Multiplicity of Rabbit Plasminogen. Physical Characterization†

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ABSTRACT: Two fractions of rabbit plasminogen have been isolated by specific affinity chromatography on Sepharose 4B-L-lysine solid supports. Each fraction migrated as a single but distinct component on polyacrylamide gel electrophoresis at pH 4.3 and each was resolved into five activatable components by polyacrylamide gel electrophoresis at pH 9.5. Examination of the isozyme pattern in the original fractions by isoelectric focusing demonstrated that the first fraction eluted from the affinity columns consisted of five isozymes with an isoelectric pH range of 6.20–7.78. The second fraction eluted from the affinity columns also consisted of five isozymes but possessed an isoelectric pH range of 6.95–8.74. The native molecular weight of each plasminogen fraction was 88,000 ± 3000 and the subunit molecular weight was 86,000 ± 3000. These results demonstrate that rabbit plasminogen consists of a single polypeptide chain. The amino acid compositions and number of amides in each original fraction were also indistinguishable. Further, on activation to plasmin by urokinase each plasminogen fraction was converted to a two-chain plasmin structure. The molecular weights of the component chains were 62,000 ● 3000 and 24,000 ± 1800 and were cross-linked in plasmin by disulfide bond(s). Studies on streptokinase activation demonstrated that each fraction of rabbit plasminogen is streptokinase sensitive with the component plasmin polypeptide chains indistinguishable in molecular weight to those produced by urokinase activation.

Plasminogen is a protein which is found in the plasma of all species tested and is the zymogen which is converted to plasmin (EC 3.4.4.14); the enzyme which functions physiologically in the lysis of the fibrin clot. The mechanism of activation of human plasminogen by proteases such as urokinase, trypsin, and tissue activators has been extensively studied by Robbins and coworkers (Summaria *et al.*, 1967a; Robbins *et al.*, 1967). They conclude that activation proceeds *via* cleavage of an arginyl–valine bond in plasminogen, resulting in the conversion of the single-chain plasminogen molecule to a two-chain plasmin structure, stabilized by one disulfide bond. Recently, the mechanism of the conversion of human plasminogen to plasmin by streptokinase, a protein possessing no inherent proteolytic activity, has been described by Reddy and Markus (1972).

The results of a survey conducted by Wulf and Mertz (1969) demonstrated that the streptokinase induced activation of plasminogen was species specific. Only the plasminogens of man, monkey, and cat plasmas were activated by catalytic quantities of streptokinase. Higher quantities of streptokinase were required for activation of dog and rabbit plasminogens. Urokinase activated the plasminogens of all species equally well (Wulf and Mertz, 1969). However, these studies were very qualitative in nature and complicated by the fact that the streptokinase preparations, as well as all of the plasminogens used, were highly impure.

Robbins and coworkers have elucidated most of the physical and chemical properties of human plasminogen, however these data are lacking on other species of plasminogen which exhibit variable sensitivities to streptokinase activation. During our investigations with various plasminogens we have noticed that there is considerable multiplicity in the structure of this plasma protein. This has been previously observed for human plasminogen (Deutsch and Mertz, 1970; Wallén and Wiman, 1970; Summaria and Robbins, 1970) and canine plasminogen (Heberlein and Barnhart, 1968). We wish to report in this manuscript the nature of the multiplicity of plasminogen which is representative of that found in rabbit plasma as well as some interesting aspects of its purification, properties and streptokinase sensitivity.

Experimental Procedure

Preparation of Rabbit Plasminogen. This procedure is a modification of the Deutsch and Mertz (1970) method for isolating human plasminogen. The starting material for the purification was whole citrated rabbit plasma. A quantity of phenylmethylsulfonyl fluoride in ethanol was added in order to give a 0.001 M solution of the inhibitor in plasma. The Sepharose 4B-L-lysine columns were prepared by adding a solution containing 20 grams of cyanogen bromide dissolved in 100 ml of water to 100 ml of previously washed Sepharose 4B (Pharmacia) at room temperature. The solution was stirred and maintained at pH 11.0 by repeated additions of 4 N NaOH. When the base uptake ceased, the resin was rapidly washed under suction with 2 l. of cold 0.1 N $NaHCO_3$. A solution containing 20 g of L-lysine in 100 ml of 0.1 M phosphate, final pH 9.2 at 5°, was then added to the resin. The suspension was stirred gently at 5° overnight. The resin was washed with 0.3 M phosphate and packed into a 2.5-cm diameter column. All subsequent affinity chromatography operations were performed at room temperature.

Approximately 300 ml of rabbit plasma was placed over a 2.5 cm × 20 cm column of Sepharose 4B-L-lysine equilibrated with 0.3 M phosphate, pH 8.2, and washed with the same solvent until the absorbance of the eluate at 280 nm was essentially zero. At this point a solution of 0.1 M phosphate–0.1 M 6-aminohexanoic acid was passed through the column at a flow rate of 30 ml/hr. Approximately 5-ml fractions were collected. This step removed the retarded

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plasminogen in a single peak. This material was then concentrated by ultrafiltration at 4°. A small volume (4 ml) of this solution was then passed through a 2.5 × 75 cm column of Sephadex G-100, equilibrated with 0.1 M NH_4HCO_3 at 4° and developed with the same solvent at a flow rate of 10 ml/hr. Approximately 3-ml fractions were collected. This step served to separate a small impurity on the frontal side of the main plasminogen fraction. The tubes containing plasminogen were pooled and again passed through a 2.5 cm × 20 cm column of Sepharose 4B-L-lysine equilibrated with 0.3 M phosphate, pH 8.2. This column was developed with a linear gradient consisting of 200 ml of 0.1 M phosphate, pH 8.2, as the starting solvent and 200 ml of 0.1 M phosphate–0.025 M 6-aminohexanoic acid, as the limit solvent. Approximately 3-ml fractions were collected at a flow rate of 20 ml/hr. The active fractions were separately pooled, concentrated by ultrafiltration at 4° and dialyzed against 0.05 M Tris·HCl, pH 8.0, prior to further use.

