Different Molecular Forms of Plasminogen and Plasmin Produced by Urokinase in Human Plasma and their Relation to Protease Inhibitors and Lysis of Fibrinogen and Fibrin

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Urokinase-activated human plasma was studied by gel electrophoresis, gel filtration, crossed immunoelectrophoresis and electroimmunoassay with specific antibodies and by assay of esterase and protease activity of isolated fractions. Urokinase induced the formation of different components with plasminogen+plasmin antigenicity. At low concentrations of urokinase, a component with a K_D value of 0.18 by gel filtration and post β_1 mobility by gel electrophoresis was detected. The isolated component had no enzyme or plasminogen activity. In this plasma sample fibrinogen was not degraded for 10 h, but when fibrin was formed, by addition of thrombin, fibrin was quickly lysed, and simultaneously a component with a K_D value of 0 and α_2 mobility appeared, which was probably plasmin in a complex with α_2 macroglobulin. This complex showed both esterase and protease activity. After gel filtration with lysine buffer of the clotted and lysed plasma another two components were observed with about the same K_D value by gel filtration as plasminogen (0.35), but β_1 and γ mobilities by gel electrophoresis. They appeared to be modified plasminogen molecules, and possibly plasmin with γ mobility. Similar processes occurred without fibrin at higher urokinase concentrations. Here a relatively slow degradation of fibrinogen was correlated to the appearance of the plasmin- α_2 macroglobulin complex. The fibrin surface appeared to catalyse the ultimate production of active plasmin with a subsequent preferential degradation of fibrin and the formation of a plasmin- α_2 macroglobulin complex. The gel filtration and electrophoresis of the plasma protease inhibitors, α_1 antitrypsin, inter- α -inhibitor, antithrombin III, and C_1 -esterase inhibitor indicated that any complex between plasmin and these inhibitors was completely dissociated. The β_1 and post β_1 components appear to lack correlates among components occurring in purified preparations of plasminogen and plasmin.

Plasmin (EC 3.4.21.7) is a serine protease originating from a precursor, plasminogen, present in animal blood plasma. The physiological function of plasmin is the degradation and dissolution of fibrin deposits in the blood vessels. Urokinase (EC 3.4.99.26) is an activator of plasminogen excreted in mammalian urine. It resembles trypsin and plasmin in its activity against low-molecular-weight substrates, but attacks only one protein, plasminogen (White & Barlow, 1970). Studies on purified plasminogen have added to the knowledge of the molecular changes involved in its activation by urokinase and other activators (Summaria *et al.*, 1967, 1973; Rickli & Otavsky, 1973; Wiman & Wallén, 1973). Urokinase is now under extensive trial as a thrombolytic agent, administered intravenously to patients (Sasahara *et al.*, 1973).

Little is known about the sequence of events that occurs when plasminogen is activated in its natural environment, plasma, by biological activators. Immunochemical techniques combined with gel electrophoresis and gel filtration have been used to study this problem. Niléhn & Ganrot (1967) studied

human plasma during intravenous streptokinase therapy, and found that plasmin was predominantly bound to α_2 macroglobulin but not to α_1 antitrypsin. Müllertz (1972a) studied human fibrinolytic post-mortem plasma. It contained no plasminogen but did contain the plasmin- α_2 macroglobulin complex and another component with plasminogen antigenicity. Complexes with the protease inhibitors, α_1 antitrypsin and inter- α -inhibitor, were not detected. Preliminary studies (Müllertz, 1972b) showed a similar pattern in normal human plasma after addition of urokinase.

Experimental

Materials

Buffers for gel electrophoresis and gel filtration are described below. GE-Tris buffer: Tris, 200 mmol/l; calcium lactate, 2 mmol/l; HCl, 52 mmol/l; pH 8.6; I 0.058. GE-lysine buffer: lysine hydrochloride, 50 mmol/l; calcium lactate, 2 mmol/l; NaOH,

1 mol/l; pH 8.6; I 0.056. GE-barbiturate buffer: sodium barbiturate, 64 mmol/l; barbituric acid, 11 mmol/l; calcium lactate, 2 mmol/l; pH 8.6; I 0.070. GF-Tris buffer: Tris-HCl, 75 mmol/l; NaCl, 75 mmol/l; NaN_3 , 3 mmol/l; NaOH, 1 mol/l; pH 7.6; I 0.15. GF-lysine buffer: Tris, 100 mmol/l; lysine hydrochloride, 50 mmol/l; NaCl, 20 mmol/l; HCl, 1 mol/l; pH 7.6; I 0.15.

Agarose was obtained from L'Industrie Biologique Française S.A., Genevilliers, Seine, France. Sephadex G-200 and Blue Dextran (relative molecular mass, $M_r = 2 \times 10^6$) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Casein, Hammarsten, from E. Merck A.-G., Darmstadt, W. Germany, was prepared as described by Müllertz (1955) and dissolved in Tris-HCl buffer, pH 7.6. α -N-Benzoyl-L-arginine ethyl ester hydrochloride and α -N-benzoyl-L-arginine hydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.) were dissolved in Tris-HCl buffer, pH 7.6.

Specific rabbit immune globulins against human α_2 macroglobulin, α_1 antitrypsin and human serum protein, dissolved in NaCl (0.1 mol/l)- NaN_3 (0.015 mol/l), were obtained from Dakopatts, Copenhagen, Denmark, and the volume fraction in agarose gel was 0.017. For specific rabbit immune serum against C_1 -esterase inhibitor and inter- α -inhibitor (from Beringwerke, Marburg Lahn, W. Germany), the volume fraction was 0.015 and 0.012 respectively and for that against antithrombin III (from Nyegaard and Co., Oslo, Norway), the volume fraction was 0.025. Sera reacting specifically with human plasminogen and plasmin (volume fraction 0.01) were produced and controlled as described by Müllertz (1972a).

