for each casein unit of plasminogen. The trypsin-activated plasmin was removed from solution by isoelectric precipitation at pH 6.2 ± 0.2 and dissolved at pH 3.7. The bulk of the trypsin was precipitated with the plasmin at pH 6.2.

Further Purification of Urokinase-activated Plasmin by Gel Filtration—Urokinase-activated plasmin could be purified by gel filtration through Sephadex G-50, medium, columns equilibrated with 0.0005 N HCl, pH 3.5, at 4°. The experimental conditions were the same as those used to further purify plasminogen by gel filtration. The result of a typical experiment is shown in Fig. 3. The preparation was separated into two separate, distinct groups of components. All of the enzyme was found in the second minor peak. The specific activities of the fractions throughout the main portion of the peak were similar, approximately 4.3 units per absorbancy unit. The specific activity of the pool of Fractions 52 to 68 was 4.3 units per absorbancy unit. The purification factor was 1.3 and the recovery was 32% (see Table II). The behavior of urokinase-activated plasmin and plasminogen on Sephadex G-50 under the same conditions of gel filtration was similar (see Fig. 1). The specific activities of gel-filtered plasminogen preparations were always higher than those found for the corresponding gel-filtered plasmin preparations. The salt-free gel-filtered preparations were dried from the frozen state.

Further Purification of Plasmin by Chromatography on DEAE-Sephadex—Urokinase activated plasmin, after isoelectric precipitation, was dissolved in ice-cold 0.05 M Tris-0.02 M lysine-0.10 M NaCl buffer, pH 9.0, and dialyzed against 10 volumes of this buffer at 4°. The enzyme was chromatographed on DEAE-Sephadex under the same conditions as used for plasminogen.

The result of a typical experiment is shown in Fig. 4. The $R_f$ value of the peak was close to 1.0. The total enzyme recovery was 65%. The highest specific activity obtained in an individual tube was 14.3 units per absorbancy unit. The enzyme coming through with the leading edge had the highest specific activity. The purification factor calculated from the fractions with the highest specific activity was 3.3. The specific activity of the pool of Fractions 33 to 48 was 13.0 units per absorbancy unit. The recovery of this pool was 34%. The pool was adjusted to pH 3.7 with N HCl and dried from the frozen state. The dried enzyme preparation was dissolved in a small volume of 50% glycerol. The plasmin preparation had a specific activity of 11.0 units per absorbancy unit. The solution contained 73.3 units per ml and 3.7 mg of protein per ml. The specific activity was similar to the specific activity of the highly purified chromatographed plasminogen preparation obtained under the same conditions. Although plasmin has been obtained with specific activities between 13 to 14 units per absorbancy unit, the preparations lose activity on handling, and on storage in the deep freeze at −25°. The higher specific
activities obtained with plasmin, as compared to plasminogen, would agree with the splitting off of a peptide during the activation process (21, 22) with subsequent removal of the inactive material. The best preparations have been purified 33-fold relative to the acid extract of Fraction III2,3 (see Table II). Urokinase cannot be detected by enzymatic methods in any of the plasmin preparations. Urokinase is not adsorbed under the chromatographic conditions described above. Rechromatography of the plasmin under the same conditions did not increase its purity.

Trypsin-activated plasmin was chromatographed by adsorption and elution methods on DEAE-Sephadex columns. In a typical experiment (Fig. 5) plasmin was adsorbed in 0.05 M Tris-0.02 M lysine, pH 9.0, and eluted with the Tris-lysine buffer containing 0.15 M NaCl. The residual trypsin, which was coprecipitated or adsorbed during isoelectric precipitation of the plasmin, was not adsorbed under these conditions, whereas the plasmin was quantitatively adsorbed. The plasmin was eluted by increasing the ionic strength to 0.22 with NaCl. This ionic strength was higher by 0.05 than that used for the starting agent development chromatography of plasmin prepared by urokinase activation. The highest specific activity obtained in a single fraction was 8.3 units per absorbancy unit. A pool of Fractions 95 to 106 gave a preparation with a specific activity of 7.0 units per absorbancy unit with a recovery of 40% of the enzyme chromatographed. This method will separate unbound or coprecipitated trypsin from plasmin.