Polyacrylamide Gel Electrophoresis. Analytical gels were performed at pH 4.3 (Reisfield *et al.*, 1962), pH 9.5 (Davis, 1964), and molecular weight estimations were performed on 7% gels in SDS[1] (Weber and Osborn, 1969) using appropriate molecular weight markers.

Isoelectric focusing experiments were performed using an LKB 110 ml column with LKB carrier ampholytes. The procedure was essentially that recommended by the manufacturer with a few exceptions. The electrode solutions were 0.4 ml of concentrated phosphoric acid, 28 ml of H_2O, and 24 g of sucrose for the anode, and 0.1 g of NaOH and 10 ml of H_2O for the cathode. After adding the anode solution a linear gradient was prepared. The starting solvent consisted of 1.88 ml of pH 3–10 ampholines in 40% sucrose, 40.12 ml of H_2O and 28 g of sucrose. The limit solvent consisted of 0.63 ml of pH 3–10 ampholines in 40% sucrose dissolved in 59.37 ml of H_2O. The gradient was layered directly above the anode solution with the aid of a peristaltic pump. After approximately 15 ml of the gradient emerged, 1 ml of the protein solution (7 mg/ml) was added directly to the starting solvent. This was done to avoid contact of the protein with the electrode solution. The remainder of the gradient was then applied. At this point a few extra milliliters of the limit solution was added, followed by the cathode solution. The run was then started and allowed to proceed for 48 hours at 4°. At the conclusion of the run, 1-ml fractions were collected from the column and the tubes were analyzed for protein by absorbance at 280 nm. pH determinations on each tube were performed at 22° with a Radiometer 26 pH meter, employing the expanded scale capabilities of the instrument. Plasmin activities on each pool were determined after conversion of plasminogen to plasmin by urokinase.

Ultracentrifugation. Molecular weights of native plasminogen were performed on well-dialyzed preparations in 0.05 M Tris·HCl, pH 8.0 at 20°, by the meniscus depletion technique of Yphantis (1964). Rotor speeds of 20,000 rpm and column heights of 3 mm were used. Traces of the protein distribution at equilibrium were obtained with scanner optics on the Beckman Model E analytical ultracentrifuge at 280 nm. The protein concentrations in each run were of the order of 0.1 mg/ml. The results were calculated in the usual manner (Yphantis, 1964). Partial specific volumes of each plasminogen fraction were calculated from the amino acid compositions.

Sedimentation coefficients ($s^0_{20,w}$) were also performed with ultraviolet scanner optics at 20° and 280 nm. The protein was dialyzed against the desired buffer and diluted with the final dialysate to a concentration of approximately 0.2 mg/ml. The sedimentation coefficient was calculated in the usual manner and corrected to the density and viscosity of H_2O at 20° (Schachman, 1959).

Plasmin assays were performed on column eluates by removing a sufficient aliquot of the desired fraction such that approximately 25 μg of plasminogen was present. Tris·HCl buffer (0.1 M) was added to each tube in order to adjust the volumes at this stage to 0.2 ml. Urokinase (20 μl; 180 Plough units) was placed in each assay sample and the tubes were incubated at 30° for 10 min. Tos-Arg-OMe (30 μl; 5 μmoles) was then added to the incubation mixture and the tubes were allowed to further incubate for 10 min. The amount of remaining Tos-Arg-OMe was assayed by modifying Hestrin's (1949) procedure as we have previously described (Brockway and Castellino, 1971). Control experiments in the absence of urokinase, in the absence of plasminogen, and in the absence of all proteins were performed. Specific activities (V_{max}) were performed in the usual manner.

Amino Acid Analyses. The amino acid compositions of rabbit plasminogen fractions 1 and 2 were determined by hydrolyzing 0.5-mg samples of the protein in 6.0 N HCl for 24, 48, and 72 hr at 110° and performing the analysis on a Beckman 117 amino acid analyzer. Duplicate samples were analyzed at each time interval.

The total half-cystine content of each protein was determined as cysteic acid by analyzing 6.0 N HCl hydrolysates of samples previously treated with performic acid (Moore, 1963). The tryptophan content of each sample was determined by the method of Edelhoch (1967) and the sulfhydryl content was determined by Ellman's (1959) method.

The total amide content (glutamine + asparagine) of each plasminogen fraction was determined by measuring the amount of glycine incorporated into the free carboxyl groups of the proteins by amino acid analysis. The carboxyl groups of each plasminogen were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in the presence of glycine methyl ester (Hoare and Koshland, 1967). Each plasminogen, as well as all reagents, were dissolved in 8 M urea. Samples were withdrawn at various times and diluted 5-fold with 1 M sodium acetate, pH 4.75. The samples were then exhaustively dialyzed against water and lyophilized. Blanks were run in the absence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in order to ensure that all unreacted glycine methyl ester was removed from the samples after dialysis. The proteins were then hydrolyzed and subjected to amino acid analysis. The number of glutamine + asparagine residues present in the sample is equal to the total of the aspartic and glutamic acid residues obtained by amino acid analysis of the blanks minus the glycine incorporated into the modified samples. One additional glycine was subtracted due to incorporation into the carboxy-terminal residue.