Bovine protease inhibitor (Trasylol, from Bayer, Leverkusen, W. Germany), 5×10^6 kallikrein inhibitor units/l (Vogel *et al.*, 1968), was dissolved in NaCl (0.15 mol/l). 6-Aminohexanoic acid (ϵ -aminocaproic acid) was obtained from Fluka A.-G., Chemische Fabrik, Buchs, Switzerland. Urokinase 'Reagent' was from Leo, Copenhagen, Denmark, with approx. 7700 Ploug units/mg equivalent to 10000 CTA units/mg (Kok & Astrup, 1969). The specific activity data and molecular mass of purified urokinase (White & Barlow, 1970) indicated that 5500 CTA units are approximately equivalent to 1 nmol. Bovine thrombin (Thrombine, from Hoffman-La Roche, Basel, Switzerland) contained 50 NIH units/mg. The two reagents were dissolved in NaCl (0.15 mol/l).

Plasma and serum were from healthy human subjects. Plasma was obtained from blood stabilized by EDTA (dipotassium salt; 4 mmol/l of blood). Serum was obtained from blood left at room temperature for spontaneous clotting. The samples were used at once or stored at -20°C .

Plasminogen was prepared by the method of Deutsch & Mertz (1970) essentially as described by

Wiman & Wallén (1973) from out-dated human donor plasma. The preparation only contained traces of other proteins as controlled by crossed immunoelectrophoresis in agarose gel with immune globulins against human serum protein. It contained 143.2 mg of protein (10 ml) and $87.2 \pm 1.2 \mu\text{mol}$ (mean $\pm$ S.E.M.; $n = 2$ regressions) of activatable plasminogen as determined by active-site titration with *p*-nitrophenyl-*p*-guanidinobenzoate (Chase & Shaw, 1969) and $96.4 \pm 2.4 \mu\text{mol/l}$ (mean $\pm$ S.E.M.; $n = 2$ regressions) by titration with bovine protease inhibitor (Christensen & Müllertz, 1974) after activation in glycerol as described below. After gel electrophoresis it showed β_2 - γ mobility with GE-Tris buffer.

Plasmin was prepared by complete activation of plasminogen by traces of urokinase, i.e. at a molar ratio of plasminogen/urokinase of approx. 1×10^3 at a plasminogen concentration of approx. $50 \mu\text{mol/litre}$ of water and under conditions at which plasmin is known to be stable (lysine, 0.2 mol/l; pH 7.6; glycerol, 6.8 mol/l; 4°C ; 48 h). This preparation was used (diluted) for the recovery experiments. To avoid stabilizing agents other than lysine in the preparation used in the stability experiments, plasmin was produced by activation of plasminogen without glycerol under the following conditions: plasminogen, approx. $22 \mu\text{mol/l}$; urokinase, approx. $1.4 \mu\text{mol/l}$; lysine, 0.2 mol/l; pH 7.6; 25°C ; 15 min. After gel electrophoresis at pH 8.6, plasmin showed γ mobility, moving slightly cathodically with GE-lysine buffer, and slightly anodically with GE-Tris buffer.

The concentration of plasminogen in normal human plasma was reported to be $204 \pm 35 \text{ mg/l}$ (mean $\pm$ S.D.) by radioimmunoassay (Rabiner *et al.*, 1969), which is equivalent to $2.5 \pm 0.4 \mu\text{mol/l}$, with an M_r of 81 000 for the plasminogen used as standard.

Methods

Fibrinogen concentration. This was determined as described by Blombäck & Blombäck (1956).

Immunochemical techniques. The concentration of the plasma protease inhibitors and plasminogen+plasmin was determined by electroimmunoassay in agarose gel containing specific antibodies, as described by Laurell (1972). Crossed immunoelectrophoresis was performed in agarose gel with GE-barbiturate buffer, essentially as described by Ganrot (1972). Radial immunodiffusion was done essentially as described by Clausen (1969) in agarose gel.

Analytical gel filtration. This was done in Sephadex G-200 at $3.0 \pm 0.5^\circ\text{C}$ and the column parameters were determined as described by Müllertz (1972a). Both GF-lysine and GF-Tris buffers were used for equilibration and elution. Lysine stabilizes any plasmin present, whereas Tris has no such effect. On the other hand, lysine is known to interact both with plasminogen and plasmin, whereas Tris is neutral in this respect.

Preparative gel filtration. This was done essentially as the analytical gel filtration. The column (Pharmacia, K 100/100) was $1.00\text{m} \times 7.85 \times 10^{-3}\text{m}^2$ with a total volume of 6 litres. The sample volume was 150ml, the flow rate was 60ml/h. The processing time was 72h.

Analytical agarose-gel electrophoresis. This was performed essentially as described by Johansson (1972). GE-barbiturate buffer was used.