Specific Activity Parameters: Relationship Between Absorbancy, Protein Nitrogen, and Solids—The specific activity of plasminogen and plasmin, prepared by chromatography of modified Kline method preparations, has been defined in terms of units per absorbancy unit. The best preparations of plasminogen and plasmin, after dialysis at 4°, at pH 3.7, for 72 hours, were analyzed for total nitrogen by micro-Kjeldahl, and for total solids, by drying from the frozen state. The analytical data obtained on the preparations are summarized in Table IV. The best

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**Table IV**

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Dialyzed</th>
<th>Nondialyzed</th>
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<tbody>
<tr>
<td></td>
<td>Solids</td>
<td>Total Nitrogen</td>
</tr>
<tr>
<td></td>
<td>mg/ml</td>
<td>mg/ml</td>
</tr>
<tr>
<td>Plasminogen 46</td>
<td>8.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Plasminogen 63A1</td>
<td>2.4</td>
<td>0.38</td>
</tr>
<tr>
<td>Plasminogen 63B1</td>
<td>2.0</td>
<td>0.49</td>
</tr>
<tr>
<td>Plasmin 47</td>
<td>3.4</td>
<td>0.62</td>
</tr>
<tr>
<td>Plasmin 65</td>
<td>1.4</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* At 280 μm; in 0.01 N HCl.
† Conversion by factors obtained after dialysis of preparations.
‡ The A fraction is the ascending and the B fraction is the descending portion of the plasminogen peak obtained by chromatography on DEAE-Sephadex.
plasminogen preparations contained 15.7 to 16.9% nitrogen whereas the best plasmin preparations contained 14.3 to 15.3% nitrogen. The extinction coefficient, $E_{\text{abs}}$, was approximately 20 for both plasminogen and plasmin. The plasminogen preparations had a specific activity of 9.0 to 10.0 units per absorbancy unit, or 18.2 to 19.5 units per mg, or 108 to 123 units per mg of nitrogen. The plasmin preparations had a specific activity of 9.3 to 11.0 units per absorbancy unit, or 18.6 to 21.4 units per mg, or 130 to 140 units per mg of nitrogen. There appear to be some differences in the specific activity parameters on analysis of the ascending and descending portions of the plasminogen peak as seen in plasminogen No. 63A and 63B (Table IV). Although the specific activities defined in terms of units per absorbancy unit or units per mg of solids are similar, the specific activity defined in terms of units per mg of nitrogen is different.

A comparison of these specific activities with those published by other investigators necessitates the use of other conversion factors for proteolytic activity. The Klune and Fishman (16) and Sgouris et al. (7) factors would increase the specific activity of both plasminogen and plasmin.

**Purification Factors**—The purification factor for both plasminogen and plasmin relative to plasma and serum calculated from the various specific activity parameters is about 900. The plasminogen concentration of both human plasma and serum was found to be the same, approximately 1.7 units per ml, determined by methods described by Alkjaersig, Fletcher, and Sherry (23) who obtained similar values. These data confirm the observations of Fantl (24) that serum has the same amount of plasminogen as plasma. The value is a measure of the streptokinase-activatable proteolytic activity only. Plasma and serum both contain a nearly equal amount of active protease. The best plasminogen preparations contain small amounts of active protease, about 4.9% of the total potential proteolytic activity. The specific activity of plasminogen in plasma or serum is approximately 0.023 units per mg of protein, whereas the specific activity of the best preparations of plasminogen and plasmin is about 20 units per mg. This would give a purification factor of close to 900, relative to plasma or serum. As already described, the purification factor for plasminogen is 30-fold, and the purification factor for plasmin is 33-fold, relative to plasma Fraction IIIa.

**Physical Characterization of Plasminogen and Plasmin**—Electrophoretic analyses of plasminogen (No. 46) and plasmin (No. 47), chromatographed on DEAE-Sephadex, were carried out in a pH 2.9 glycine buffer, ionic strength of 0.16. The electric field strength was 5.5 volts per cm and the time was 120 minutes. The patterns are shown in Fig. 6 and the data obtained from these patterns are summarized in Table V. These studies show that highly purified proenzyme and enzyme are heterogeneous, with two peak patterns. They both show the same component, between 75 and 80%, calculated from an average of both ascending and descending patterns. The mobility of this major component was 9.7 x 10^{-5} cm² per volt per second, as average descending values. A minor, slower moving component, between 20 and 25%, was present with a mobility of 8.6 to 8.9 x 10^{-5} cm² per volt per second, as average descending values.

Sedimentation analyses were made on DEAE-Sephadex chromatographed plasminogen (No. 46) and plasmin (No. 47) in 0.001 N HCl containing 0.1 M NaCl, pH 2.8, at 20°. The sedimentation constants were corrected to water ($\bar{s}_{20,w}$). Plasminogen (Fig. 7) and plasmin were homogeneous with sedi-
gmentation constants of 3.9 S and 4.4 S, respectively, uncorrected for protein concentration. Sedimentation analysis of gel-filtered plasminogen, with a specific activity of 8.3 units per absorbancy unit, was also found to be homogeneous but with a sedimentation constant of 4.4 S, uncorrected for protein concentration.