Streptokinase Sensitivities. Stock solutions of rabbit plasminogens, streptokinase, and 0.05 M Tris·HCl, pH 8.0, were employed in this procedure. Rabbit fraction 1 from affinity chromatography was prepared at 6.95 mg/ml and rabbit fraction 2 from affinity chromatography was prepared at 10.8 mg/ml, each in Tris buffer. Streptokinase was diluted to either 100,000 units/ml or 1250 units/ml. Incubation solutions were prepared by adding 0.120 ml of plasminogen, 0.040 ml of Tris buffer, and 0.100 ml of streptokinase for fraction 1 and 0.075 ml of plasminogen, 0.080 ml of Tris buffer, and 0.100 ml of streptokinase for fraction 2. Each solution was incubated

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; Tos-Arg-OMe, α-*N*-tosyl-L-arginine methyl ester.

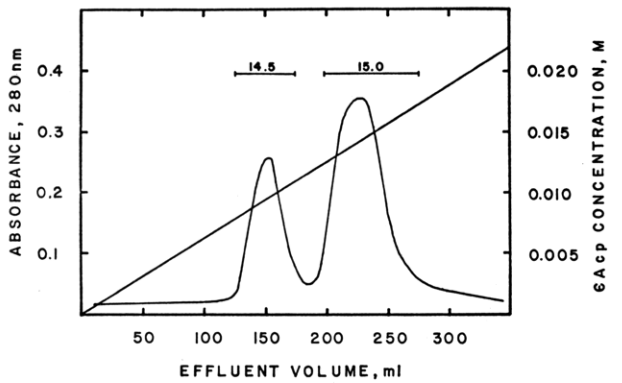

FIGURE 1: Elution profile of rabbit plasminogen produced by gradient elution from Sepharose 4B–L-lysine affinity columns. The absorbance at 280 nm and the concentration of 6-aminohexanoic acid (ε-Acp), as a function of the effluent volume are presented on the graph. The numbers above each peak represent the specific activities of each pool toward Tos-Arg-OMe after conversion of the plasminogen to plasmin by urokinase. The activity units are μmoles of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of plasminogen originally added prior to activation.

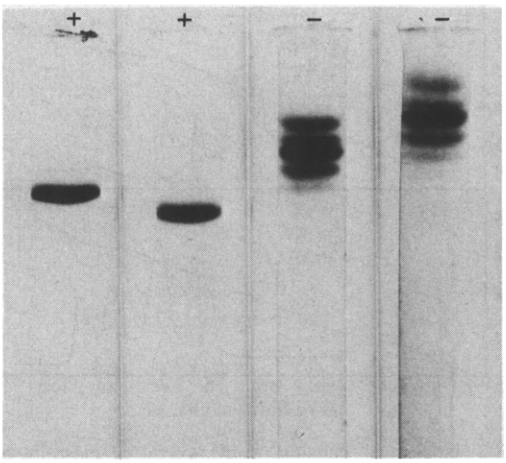

FIGURE 2: Polyacrylamide gel electrophoretograms of rabbit plasminogen. Left to right: rabbit fraction 1, pH 4.3; rabbit fraction 2, pH 4.3; rabbit fraction 1, pH 9.5; rabbit fraction 2, pH 9.5. Fraction 1 and fraction 2 refer to the elution positions in Figure 1. Electrode polarities are indicated on each gel.

at 30°. At desired intervals, 0.010 ml of the incubation mixture was added to a solution containing 0.190 ml of Tris buffer and 0.050 ml of a 0.05 M Tos-Arg-OMe solution. This mixture was incubated for 10 min at which time a 0.200-ml aliquot was removed for assay of the remaining Tos-Arg-OMe, as described above. Control experiments in the absence of plasminogen, in the absence of streptokinase, and in the absence of all proteins were continually performed. Experiments were also performed with various levels of streptokinase in the same manner.

Concurrent with the withdrawing of samples for Tos-Arg-OMe assays, samples were removed for monitoring conversion of plasminogen into plasmin by SDS gel electrophoresis. For these experiments 0.015-ml aliquots were taken from the plasminogen–streptokinase mixtures and rapidly frozen in a Dry Ice–ethanol bath. The samples were then lyophilized and dissolved in SDS protein buffer (Weber and Osborn, 1969)–5% 2-mercaptoethanol. The solutions were incubated overnight at 37° and were analyzed by SDS polyacrylamide gel electrophoresis.

Materials. Whole rabbit blood was either withdrawn by us in the laboratory or purchased from Pel-Freez or Research Products, Inc. In all cases the blood was 5% in sodium citrate.

Streptokinase was highly purified by Dr. Hugo Nihlen of AB Kabi, Stockholm, Sweden and very generously donated by him. This material exhibited no independent proteolytic activity and produced 1 major and 1 very minor band on SDS gel electrophoresis. The specific activity was approximately 100,000 units/mg which is equivalent to the highest specific activity prepared to date (DeRenzo *et al.*, 1967).

Urokinase was purchased from Calbiochem and diluted to 9000 Plough units/ml.

All polyacrylamide gel electrophoresis reagents were purchased from Canalco, Inc., and all electrofocusing reagents were purchased from LKB. Other reagents were the best commercially available.