Preparative agarose-gel electrophoresis. The procedure described by Johansson (1972) was modified as follows. Agarose gel (0.5g/l) was used in a 3mm thick layer and troughs ($5\text{mm} \times 20\text{mm} \times 3\text{mm}$; length $\times$ breadth $\times$ depth) were cut out of the gel. The material was mixed with equal volumes of preheated, liquid agarose gel (1g/l, temperature of gelation, 37°C) on a water bath at 40°C , and then $300\mu\text{l}$ of the mixture was quickly added to the troughs by using a constriction pipette. The (small) deficit occurring by the pipetting of the viscous solution was determined and corrected for. The mean temperature in the gel, measured by a thermistor probe, was about 15°C . Bromphenol Blue was added to all plasma specimens as an albumin marker to ensure almost identical distances of migration of the proteins in the preparations separated. The localization of various components was carried out by performing a crossed immunoelectrophoresis on 2mm-broad bars of gel cut out of the gel containing the separated proteins. During the procedure the electrophoretic preparations were kept at 4°C in a moist chamber. Crossed immunoelectrophoresis performed on bars cut out in the longitudinal and transverse direction at zero time and after 24h at 4°C proved that no noticeable change of distribution of plasminogen+plasmin antigenic components took place in the gel during 24h at 4°C . The section that corresponded to a component was cut out of the gel. The gel sections were weighed and comminuted by a motor-driven sharp stainless-steel propeller, and centrifuged at $18000g$ at 4°C for 30min. The supernatant gel liquid (about 0.5 of the total volume) was used for the assay. The dilution was calculated from the mass of the gel section, the density of the gel and the volume of the material applied.

Determination of enzymic activity. Benzoylarginine ethyl esterase activity was determined at 37°C as described by Worning & Müllertz (1966). The final solution of the assay contained in Tris-HCl buffer: benzoylarginine ethyl ester, 0.67mmol/l ; lysine, 33mmol/l (lysine expts.); pH 7.6, $I 0.15$. Caseinolytic activity was determined as described by Müllertz (1955), except in the experiments in Table 6 where the method described by Lauritsen (1968) was used. The final solution of the assay contained in Tris-HCl buffer: casein, 15g/l ; lysine, 17mmol/l (lysine expts.); pH 7.6, $I 0.15$. Lysine had no inhibitory effect at the applied concentrations. This was shown by measuring the enzyme activity of dilutions (1/20) prepared from

a stock solution of plasmin in lysine (0.1mol/l). The final lysine concentrations were 95, 45, 20 and 5mmol/l . The activity did not increase significantly with decreasing lysine concentration.

The gel liquid obtained from the preparative gel electrophoresis was mixed with 0.1 vol. of appropriate solutions to give the final values: Tris, 182mmol/l ; lysine, 45mmol/l ; calcium lactate, 1.8mmol/l ; pH 7.6. Plasminogen was determined by assaying the increase of enzyme activity after addition of an excess of urokinase.

Preparation of urokinase-activated plasma samples. Human plasma was diluted to 1.15 vol. as described below. Sample A: plasma+NaCl soln. (0.15mol/l). Samples B and C: plasma+urokinase. Samples Bcl and Ccl: plasma+urokinase+thrombin (cl, clotted and lysed). Samples B and Bcl contained urokinase, 22×10^3 Ploug units/l (approx. 5nmol of enzyme/l), and samples C and Ccl contained 104×10^3 Ploug units/l (approx. 25nmol of enzyme/l). Taking the plasminogen concentration of normal plasma to be approx. $2.5\mu\text{mol/l}$, the final molar ratio of urokinase/plasminogen was approx. 2×10^{-3} and 1×10^{-2} . The final concentration of thrombin in samples Bcl and Ccl was 9×10^3 NIH units/l. Samples Bcl and Ccl clotted in 8 and 30s and lysed in 1.8 and 0.6ks (30 and 10min) respectively. After different periods of time at 37°C samples were removed for analysis. In the experiments shown in Plate 1 only, $11\mu\text{mol}$ of bovine protease inhibitor/litre was added to prevent changes caused by plasmin.

Preparation of concentrated pooled fractions from gel filtration of plasma. The results of the gel-filtration experiments suggested the development of two (in sample B) and three (in samples Bcl and C) components with plasminogen+plasmin antigenicity (Fig. 1). The appearance of the immunoprecipitate peaks differed distinctly, enabling a division of the fractions into three pools, one comprising most of the 19S fractions with α_2 macroglobulin and benzoylarginine ethyl esterase activity (samples Bcl and C), another comprising the early half of 7S fractions ($7S_1$) and the third comprising the late half of 7S fraction ($7S_2$). The fractions from plasma without urokinase (A) were divided into three similar pools according to their K_D values. The $7S_2$ pool (A) contained almost all plasminogen+plasmin antigenic material (plasminogen). The 19S, $7S_1$ and $7S_2$ pools were prepared by preparative gel filtration of samples A, B and Bcl (30min, 37°C) and C (5h, 37°C). For the experiments on electrophoretic mobility (Plates 4 and 5, and Table 2) the pooled fractions were concentrated in an Amicon stirred ultrafiltration cell, model 52, with a PM 10 membrane. For the experiments on enzymic properties the pooled fractions were freeze-dried in a freeze-drying apparatus (type CD from Heto, Birkerød, Denmark), dissolved in a small volume of water and desalted by gel filtration in

columns (Pharmacia K 15/30; $0.30\text{m} \times 177\text{mm}^2$) of Sephadex G-25 after a preliminary calibration with Blue Dextran and K_2CrO_4 . Fractions (5 ml) were collected and the E_{280} was measured. GF-lysine was used as the elution buffer. In all cases the final volume was adjusted to one-third of the original plasma volume.

Results

Transformations of plasminogen in plasma induced by urokinase

Samples of normal plasma and urokinase-activated plasma were prepared as described under 'Methods'. After defined periods of time samples were removed for analysis. The migration of the plasminogen+plasmin antigenic components was determined by crossed immunoelectrophoresis (Plate 1). Unactivated plasma (A) contained one component with β_2 mobility (plasminogen). In activated plasma (B and C) a pattern of two or three components with plasminogen+plasmin antigenicity gradually developed. In plasma with a high concentration of urokinase (C) a new component with post β_1 mobility developed, whereas the β_2 component decreased and almost disappeared after 18 ks (5h). After 1.8 ks (30min) a component with α_2 mobility appeared. At 36 ks (10h) most of plasminogen apparently had

been converted into the α_2 component and the post β_1 component had also decreased somewhat. In the sample with a low concentration of urokinase (B) a post β_1 component was apparent after 0.6ks and the corresponding peak increased until about 18 ks. In this case no α_2 component appeared within 36 ks (10h). Coagulation and subsequent fibrinolysis accelerated the transformations considerably, both at low and high urokinase concentrations (cf. Plates 1 and 2). Thus a distinct α_2 component developed in 1.8 ks in sample Bcl and in 0.6 ks in sample Ccl (Plate 2).