**Gel Diffusion and Immunoelectrophoresis**—Gel diffusion and immunoelectrophoretic methods were used to study and characterize plasminogen and plasmin prepared by the various methods described above. The purified proenzyme and enzyme preparations studied were plasminogens No. 63A (0.33% protein), No. 63B (0.39% protein), No. 75 (0.20% protein; 6.7 units per

![Fig. 8. Immunoelectrophoretic pattern of plasminogen and plasmin with antiplasmin serum No. 65. Top well, plasminogen No. 63A; bottom well, plasmin No. 65.](image)

![Fig. 9. Gel diffusion analysis of plasmin and plasminogen. Left slide, antiplasmin serum No. 65: Well 1, plasmin No. 65; Well 2, plasminogen No. 63A; Well 3, plasmin No. 65; Well 4, plasminogen No. 75; Well 5, plasmin No. 65; Well 6, plasminogen No. 63B. Right slide, antiplasminogen serum No. 75: Well 1, plasminogen No. 75; Well 2, plasminogen No. 63A; Well 3, plasminogen No. 75; Well 4, plasmin No. 65; Well 5, plasminogen No. 75; Well 6, plasminogen No. 83.](image)
FIG. 10. Left slide, gel diffusion analysis of different plasmins with antiplasmin serum No. 65. Well 1, urokinase-activated plasmin, crude, 0.36% protein; Well 2, streptokinase-activated plasmin, crude, 0.67% protein; Well 3, plasminogen No. 66; Well 4, trypsin-activated plasmin, crude, 0.66% protein. Right slide, absorbancy unit), and No. 83 (0.36% protein, 7.3 units per absorbancy unit), and plasmin No. 65 (0.17% protein). Plasminogens No. 75 and No. 83 were prepared by direct chromatography of Fraction III,3 extracts on DEAE-Sephadex.

Immunoelectrophoretic studies on the highly purified preparations show that plasminogen and plasmin are β-globulins (Fig. 8). The proenzyme and enzyme prepared by chromatography of the modified Kline method material give single, strong, broad bands in the β- and γ-globulin zones. Plasminogen differs from plasmin by appearing in the β-2 region whereas plasmin is a β-1 globulin. The same results are obtained with either antiplasminogen or antiplasmin sera. Proenzyme and enzyme are identical on Ouchterlony analysis (Fig. 9). They appear to be immunochemically homogeneous. Plasminogen prepared by the direct column chromatography method gives a strong, broad band in the β-2 region but the band is not as long as that observed with the chromatographed, modified Kline method preparation and, in addition, light β-1 and γ-globulin bands could be seen. Plasminogens prepared either by the direct chromatographic method or by chromatography of modified Kline method preparations are identical on Ouchterlony analysis (Fig. 9). Also, in gel diffusion, urokinase-activated plasmin is identical to streptokinase-activated and trypsin-activated plasmins (Fig. 10). Streptokinase-activated plasmin, containing activator, has the same electrophoretic mobility, gel diffusion characteristics, and immunoelectrophoretic behavior as the other types of plasmin. The major antigenic determinant groups of these plasmins, prepared by activation of plasminogen with three different types of activators, appear to be the same.

The antihuman plasminogen and antihuman plasmin sera react with both undiluted human plasma and serum but not with fibrinogen, indicating minor antigenic components in plasminogen and plasmin which are not detectable in the plasminogen or plasmin immunoelectrophoretic patterns. The rabbit and horse antihuman serum sera react only with directly chromatographed plasminogen (Fig. 10). The antihuman fibrinogen serum reacts only with the directly chromatographed plasminogens, showing a small, weak, single band in the β-2 region.

DISCUSSION

Plasminogen and plasmin of varying degrees of purity have been prepared by other investigators (1-14) with a variety of methods with different types of plasma and serum fractions. The availability of Fractions III,3 in large quantities made this preparation the starting material of choice in the development of a new method for the preparation of plasminogen. The Kline method (3) was modified at the last step to include an isoelectric precipitation of the proenzyme at pH 6.0 with NaH₂PO₄. This modification gave plasminogen preparations in a concentrated form, with good yields.