Results

The profile obtained with the gradient elution of plasminogen bound to Sepharose 4B–L-lysine columns is presented in Figure 1. Clearly two peaks are obtained, both of which are activatable to plasmin by urokinase. The first two steps of the purification (*i.e.*, batch elution of plasminogens from Sepharose 4B–L-lysine and Sephadex G-100 chromatography) are relatively routine and elution profiles are not presented. The overall combined yield of plasminogen is approximately 70% in terms of both protein and total activity from plasma in this purification scheme.

Figure 2 shows examples of polyacrylamide gel electrophoresis at pH 4.3. Each isolated fraction migrates as a single component but possesses a distinct electrophoretic mobility. Figure 2 also presents examples of polyacrylamide gels performed at pH 9.5. Each apparently homogeneous component at pH 4.3 is separated into five subfractions at pH 9.5. As is evident, the electrophoretic distribution of the subforms obtained from the two original fractions is also distinct at pH 9.5 although the more anodic migrating subforms of fraction 2 overlap the more cathodic migrating subforms of fraction 1. At this stage, gel slicing experiments indicated that all subforms from each fraction were activated to plasmin by urokinase.

In order to further characterize the subforms of each affinity chromatography isolated plasminogen, preparative isoelectric focusing experiments were performed. Profiles from these experiments are shown for each original plasminogen fraction in Figure 3. Both fraction 1 and fraction 2 are composed of five activatable subforms. The isoelectric points of the subforms are listed in Table I. Polyacrylamide gel electrophoresis photographs of each subform at pH 9.5 are shown in Figure 4. These gels were run after exhaustively dialyzing each subform against water and lyophilizing the contents of the dialysis tubing.

In an attempt to elucidate possible structural differences between the two plasminogen fractions and to further characterize the general properties of rabbit plasminogen we have performed other physical and chemical measurements. Molecular weights of native plasminogen were performed by analytical ultracentrifugation. Plots of the natural logarithm of the change in absorbance *vs.* the square of the radial distance from the center of rotation for each plasminogen fraction were linear at several speeds, indicating reasonable homogeneity in the molecular weights of the isozymes within each fraction.

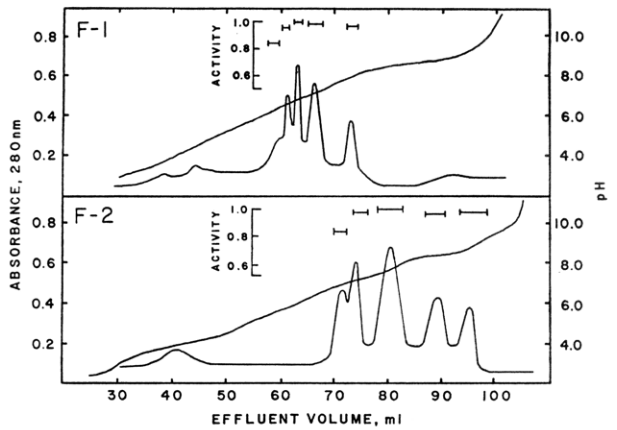

FIGURE 3: Isoelectric focusing profiles of rabbit plasminogen. Top: rabbit fraction 1 focused at 4° on a pH 3–10 gradient. The absorbance at 280 nm, the experimental pH gradient, and activities of each pool after dialysis, concentration and activation to plasmin by urokinase are indicated on the graph. Specific activities are indicated on the whole pool on the insert, relative to the central fraction having a specific activity of 1.0, by a bar above each fraction. The V_{max} of the central fraction was 13.4 ± 0.4 μmoles of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of plasminogen originally added prior to activation. Bottom: as top except that rabbit fraction 2 was employed. The V_{max} of the central fraction was 13.7 ± 0.4 μmoles of Tos-Arg-OMe cleaved min^{-1} mg^{-1} of plasminogen originally added prior to activation.

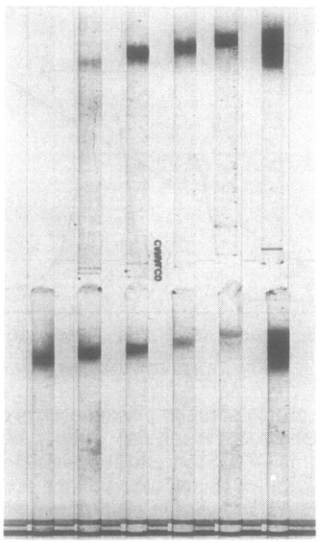

FIGURE 4: Polyacrylamide gel electrophoretograms at pH 9.5 of each isozyme obtained after pooling and concentrating the peaks from Figure 3. Intact fraction 1 and 2 are also shown for illustration. Top: fraction 1 isozymes; left to right, pH 6.56 isozyme, pH 6.85 isozyme. pH 7.24 isozyme, pH 7.78 isozyme, and intact fraction 1. The pH 6.20 isozyme was not isolated in pure form. Bottom: fraction 2 isozymes; left to right, pH 6.95 isozyme, pH 7.18 isozyme, pH 7.89 isozyme, pH 8.42 isozyme, pH 8.74 isozyme and intact fraction 2.

Similar data were obtained for each diisopropyl fluorophosphate inhibited native plasmin. Subunit molecular weights of each plasminogen were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and photographs of the results are presented in Figure 5. Evaluation of molecular weights from these gels were performed according to the method suggested by Weber and Osborn (1969) using as molecular weight standards; human plasminogen, bovine serum albumin, catalase, ovalbumin, rabbit muscle aldolase, malate dehydrogenase, trypsin, and bovine α-lactalbumin. Bomophenol Blue was used as a marker dye.