The immunoelectrophoresis of samples A, B, C, Bcl and Ccl was repeated five times on the samples kept at -20°C . A different plasma specimen gave similar results (see Plates 4 and 5).

The plasma samples were also studied by analytical gel filtration on Sephadex G-200. Two different specimens of normal plasma were analysed by using GF-Tris and GF-lysine buffers, i.e. four complete gel-filtration series each comprising four gel filtrations. The results were essentially similar. Each plasma was used to prepare the samples A, B and Bcl after 30min and C after 5h. The samples were kept at -20°C until gel filtration. After gel filtration the concentrations of protein, plasminogen+plasmin, plasma protease-inhibitors and the benzoylarginine ethyl ester concentration of the fractions were determined as described under 'Methods'.

Fig. 1. Gel-filtration distribution of plasminogen+plasmin, plasma protease-inhibitors and benzoylarginine ethyl esterase activity in unactivated and urokinase-activated plasma

Systems: A is the eluted material from a gel filtration on Sephadex G-200 of normal human plasma without urokinase (sample A); B is the eluted material from plasma with low urokinase concentration (sample B); Bcl is the eluted material from plasma with a low urokinase concentration after coagulation and lysis (sample Bcl). In all cases after an incubation time of 1.8 ks at 37°C . The concentration of the antigens in each fraction was determined by electroimmunoassay in agarose gel with the appropriate specific antibodies: $\circ$, protein; $\bullet$, α_2 macroglobulin; $\blacktriangle$, plasminogen plus plasmin; $\triangle$, inter- α -inhibitor; $\blacksquare$, α_1 antitrypsin. $\square$, Enzyme concentration of each fraction determined with benzoylarginine ethyl ester.

EXPLANATION OF PLATE I

Transformation of plasminogen in human plasma, induced by urokinase, demonstrated by crossed-gel immunoelectrophoresis

Systems: A, human plasma without urokinase (sample A); B, human plasma with low urokinase concentration (sample B); C, human plasma with high urokinase concentration (sample C); NP, normal human plasma. The incubation time (at 37°C) of the samples before assay is stated for each row on the Plate. Each sample (5 μ l) was subjected to a crossed-gel immunoelectrophoresis. The first dimension was with a potential gradient of 2 V/mm for 2 h; the second dimension was in agarose with antiplasminogen serum and a potential gradient of 0.3 V/mm for 17 h. The bottom panel shows the first-dimension electrophoresis of normal plasma.

EXPLANATION OF PLATE 2

Transformations of plasminogen in human plasma induced by urokinase, enhanced by fibrin, demonstrated by crossed immunoelectrophoresis

Systems: A, $t = 0$, human plasma without urokinase (sample A) after an incubation time of 0 ks; Bcl, $t = 1.8$ ks, and Bcl, $t = 72$ ks, human plasma with low urokinase concentration after coagulation and lysis (sample Bcl) after incubation times of 1.8 and 72 ks; Ccl, $t = 0.6$ ks, and Ccl, $t = 36$ ks, human plasma with high urokinase concentration after coagulation and lysis after incubation times of 0.6 and 36 ks. The samples were subjected to crossed immunoelectrophoresis. NP, First-dimension electrophoresis of normal plasma. For experimental conditions see legend to Plate 1.

EXPLANATION OF PLATE 3

Electrophoretic mobility of plasma protease inhibitors, and plasminogen + plasmin antigenic components demonstrated by crossed immunoelectrophoresis

Systems: urokinase-activated plasma sample Bcl. The samples (5 μ l) were subjected to crossed immunoelectrophoresis. The first dimension was with a potential gradient of 2 V/mm for 2 h and the second dimension was with a potential gradient of 0.3 V/mm for 17 h. The second-dimension electrophoresis was run into agarose gel (1) with anti-plasminogen serum, (2) with anti- α_2 macroglobulin immune globulin, (3) with both antibodies and (4) with anti- α_1 antitrypsin immune globulin and anti-inter- α -inhibitor serum, α_1 antitrypsin was most anodic. NP, First-dimension electrophoresis of normal plasma.

EXPLANATION OF PLATE 4

Electrophoretic mobility and relation to α_2 macroglobulin of plasminogen+plasmin antigenic components isolated by gel filtration with GF-Tris buffer

Systems: 19S-B, 7S₁-B, 7S₂-B and 19S-Bcl, 7S₁-Bcl, 7S₂-Bcl are the concentrated pooled fractions after gel filtration on Sephadex G-200 of samples B and Bcl; A, B and Bcl are the unmodified plasma samples without and with urokinase. Each sample (5 μ l) was subjected to crossed immunoelectrophoresis. The first dimension was with a potential gradient of 2 V/mm for 2 h, and the second dimension was in agarose gel with anti-plasminogen serum and anti- α_2 macroglobulin immune globulin and a potential gradient of 0.3 V/mm for 17 h. NP, First-dimension electrophoresis of normal plasma. The peaks with α_2 mobility are due to α_2 macroglobulin. Plasmin associated with α_2 macroglobulin is apparent as a half-bow precipitate inside the cathodic half of the α_2 macroglobulin peak. The other peaks are due to plasminogen+plasmin antigenic components.