The introduction of Sephadex (25) and DEAE-Sephadex (26), as new tools in protein separation problems made possible the development of new methods for preparing highly purified plasminogen and plasmin. Gel filtration of plasminogen, prepared by the modified Kline method, through Sephadex gave preparations with a 2.6-fold increase in purity, whereas gel filtration of plasmin, prepared by activation of plasminogen with
urokinase, in 25% glycerol, gave preparations with a 1.3-fold increase in purity. The gel filtration method removed a group of impurities which were of higher molecular weight and which came through the columns as the first major peak. The gel filtration method is an excellent procedure for further purifying plasminogen, but not plasmin. Since this method separates proteins of different molecular sizes, it is useful in separating out both higher and lower molecular weight impurities. This method may also be used to prepare the proenzyme and enzyme in a salt-free form. Slotta, Michl, and Santos (27) recently reported on the use of Sephadex G-25 to desalt and partially fractionate plasminogen.

The use of chromatographic methods resulted in plasminogen and plasmin of a purity equal to or better than that reported by others (1-14). Also, the report by Kline and Fishman (15) that plasmin can be prepared with a specific activity higher than that found for plasminogen has been confirmed. The use of DEAE-Sephadex as the anion exchanger and the use of starting agent development conditions resulted in a simple method for preparing high purity plasminogen and plasmin. Once the chromatographic method was worked out for plasminogen, it was possible to use the same conditions for plasmin. Finally, it was possible to directly chromatograph Fraction III, extracts giving preparations of a higher specific activity than those obtained with the modified Kline method. The direct chromatographic method was a very simple procedure for preparing high purity preparations from extracts of Fraction III, without subjecting plasminogen to the acid and alkaline conditions used in the modified Kline method. The chromatographic method gave specific activities which were 3-fold over the best specific activities obtained with the modified Kline method. Also, the method was reproducible and the yields were excellent. Both DEAE-cellulose (8, 10, 13, 14, 28) and DEAE-Sephadex (29) have been used to separate and purify plasminogen from various starting materials.

The method for measuring proteolytic activity used in this study is a modification of the Remmert and Cohen method. Unfortunately, all investigators using the Remmert and Cohen method do not use the same conditions for assay. The casein unit described in this paper is equal to approximately 1.4 Kline and Fishman casein units (16) and 1.1 National Institutes of Health tentative standard casein units and 1.1 Sgouris et al. casein units (7) and 2.5 Alkjaersig, Fletcher, and Sherry (30) casein units. Other investigators (3, 10) have defined the casein unit by other parameters. The comparison of specific activities of preparations reported in the literature is difficult because of the differences in measurement of proteolytic activity and definition of unit.

The most highly purified plasminogen preparation described in this paper appears to be similar in purity only to the preparation recently described by Kline and Fishman (4) and prepared by an improvement in the Kline method. However, there are several real differences between the two preparations, particularly on ultracentrifugal and electrophoretic analyses. In this study, it was found that the specific activity of plasmin was higher than the specific activity of plasminogen. Also, plasminogen was found to contain more nitrogen than plasmin. The splitting off of high nitrogen-containing peptides such as lysine and arginine could account for this difference. The nitrogen values were similar to those reported by Shulman, Alkjaersig, and Sherry (31), but Kline and Fishman (4) reported a lower value, 14.9%, for plasminogen. An unusual difference between plasminogen and plasmin as prepared by the methods described in this paper and the preparations reported by Shulman, Alkjaersig, and Sherry (31) is in the extinction coefficient, \( \varepsilon^\text{plasmin}_\text{cm}^{-1} \text{mM}^{-1} \), namely 20 as compared to 14.

The purification factor, relative to plasma Fraction III, was 30 fold for plasminogen whereas for plasmin it was 33 fold. The purification factors, relative to plasma or serum, were close to 900. Purification factors between 135 and 600 have been reported (1, 4-6, 10).

The homogeneity of plasminogen and plasmin was evaluated by physical and immunological methods. The plasminogen and plasmin preparations prepared by chromatography on DEAE-Sephadex and the plasminogen prepared by gel filtration on Sephadex were found to be homogeneous on ultracentrifugal analysis at pH 2.8. The sedimentation constant, \( s_{20,w} \), was found to be 3.9 S for chromatographed plasminogen, 4.4 S for gel-filtered plasminogen, and 4.8 S for chromatographed plasmin. The sedimentation constant for chromatographed plasminogen may be close to 4.4 when corrected for protein concentration since the \( s_{20,w} \) for plasminogen is dependent on protein concentration (31, 32). Ultracentrifugal analysis does not appear to be decisive as a criterion of purity since the specific activities of the preparations varied between 17 and 21 units per mg. Other investigators (4, 5, 31) also found a single molecular species on ultracentrifugal analysis with specific activities much less than that reported in this paper. Electrophoretic analysis of plasminogen and plasmin, at pH 2.9, showed two peaks, the major component constituting between 75 and 80% of the total. The mobility of the major component was 9.7, whereas the mobility of the minor component was 8.6. Both plasminogen and plasmin gave the same mobilities for major and minor components. The two components may represent multiple molecular forms of the enzyme. The separation of the two species by electrophoretic methods may help to resolve the problem. Kline and Fishman (4) reported that electrophoretic analysis was not decisive as a criterion of purity since preparations of varying degrees of purity showed single peaks. Electrophoretically homogeneous preparations have been obtained in other studies (31, 32).