Sedimentation coefficients of each plasminogen fraction were determined and similar results to those previously reported were obtained (Brockway and Castellino, 1972). The gross alteration in the $s^0_{20,w}$ induced by 6-aminohexanoic acid was also observed, as before. We have titrated the alteration in the $s^0_{20,w}$ with 6-aminohexanoic acid in order to determine the dissociation constant of this fibrinolytic inhibitor to each plasminogen fraction (Brockway and Castellino, 1972). A summary of these physical measurements is given in Table II.

The amino acid composition of each plasminogen fraction is given in Table III. All values represent the average of 24-, 48-, and 72-hr hydrolysis times except serine and threonine which were extrapolated to zero time and valine, isoleucine, and leucine which are given by the 72-hr hydrolysis

TABLE 1: Isoelectric Points of the Isozymes of Rabbit Plasminogen at 22°.

	p*I*	
Isozyme[a]	Rabbit Fraction 1[b] ±0.1	Rabbit Fraction 2[b] ±0.1
1	6.20	6.95
2	6.56	7.18
3	6.85	7.89
4	7.24	8.42
5	7.78	8.74

[a] In the numbering of isozymes the more anodic migrating species is given the lower number (*Biochemistry 10*, 4825 (1971)). [b] Fraction 1 and 2 for rabbit plasminogen is identified solely by its elution position from the Sepharose 4B-L-lysine columns with a 6-aminohexanoic acid gradient.

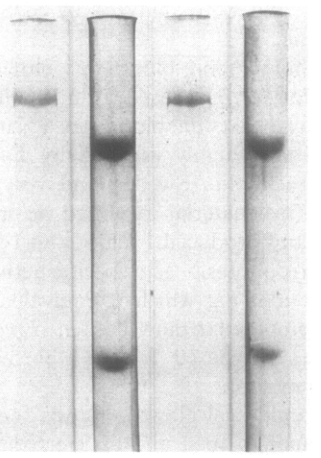

FIGURE 5: Polyacrylamide gel electrophoretograms in sodium dodecyl sulfate of rabbit plasminogens and urokinase activated rabbit plasmins. Molecular weights of the bands were evaluated by concurrently analyzing the molecular weight standards listed in Results. The procedure was that of Weber and Osborn (1969) in the presence of mercaptoethanol. Left to right, rabbit plasminogem fraction 1, rabbit plasmin fraction 1, rabbit plasminogen fraction 2, rabbit plasmin fraction 2.

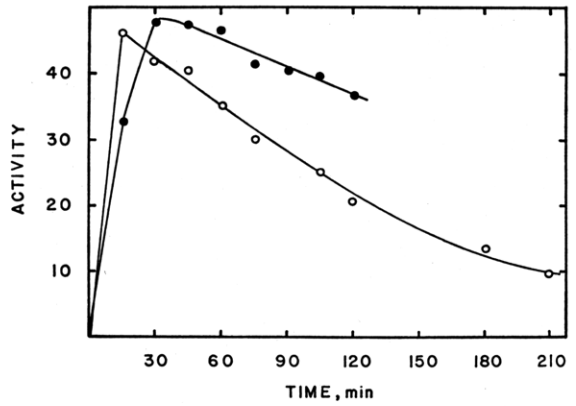

FIGURE 6: Tos-Arg-OMe activity produced with time upon incubating rabbit plasminogen with streptokinase (1 SK:5 Pg). (○) Rabbit plasminogen fraction 1; (●) rabbit plasminogen fraction 2. The activity units are μmoles of Tos-Arg-OMe cleaved 10 min^{-1} mg^{-1} of plasminogen in the original aliquot.

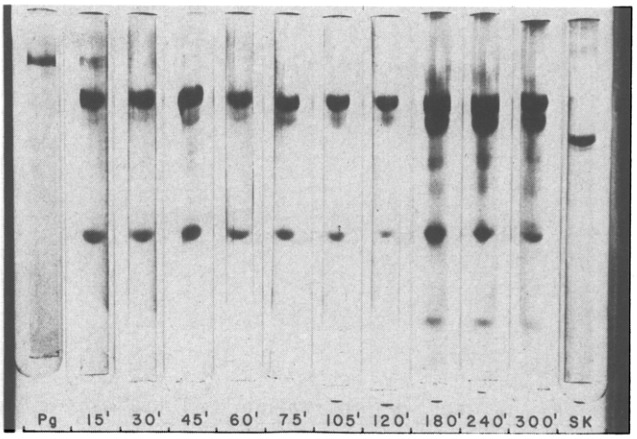

FIGURE 7: Sodium dodecyl sulfate gel electrophoretic photographs of rabbit plasminogen fraction 1 (Pg) as a function of the indicated times of incubation with 10,000 units of streptokinase (SK). The mole ratio is approximately 1 SK:5 Pg.

times. Tryptophan values were determined in 6 M guanidinium chloride by the method of Edelhoch (1967) and average values of 19 residues/molecule were obtained for each plasminogen. The total half-cystine determined after performic acid treatment was 48 residues/molecule. Titrations of each plasminogen with Ellman's reagent showed that no free sulfhydryl residues were present. The total free carboxyl residues of each plasminogen, as determined by glycine incorporation, was found to be approximately 70 residues/molecule at time periods which indicated that glycine incorporation was complete.

In order to test whether rabbit plasminogen is activated by streptokinase we have incubated the two proteins and removed aliquots for Tos-Arg-OMe assays and sodium dodecyl sulfate gel electrophoresis. Time studies on the activation at 10,000 units (0.100 mg) of streptokinase/0.83 mg of each plasminogen (1:5 molar ratio) were performed. Aliquots were taken for Tos-Arg-OMe assays at various times on each fraction and the results are shown in Figure 6. Parallel exper-

TABLE II: Physical Properties of Rabbit Plasminogen.