EXPLANATION OF PLATE 5

Electrophoretic mobility and relation to α_2 macroglobulin of plasminogen+plasmin antigenic components isolated by gel filtration with GF-lysine buffer

Systems and symbols are as described in the legend to Plate 4.

Table 1. Concentration of fibrinogen in human plasma as a function of urokinase concentration and time

The concentration of fibrinogen in plasma at different enzyme concentrations (final) of urokinase is shown after different incubation times at 37°C. Values are means $\pm$ S.E.M. for three experiments.

Incubation time (ks)	Urokinase in plasma (Ploug units/l) ...	Fibrinogen concentration (mg/100ml)		
		0	25	120
0		190 $\pm$ 5.3	197 $\pm$ 2.8	197 $\pm$ 0.4
0.6		193 $\pm$ 4.3	199 $\pm$ 1.2	194 $\pm$ 2.3
1.8		206 $\pm$ 0.6	197 $\pm$ 1.2	134 $\pm$ 1.2
18		194 $\pm$ 2.0	190 $\pm$ 0.4	47 $\pm$ 0.7
36		201 $\pm$ 5.0	196 $\pm$ 2.1	31 $\pm$ 2.7

The results for samples A, B and Bcl are shown in Fig. 1. Plasminogen from unactivated plasma (A) was eluted between the 7S and 4S proteins (K_D 0.33). The pattern of sample B indicated the presence of two components with plasminogen+plasmin antigenicity, one component being eluted with the early 7S protein (K_D 0.18) distinctly earlier than plasminogen in normal plasma, and another component being eluted almost in the same fractions (K_D 0.35) as plasminogen in sample A. The pattern of sample C (not shown) and sample Bcl indicated the presence of three components with plasminogen+plasmin antigenicity. Two components were eluted with the early and late half of the 7S protein as was the case with unclotted plasma with activator (K_D values approx. 0.1–0.2 and approx. 0.3), but a third component was eluted with the macroglobulins together with α_2 macroglobulin (K_D approx. 0–0.05). The different pattern obtained with samples B and Bcl also demonstrated the enhancing effect of coagulation and lysis on the processes.

Benzoylarginine ethyl esterase activity was located in the macroglobulin fraction with α_2 macroglobulin. The activity was small in samples A and B, but considerably higher in samples Bcl and C.

Fibrinogenolysis in urokinase-activated plasma

The concentration of fibrinogen was measured in the incubated samples A, B and C (Table 1). Samples were removed at different points of time and stabilized by addition of 11 μ mol of bovine protease inhibitor/l. Controls proved that this concentration completely inhibited fibrinogenolysis in plasma at the highest urokinase concentration for 24 h at 24°C. The concentration of fibrinogen in sample C with a high urokinase concentration decreased significantly after 1.8 ks, and was decreased further after 18 and 36 ks (5 and 10 h). In this sample the decrease of the concentration of fibrinogen was correlated in time to the appearance of the α_2 component (Plate 1). No significant decrease in the fibrinogen concentration was observed in samples A and B. These samples contained no α_2 component.

Relation of plasma protease inhibitors to the components with plasminogen+plasmin antigenicity

The concentration of the plasma protease inhibitors in the fractions obtained by gel filtration of the different plasma samples was determined by electroimmunoassay (Fig. 1). In all samples the distribution of the inhibitors was the same: α_2 macroglobulin was eluted with the 19S proteins (K_D 0) together with plasmin in samples Bcl and C. Inter- α -inhibitor was eluted between the 19S and 7S proteins (K_D 0.13). α_1 Antitrypsin was eluted with the 4S proteins (K_D approx. 0.5). All antithrombin III was eluted with the 4S proteins [K_D values approx. 0.6 (samples A and B) and 0.5 (sample Bcl)] whereas the C_1 -esterase inhibitor was eluted with the early 7S proteins [K_D values 0.20 (sample A), 0.18 (sample B) and 0.16 (sample Bcl)] (not shown in Fig. 1). The mobility of the plasma protease inhibitors was also determined by crossed immunoelectrophoresis of the different samples. In all cases α_1 antitrypsin and inter- α -inhibitor were completely separated from the plasminogen+plasmin antigenic components, whereas the complex between plasmin and α_2 macroglobulin was apparent in the activated samples (Plate 3). Other experiments showed antithrombin III and the C_1 -esterase inhibitor to be α_2 proteins with about the same migration as the α_2 component. The mobilities were the same in all samples.

Thus α_2 macroglobulin was associated with the same plasmin component by both methods. C_1 -esterase inhibitor occurred together with different plasminogen+plasmin antigenic components by gel filtration and gel electrophoresis, whereas the other inhibitors occurred separated from plasminogen+plasmin antigenic component by one or both methods.

Electrophoretic mobility of the plasminogen+plasmin components isolated by gel filtration

A partial separation of the different plasminogen+plasmin antigenic components was aimed at by preparing concentrated pools of the 19S, 7S₁ (early 7S)

and 7S₂ (late 7S) fractions from preparative gel filtration of the plasma samples as described under 'Methods'. The pools were analysed by crossed immunoelectrophoresis against anti-plasminogen alone and against anti-plasminogen+anti- α_2 macroglobulin to demonstrate the relation between the plasminogen+plasmin components and α_2 macroglobulin. In each case the mobility of the components of the three pools, of the corresponding activated plasma and of normal plasma was compared (Plates 4 and 5, and Table 2).