Gel diffusion and immunoelectrophoretic methods were also used to determine purity and, in addition, to characterize the proenzyme and enzyme. Plasminogen and plasmin were found to be \( \beta \)-globulins, appearing in both the \( \beta \)- and \( \gamma \)-zones as single, strong, broad bands. Plasminogen differed from plasmin by appearing in the \( \beta\-\gamma \) region whereas the plasmin appeared in the \( \beta \)-1 region. Both proenzyme and enzyme prepared by chromatography of modified Kline method preparations were found to be homogeneous by immunoelectrophoresis and identical by Ouchterlony analysis. Plasminogen prepared by direct chromatography of plasma Fraction III extracts showed \( \beta \)-1 and \( \gamma \) impurities but was otherwise identical to plasminogen and plasmin obtained by chromatography of modified Kline method materials. The long, broad, band found with all preparations was similar to that found with \( \gamma \)-globulin, which may be due to a smoothly changing series of electrophoretically different molecular species. This could account for the two-component system found in the electrophoretic studies. Plasmins prepared by activation of plasminogen with either urokinase or trypsin or...
streptokinase are identical by Ouchterlony analysis and show the same immunoelectrophoretic behavior. Both proenzyme and enzyme contain minor plasma antigenic components since the antiplasminogen and antiplasmin sera react with undiluted plasma and serum. Broad spectrum rabbit and horse antihuman serum sera react only with plasminogen prepared by direct chromatography of plasma Fraction III2,3 extracts, indicating the high degree of purity of the proenzyme and enzyme preparations prepared by chromatography of modified Kline method materials. None of the proenzyme and enzyme preparations contain fibrinogen. Rybak (9) found by immunoelectrophoretic analysis two zones in the β-globulin region with nearly the same mobility. Hagan, Ablondi, and De Renzo (5) also found two bands on Ouchterlony analysis of preparations chromatographed on carboxymethyl-cellulose. Rife, Milgrom, and Shulman (33) recently reported that plasminogen and plasmin preparations give two zones in the β region and one zone in the γ region, with the activity residing in the β components. The proenzyme and enzyme preparations prepared from modified Kline material are homogeneous by ultracentrifugal, gel diffusion, and immunoelectrophoretic analyses. Since the electrophoretic studies indicate heterogeneity, it is possible that either the agar electrophoretic technique could not separate the two components or that they are immunologically identical.

SUMMARY

Plasminogen can be prepared in a highly purified form from human plasma Fraction III2,3 by a modification of the Kline method followed by either gel filtration through Sephadex columns or chromatography on DEAE-Sephadex columns, with starting agent development conditions. The major modification of the Kline method was to precipitate the plasminogen, in the final step, at pH 6.0, with NaH₂P0₄. Plasminogen, of the same purity, can also be prepared by directly chromatographing extracts of plasma Fraction III2,3 on DEAE-Sephadex columns. The methods are simple, reproducible, and with excellent yields. Plasmin, prepared by activation of the proenzyme with trace quantities of urokinase in glycerol, can be isolated in a high state of purity by chromatography on DEAE-Sephadex columns. Plasminogen and plasmin have been prepared with specific activities of 19.5 and 21.4 casein units per mg of protein or 123 and 140 casein units per mg of nitrogen, respectively. The purification factors were 30- and 33-fold, respectively, relative to plasma Fraction III2,3 and approximately 900-fold relative to plasma or serum. The proenzyme and enzyme were found to be homogeneous in the ultracentrifuge and by immunoochemical methods but are heterogeneous on electrophoretic analysis. On immunoelectrophoresis in agar, they give single, strong, broad bands in the β-globulin region. Plasminogen appears in the β 2 region whereas plasmin appears in the β 1 region. Gel diffusion (Ouchterlony) analysis shows identity between proenzyme and enzyme.

REFERENCES

Purification of Human Plasminogen and Plasmin by Gel Filtration on Sephadex and Chromatography on Diethylaminoethyl-Sephadex
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