Parameter	Rabbit Fraction 1	Rabbit Fraction 2
Native plasminogen Molecular weight[a]	87,000 ± 3000	88,000 ± 3000
Subunit plasminogen Molecular weight[b]	86,500 ± 3000	86,000 ± 3000
Native plasmin Molecular weight[a]	84,000 ± 3000	85,300 ± 3000
Subunit plasmin (+ mercaptoethanol) Molecular weight[b]	62,000 ± 3000 24,000 ± 1800	61,500 ± 3000 23,500 ± 1800
$s^0_{20,w}$ native (−6-amino-hexanoic acid) (S)	5.45 ± 0.06	5.48 ± 0.06
$s^0_{20,w}$ native (+6-amino-hexanoic acid) (S)	4.63 ± 0.05	4.60 ± 0.05
Dissociation constant for 6-aminohexanoic acid (M)	1.89 ± 0.1 × 10^{-3}	3.12 ± 0.2 × 10^{-3}

[a] Determined by ultracentrifugation at 20°. [b] Determined by sodium dodecyl sulfate gel electrophoresis.

TABLE III: Amino Acid Compositions of Rabbit Plasminogen Fractions 1 and 2.

Amino Acid	Calcd No. of Residues/Molecule ± 5% Rabbit[a] Fraction 1	Rabbit[a] Fraction 2
Lysine	53	50
Histidine	21	20
Arginine	55	54
Aspartic acid	80	78
Threonine	58[b]	57[b]
Serine	58[b]	58[b]
Glutamic acid	84	83
Proline	58	58
Glycine	56	57
Alanine	49	50
Valine	32[c]	34[c]
Methionine	9	10
Isoleucine	22[c]	23[c]
Leucine	40[c]	41[c]
Tyrosine	30	32
Phenylalanine	18	19
Cysteic acid	48	49
Total amides	72[d]	69[d]
Tryptophan	19[d]	19[d]
Total residues	790	792

[a] The results were calculated on the basis of a molecular weight of 88,000 for each rabbit fraction. [b] Determined by extrapolation to zero hydrolysis time. [c] Determined by the 72-hr hydrolysis time. [d] Determined separately as described in the text.

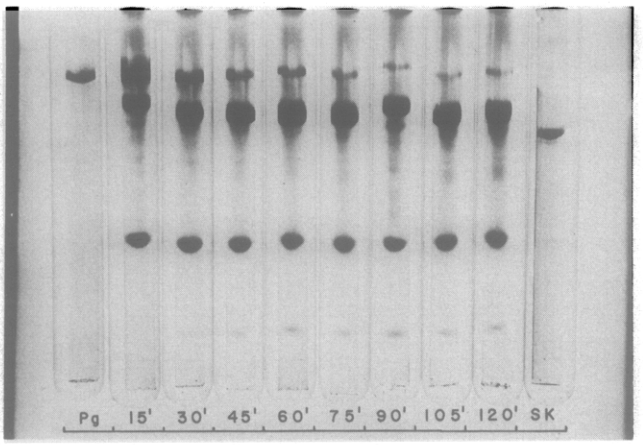

FIGURE 8: As in Figure 7 except that rabbit plasminogen fraction 2 was used.

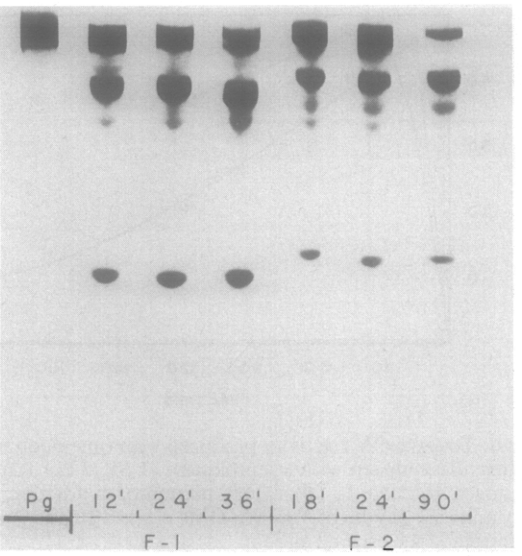

FIGURE 9: Examples of sodium dodecyl sulfate gel electrophoretic patterns of rabbit plasminogen at various times of incubation with 125 units of SK (1 SK:320 Pg). F-1 and F-2 refer to rabbit plasminogen fraction 1 and rabbit plasminogen fraction 2, respectively.

iments utilizing sodium dodecyl sulfate gel electrophoresis were performed and the results are shown in Figure 7 for fraction 1 and in Figure 8 for fraction 2. We have also performed various experiments at $^1/_2$ to $^1/_{500}$th of the above levels of streptokinase and, as expected, activation becomes slower. Representative examples of the sodium dodecyl sulfate gel electrophoretic behavior of plasminogen fractions 1 and 2 incubated with very low levels of streptokinase (1:320 molar ratio) for various times are shown in Figure 9.

Discussion

We have demonstrated in this study that two major fractions of plasminogen can be isolated from rabbit plasma by specific affinity chromatography. The fact that two major plasminogen fractions existed in human plasminogen has been earlier demonstrated by starch block electrophoresis (Wallen, 1962). Since the ligands used in this affinity chromatography technique, *i.e.*, 6-aminohexanoic acid and L-lysine, are inhibitors of the plasminogen to plasmin conversion, added significance is attached to these findings. The implication derived is that the plasminogen which exists in the plasma is not homogeneously susceptible to inhibition by these agents. It should be emphasized that a purification technique such as this lends assurance to the fact that the plasminogen isolated is representative of the plasminogen found in the plasma. If standard techniques for purifying plasminogen, which involve acid precipitation, isoelectric precipitation, *etc.*, had been used, it is doubtless that some of the fractions and subfractions found in this study would have escaped detection due to solubility differences under these conditions.