The 19S pool from sample Bcl contained a plasminogen+plasmin antigenic component with the same mobility and same co-precipitation with α_2 macroglobulin as the α_2 component in the corresponding plasma (see also Plate 3). The 7S₁ pool from samples B and Bcl both contained a homogeneous plasminogen+plasmin antigenic component with the same mobility as the post β_1 component in the

corresponding plasma samples. The 7S₂ pools isolated from gel filtration of samples B and Bcl in GF-Tris buffer both contained a main component very similar in mobility and appearance to plasminogen (Plate 4). The corresponding 7S₂ pools isolated from gel filtration in GF-lysine buffer contained a main component with β_1 mobility, definitely more anodic than that of the components in the 7S₁ pools and in the plasma samples (Plate 5). They also contained a γ component not apparent in activated or normal plasma. The 7S₂ pool from gel filtration of sample B in GF-lysine buffer also contained some material with α_2 mobility. The mobility of the components with plasminogen+plasmin antigenicity in pools prepared from gel filtration in GF-Tris buffer was unchanged by the addition of lysine (0.05 mol/l) to the pool.

Enzymic properties of the plasmin and plasminogen components

Stability of plasmin. Plasmin is stabilized both by proteins and lysine. The mean protein concentration in the agarose gel after preparative gel electrophoresis and in the eluates of preparative gel filtration was about 2.5 g/l. The stability of plasmin was studied at the applied lysine concentration (50 mmol/l), both with and without the inert protein, gelatin, and at pH, temperature and time-periods equal to or less favourable than those applied at preparative gel electrophoresis and gel filtration (Table 3). Small losses were observed by long-term storage at 3°C; apart from that no significant decrease in activity occurred.

Recovery of plasminogen and plasmin by preparative electrophoresis. The active plasmin and activatable plasminogen could be recovered only by using purified preparations, and not in experiments with plasma because of the high inhibitor content of this material. The preparations were subjected to preparative gel electrophoresis in GE-Tris (plasminogen) and

Table 2. *Electrophoretic mobility of plasmin+plasminogen antigenic components in concentrated pools, after gel filtration on Sephadex G-200 of human plasma with urokinase, uncoagulated (sample B) and with urokinase, coagulated and lysed (sample Bcl)*

For details see the text.

Plasma sample	Fraction	Gel filtration buffer	
		Tris	Lysine
B	19S	0	0
Bcl	19S	α_2^*	α_2^*
B	7S ₁	Post β_1	Post β_1
Bcl	7S ₁	Post β_1	Post β_1
B	7S ₂	$\beta_2^\dagger$	$\gamma, \beta_1, (\alpha_2)$
Bcl	7S ₂	$\beta_2^\dagger$	γ, β_1

* Plasmin- α_2 macroglobulin complex.

† Appearance of peak similar to that of plasminogen in sample A 7S₂ fraction.

Table 3. *Stability of purified plasmin at different conditions*

Plasmin was diluted by the appropriate buffers, samples were removed at zero time and after the indicated periods and stabilized by addition of glycerol to a concentration of 6.8 mol/l and stored at -20°C, until assay of the enzyme activity. Both benzoylarginine ethyl ester and casein were used as substrates. Buffers: (a), GE-lysine; (b), GE-lysine+gelatin (2.5 g/l); (c), GF-lysine; (d), GF-lysine+gelatin (2.5 g/l); (e), GF-Tris; (f), GF-Tris+gelatin (2.5 g/l). Values are means $\pm$ S.E.M. for four experiments. EC(*t*), Enzyme concentration at stated time; EC(0), enzyme concentration at zero time. *P*, Probability of the deviation of EC(*t*)/EC(0) from 1.00.

Buffer	Temp. (°C)	Time (h)	Benzoylarginine ethyl ester	<i>P</i>	Casein	<i>P</i>
			[EC(<i>t</i>)/EC(0)]		[EC(<i>t</i>)/EC(0)]	
(a)	22	4	0.99 $\pm$ 0.05	>0.40	0.98 $\pm$ 0.02	>0.15
(b)	22	4	0.93 $\pm$ 0.04	>0.25	1.00 $\pm$ 0.04	>0.50
(a)	3	24	0.94 $\pm$ 0.04	>0.10	0.98 $\pm$ 0.02	>0.15
(b)	3	24	0.99 $\pm$ 0.03	>0.35	0.98 $\pm$ 0.03	>0.25
(c)	3	144	0.86 $\pm$ 0.03	<0.01	0.95 $\pm$ 0.08	>0.25
(d)	3	144	0.91 $\pm$ 0.03	<0.05	0.90 $\pm$ 0.04	<0.05
(e)	3	144	0.86 $\pm$ 0.06	<0.10	0.78 $\pm$ 0.02	<0.01
(f)	3	144	0.83 $\pm$ 0.04	<0.02	0.81 $\pm$ 0.02	<0.01

GE-lysine (plasmin) buffers at pH 8.6, with and without gelatin at 2.5 g/l. The controls were kept undiluted at -20°C (Table 4). The recovery both of plasminogen and plasmin was much greater with gelatin than without. As plasmin was stable under gel-electrophoresis conditions without gelatin, the effect was probably due to a decreased absorption of plasminogen and plasmin to the gel.

Immunochemical recovery of plasminogen+plasmin antigenic components by gel electrophoresis. Plasma samples at different stages of plasminogen conversion (samples A, B and Bcl, all after 1.8 ks) were separated by electrophoresis, and gel sections were isolated which corresponded to the localization of the α_2 component (in sample Bcl), the β_1 component (in

samples B and Bcl) and the β_2 component (in all samples). The gel liquid and the corresponding equally diluted plasma were analysed by immunochemistry (Table 5). The recovery of plasminogen from sample A plasma corresponded well with the relatively high recovery of purified plasminogen in experiments with gelatin buffers (Table 4).

The formation of immunoprecipitates by plasmin bound to α_2 macroglobulin was impeded even with undiluted plasma (Plates 1, 2, 3, 4 and 5), possibly through a competition of antibodies and α_2 macroglobulin for plasmin, and precipitates were not detectable in experiments with dilute gel liquid (Table 5).

Enzymic properties of plasminogen+plasmin antigenic components in urokinase-activated plasma. The stability and recovery experiments ensured the validity of a study of the enzymic properties of these components isolated by gel electrophoresis and gel filtration. Preparative gel electrophoresis was performed as described above and the enzymic activity of the gel liquid against benzoylarginine ethyl ester and casein was determined directly or after activation by an excess of urokinase (Table 6). The β_2 sections of the activated samples contained both enzyme and plasminogen. Coagulation and fibrinolysis resulted in a significant and considerable decrease of plasminogen and of the total plasminogen plus enzyme activity (sample Bcl as compared with sample B). The α_2 sections showed a significant increase in esterase activity from sample A through B to Bcl and no caseinolytic activity, as might be expected from the formation of a plasmin- α_2 macroglobulin complex. The β_1 sections of samples B and Bcl showed little or no spontaneous caseinolytic activity, and the esterase activity was lower than that of the control sections of sample A. The β_1 sections of sample Bcl contained some plasminogen.

The crossed immunoelectrophoresis showed that the 19S- α_2 component was present in considerable amounts in the 19S pool from sample C (Plate 1), whereas the 7S₁-post β_1 component was present in

Table 4. Recovery of purified human plasminogen and plasmin by preparative agarose-gel electrophoresis

Purified preparations of plasminogen and plasmin were used at an active-site concentration of approx. $3\ \mu\text{mol/l}$ with lysine (0.05 mol/l), pH 7.6. After preparation of the gel liquids, controls were diluted equally with the gel-electrophoresis buffers. The enzyme concentration of plasmin was estimated with benzoyl-arginine ethyl ester and casein. In the plasminogen experiments, the plasminogen was activated by an excess of urokinase before the assay of plasmin. This was done at lysine, 50 mmol/l; urokinase, 400 Ploug units/l; pH 7.6 (final values), 1 h at 22°C . Buffers: (a), GE-lysine; (b), GE-lysine+gelatin (2.5 g/l); (g), GE-Tris; (h), GE-Tris+gelatin (2.5 g/l). A, Enzyme concentration of plasminogen or plasmin in the gel liquid; B, corresponding concentration in the applied solution diluted equally. Values are means $\pm$ S.E.M. with the number of experiments in parentheses.

Buffer	Material	Benzoyl-arginine ethyl ester (A/B)	Casein (A/B)
(a)	Plasmin	0.45 ± 0.03 (10)	0.49 ± 0.02 (10)
(b)	Plasmin	0.76 ± 0.01 (12)	0.85 ± 0.01 (12)
(g)	Plasminogen	0.44 ± 0.02 (8)	0.57 ± 0.02 (8)
(h)	Plasminogen	0.85 ± 0.01 (8)	0.92 ± 0.02 (8)

Table 5. Recovery of plasminogen+plasmin antigenic components in gel liquid after preparative gel electrophoresis

Results are expressed as: concentration of (plasminogen+plasmin) in gel liquid/concentration of (plasminogen+plasmin) in dilute plasma. The two methods of assay and the two buffers used for the electrophoresis are as shown. The corresponding plasma and gel liquids were equally diluted. The α_2 fractions and β_1 fraction from sample A plasma gave 0 values. See under 'Methods' for further details. Values are means $\pm$ S.E.M. with the number of experiments in parentheses.

Plasma sample	Fraction	Electroimmunoassay		Immunodiffusion	
		GE-lysine buffer	GE-Tris buffer	GE-lysine buffer	GE-Tris buffer
A	β_2	0.68 ± 0.01 (7)	0.90 ± 0.01 (8)	0.67 ± 0.03 (8)	0.88 ± 0.03 (8)
B	β_1	0.22 ± 0.01 (4)	0.18 ± 0 (4)	0.27 ± 0.03 (4)	0.29 ± 0.06 (4)
B	β_2	0.53 ± 0.02 (4)	0.59 ± 0.04 (4)	0.45 ± 0.01 (4)	0.52 ± 0.03 (4)
Bcl	β_1	0.32 ± 0.02 (4)	0.32 ± 0.01 (4)	0.27 ± 0.01 (4)	0.31 ± 0 (4)
Bcl	β_2	0.27 ± 0.02 (4)	0.22 ± 0.02 (3)	0.30 ± 0 (4)	0.31 ± 0 (3)

Table 6. Enzyme and plasminogen concentration of gel liquid recovered after preparative gel electrophoresis of human plasma without urokinase (sample A) and with urokinase, uncoagulated (sample B) and after coagulation and lysis (sample Bcl)

The plasma samples were activated for 30 min at 37°C. The spontaneous activity and the amount of plasminogen (increase in activity after activation by an excess of urokinase) was measured in gel liquid after electrophoresis in GE-lysine and GE-Tris buffers respectively. The activity against benzoylarginine ethyl ester and casein was expressed in arbitrary units by setting the total activity (with urokinase) of the β_2 fraction of sample A plasma equal to 100 arbitrary units. Values not significantly different from zero ($P > 0.05$) were recorded as 0. Values are expressed as means $\pm$ s.e.m. with the number of experiments in parentheses.