Since plasma contains many different types of proteases, it can be argued that the two forms isolated are perhaps artifacts of the manner in which the blood was handled and arise from proteolysis of one of the fractions. This is especially valid in studies with commercial blood, in which the history of the blood is even more variable. On the other hand, the artifactual nature of the two forms can be argued against, in what follows. The same purification results are obtained whether fresh plasma or commercial plasma is used; it does not seem to matter whether or not a proteolytic inhibitor is added to the plasma prior to purification; the technique for purification is very rapid and involves no extremes in environmental conditions of the plasma. Further evidence can be implied from data concerning the subforms of each original fraction. Gel scanning experiments always indicate the same distribution of subfractions in several different plasminogen preparations. This distribution in each case appears to be Gaussian. If proteolysis occurred on one of the fractions, it seems unlikely that such a reproducible pattern in the product should be obtained, independent of the history of the blood. Although the above evidence is speculative, it requires consideration.

Polyacrylamide gel electrophoresis at pH 4.3 indicated that each fraction migrated as a single component but each possessed a distinct electrophoretic mobility. Upon performing these experiments at pH 9.5 each fraction was resolved into five separate subforms. In order to rule out the possibility that the pH 9.5 data was an artifact produced by the high pH employed, preparative isoelectric focusing experiments were performed on sucrose density columns. Each fraction of plasminogen possessed five activatable subforms by this technique. When each subform was separately applied to pH 9.5 gels, it migrated as a single component and aligned with one of the bands produced in the original fraction. Refocusing the bands on narrower pH gradients did not result in interconversion of isozymes. Further, upon repeated freezing and thawing, temperature and pH alterations, one subform was not converted to any other. Although the structural basis of the multiplicity is not obvious at this time, current nomenclature rules require that the subforms be classified as isozymes. Therefore, rabbit plasma contains two types of plasminogen and each plasminogen consists of at least five isozymes. Recently, Summaria *et al.* (1972) have isolated eight isozymes from human plasminogen. The p*I* range of the human isozymes was 6.4–8.5, clearly compatible with the values obtained in this manuscript. However, their number of isozymes is minimal since these authors did not initially separate the two fractions which also exist in human plasminogen (Brockway and Castellino, 1972).

Molecular weight studies on native plasminogen by analytical ultracentrifugation indicate that each major rabbit plasminogen fraction possesses a molecular weight of approximately

88,000, a value slightly higher than the value for human plasminogen (Barlow *et al.*, 1969). This same value was obtained for each plasminogen by sodium dodecyl sulfate gel electrophoresis in the presence of mercaptoethanol, suggesting a single subunit for rabbit plasminogen. Upon activation of rabbit plasminogen by urokinase the molecular weight decreased slightly to 84,000 for each fraction. However this change in itself is inconclusive in terms of an activation mechanism since at these molecular weights the difference is within experimental limits. Studies of plasmin, produced by urokinase activation of each plasminogen, by sodium dodecyl sulfate gel electrophoresis in the *absence* of mercaptoethanol, indicate that initially a single species is obtained. Visually, the molecular weight appears smaller than plasminogen, however, an accurate value cannot be assigned due to the dependence of this method on the presence of mercaptoethanol. Upon addition of mercaptoethanol to the plasmin, molecular weights of 62,000 and 24,000 are obtained. There is no remaining plasmin or plasminogen. These studies indicate that as a final consequence of urokinase activation the single chain plasminogen molecule is cleaved to a two-chain plasmin molecule stabilized by disulfide bond(s). Further, we have noted an additional peptide(s) in the vicinity of the marker dye which may be an activation peptide(s).

The $s^0_{20,w}$ values for each native plasminogen appear to be in line with what would be expected from a reasonably globular molecule of this size and this value is greatly decreased upon binding of the fibrinolytic inhibitor, 6-aminohexanoic acid. We have reported this observation earlier (Brockway and Castellino, 1972) and we wish to report here that we have titrated the alteration in the $s^0_{20,w}$ of each rabbit plasminogen fraction with 6-aminohexanoic acid in order to obtain binding (dissociation) constants. The binding of this amino acid appeared to be stronger to fraction 1 than to fraction 2, as would be expected from their elution positions on affinity chromatography, using this amino acid as the eluent. Further, the binding of 6-aminohexanoic acid to each rabbit plasminogen fraction is weaker than its binding to either human or duck plasminogen (Brockway and Castellino, 1972).

The amino acid composition of each plasminogen is not striking except that they are very similar to each other. There is a high number of proline residues in each, which would not lead one to expect a high percentage of α helix in these proteins.

Each molecule is highly cross-linked by approximately 24 disulfide bonds and there are no free sulfhydryl groups present. Surprisingly, the number of amides in each fraction is identical, within the expected precision, apparently ruling this out as a possible basis for the multiplicity in structure of rabbit plasminogen. The amino acid difference index (Metzger *et al.*, 1968) between rabbit fraction 1 and *human* plasminogen is 7.04 and the value for rabbit fraction 2 and *human* plasminogen is 6.61, indicating a high degree of compositional relatedness between rabbit and human plasminogen.

Finally, we have studied the streptokinase sensitivity of rabbit plasminogen since there appears to be conflicting reports on this subject. Wulf and Mertz (1969) indicated that, qualitatively, rabbit plasminogen is weakly susceptible to streptokinase activation whereas Kline and Bowlds (1971) and Davidson (1960) report that streptokinase is inactive with this species. We have correlated the kinetics of activation, as determined by Tos-Arg-OMe hydrolysis, with physical evidence for activation, as determined by sodium dodecyl sulfate electrophoretic analysis. We find that streptokinase, at four different catalytic levels, is indeed capable of activating rabbit plasminogen. As can be seen from Figures 7 and 8, fraction 1 is completely activated by streptokinase (1:5 molar ratio) whereas fraction 2 is almost completely activated. There appears to be a double band in Figure 7 in the remaining plasminogen at the 15-min incubation times. However, this is difficult to see in the reproduction. This behavior could be due to one of the isozymes possessing a smaller plasminogen molecular weight, undetected by other techniques and only visible after the bulk of the plasminogen is activated. It is also possible that this behavior could be a result of non-specific proteolysis in the activation scheme. The rate of appearance of this double band in plasminogen fraction 1 is more evident in Figure 9 due to the slower activation rate. This behavior is not noted with plasminogen fraction 2, as can be seen from Figures 8 and 9. In further experimentation with streptokinase levels we have found that very small quantities of streptokinase (1:320) do activate human plasminogen more rapidly than rabbit plasminogen lending credence to the early qualitative observations of Wulf and Mertz (1969). However, catalytic amounts of streptokinase definitely function in rabbit plasminogen, as well as in human plasminogen.

Further examination of Figures 6–8 allow some conclusions to be made concerning the inactivation of rabbit plasmin. At lengthy periods of incubation, as plasmin loses its Tos-Arg-OMe activity, it can be observed that marked autodigestion of plasmin is not occurring. The light chain remains relatively intact whereas the heavy plasmin chain appears to undergo proteolysis to a slightly smaller species (55,000 daltons molecule^{-1}). Although the loss in plasmin activity may be due more to conformational alterations than to autodigestion, it appears that the autodigestion which does take place is relatively specific, occurring initially in the heavy chain of plasmin.

Also, at all periods of incubation with the two indicated levels of streptokinase, a small peptide(s) is visible in the vicinity of the dye marker lending some credibility to earlier observations on urokinase activation which suggested that an activation peptide(s) is released at some stage of the conversion of plasminogen to plasmin.

In summary, there is a complex distribution of plasminogens in rabbit plasma. At this point there is no obvious structural reason(s) for this distribution. We have elucidated and partially characterized this multiplicity and are currently involved in determining its possible structural basis and significance.

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Characterization of Two Forms of Beef Heart Cyclic Nucleotide Phosphodiesterase†

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ABSTRACT: A method has been developed to isolate two forms of cyclic nucleotide phosphodiesterase from beef heart. Using a 13,500*g* supernatant fraction as the starting point, the method involves ammonium sulfate fractionation, protamine precipitation, Sephadex G-200 gel filtration, and DEAE-cellulose chromatography. The total enzymatic activity could be accounted for at all stages in the procedure. There were no indications that a cofactor, protein or otherwise, was removed during the isolation. Although the two forms of phosphodiesterase could not be effectively separated by gel filtration, they could be resolved upon DEAE-cellulose into two active species, designated phosphodiesterase I and II. Phosphodiesterase I, representing 70–80% of the the total activity recovered from DEAE-cellulose, has been purified more than 700-fold. This form has a molecular weight of approximately 121,500 g/mol as determined by sedimentation equilibrium measurements. Both enzymes catalyzed the formation of 5′-mononucleotides from the two naturally occurring cyclic nucleotides, cyclic 3′,5′-adenosine monophosphate and cyclic 3′,5′-guanosine monophosphate, although the former was the preferred substrate of both forms. The K_m for cyclic 3′,5′-adenosine monophospate of the major fraction I is 3.6×10^{-5} M; that of the minor fraction II is 6.9×10^{-5} M. Both forms of the enzyme exhibited several active bands on polyacrylamide disc electrophoresis. Although the two forms of the enzyme have been obtained reproducibly upon DEAE-cellulose chromatography by both stepwise and gradient elution techniques, preliminary considerations do not preclude the possibility that the two forms may represent a complex distribution of a single enzymatic species.

Much research concerning the metabolism of cyclic 3′,5′-adenosine monophosphate (cAMP[1]) has centered upon its synthesis from ATP by the enzyme adenylate cyclase (Sutherland *et al.*, 1962; Robison *et al.*, 1970; Levey, 1970; Klainer *et al.*, 1962). However, the extent and duration of a cAMP-induced response also depends upon cyclic nucleotide phosphodiesterase, the enzyme which catalyzes the hydrolysis of cAMP to AMP. Phosphodiesterase has now been studied in heart (Butcher and Sutherland, 1962; Nair, 1966; Beavo *et al.*, 1970; Goren and Rosen, 1971), brain (Cheung, 1970, 1971; Kakiuchi *et al.*, 1971; Thompson and Appleman, 1971a,b); liver (Menahan *et al.* 1969), slime molds (Chang, 1968; Murray *et al.*, 1971), and frog erythrocytes (Rosen, 1970). How-

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[1] Abbreviations used are: cAMP, cGMP, cIMP, cUMP, cTMP, and cCMP for cyclic 3′,5′-adenosine, guanosine, inosine, uridine, thymidine, and cytidine monophosphates, respectively.