Plasma sample	Fraction	Benzoylarginine ethyl ester		Casein	
		Spontaneous	Plasminogen	Spontaneous	Plasminogen
A	α_2	0	0	0	0
	β_1	9.3 $\pm$ 0.1 (2)	5.0 $\pm$ 0.8 (2)	0	0
	β_2	5.6 $\pm$ 2.3 (2)	87.2 $\pm$ 2.7 (2)	0	91.5 $\pm$ 8.5 (2)
B	α_2	4.0 $\pm$ 0.6 (4)	0	0	0
	β_1	3.4 $\pm$ 0.5 (4)	2.8 $\pm$ 0.6 (4)	0	0
	β_2	22.2 $\pm$ 0.4 (4)	54.3 $\pm$ 2.2 (4)	14.6 $\pm$ 2.1 (4)	70.1 $\pm$ 1.9 (4)
Bcl	α_2	13.2 $\pm$ 0.7 (4)	0	0	0
	β_1	3.9 $\pm$ 0.9 (4)	8.9 $\pm$ 2.8 (4)	1.9 $\pm$ 0.7 (4)	11.1 $\pm$ 0.8 (4)
	β_2	22.6 $\pm$ 1.0 (4)	14.0 $\pm$ 1.2 (4)	12.2 $\pm$ 1.3 (4)	19.8 $\pm$ 1.5 (4)

Table 7. Enzyme and plasminogen concentration of fractions obtained by preparative gel electrophoresis of a pool obtained by gel filtration of urokinase-activated human plasma

The plasma was primarily activated at a low urokinase concentration for 30 min at 37°C (sample B). The 7S₂ pool was prepared after gel filtration of the sample on Sephadex G-200 with GF-lysine buffer and the β_1 and γ fractions were isolated by preparative gel electrophoresis of the pool. The activity against benzoylarginine ethyl ester and casein was expressed in arbitrary units, setting the activity, with excess of urokinase, of the 7S₂ fraction from unactivated plasma (sample A) equal to 100 arbitrary units. Results not significantly different from zero ($P > 0.05$) were recorded as 0. Values are means $\pm$ s.e.m. for five experiments.

Fraction and component	Benzoylarginine ethyl ester		Casein	
	Spontaneous	Plasminogen	Spontaneous	Plasminogen
Sample B, 7S ₂ , β_1	0	16.3 $\pm$ 1.9	0	4.2 $\pm$ 0.8
Sample B, 7S ₂ , γ	21.3 $\pm$ 2.0	13.5 $\pm$ 2.0	21.4 $\pm$ 0.9	17.5 $\pm$ 1.1

the 7S₁ pool of sample B (Plate 5). Both the 7S₂- β_1 and 7S₂- γ components were present in the 7S₂ pool prepared from gel filtration of samples B and Bcl in GF-lysine buffer (Plate 5). These pools contained little of the other components.

A preparative gel electrophoresis would not eliminate possible unknown strong inhibitors in complex with plasmin. An acid treatment removes most of the inhibitory effect of plasma on plasmin (Lauritsen, 1968, and references therein) whereas both plasminogen and plasmin are very stable at acid reaction (Müllertz, 1955). Therefore the spontaneous enzyme activity and the amount of activatable plasminogen in the pools prepared from gel filtration in GF-lysine buffer were assayed directly and after incubation at pH 2.0 $\pm$ 0.1 for 60 min at 22°C and subsequent neutralization. In both cases the final solutions contained: Tris, 0.06 mol/l; lysine, 0.05 mol/l; pH 7.6, I 0.15. All values were put into relation to the maxi-

mum plasmin activity (100 arbitrary units) of the plasminogen fraction of the original plasma.

The 19S pool from sample C showed a significantly higher proteolytic (control = 0) and esterase activity than the control, the 19S pool from sample A. Acid treatment increased the proteolytic activity of the pool about four times. The 7S₁ pool showed no significant enzyme or plasminogen activity with or without acid treatment. The 7S₂ pool from sample B, which contained both β_1 and γ components, showed spontaneous and plasminogen activity by both assays (plasmin approx. 24, plasminogen approx. 18 arbitrary units by casein assay). These results were not changed much by acid treatment.

The β_1 component and the γ component in the 7S₂ pool, prepared from gel filtration of sample B in GF-lysine buffer, were isolated by preparative gel electrophoresis with lysine buffer, and assayed for enzyme and plasminogen activity (Table 7). The β_1 fraction

showed no spontaneous activity, but contained plasminogen, although relatively less by the casein than by the esterase assay. The γ fraction contained, by both assays, about equal catalytic amounts of spontaneous activity and plasminogen, in total about 0.3–0.4 of the original amounts of plasminogen in the sample.

Discussion

The use of gel filtration and gel electrophoresis combined with immunochemistry with specific antibodies has disclosed a rather complex pattern of the products of activation of plasminogen in human plasma. Niléhn & Ganrot (1967) studied the processes in human plasma before, during and after streptokinase therapy. Müllertz (1972a) studied fibrinolytic post-mortem human plasma. In both cases the distribution of plasminogen+plasmin antigenic material in gel filtration analysis of plasma was very similar to that observed in the present paper in urokinase-activated plasma, in which the reactions had progressed most. Crossed immunoelectrophoresis disclosed a plasmin- α_2 macroglobulin complex and a component with β_1 mobility in post-mortem plasma.

In the present study several plasminogen+plasmin antigenic components were observed in plasma after activation by urokinase, a physiological activator excreted in urine. Two molar ratios of urokinase/plasminogen were chosen to study the relation of the activation processes to the lysis of fibrin and fibrinogen, the natural substrates of plasmin. At a low ratio (2×10^{-3}) degradation of fibrinogen was low or absent, but fibrinolysis occurred within 30 min. At the higher ratio (1×10^{-2}) fibrinolysis occurred in 10 min, fibrinogenolysis progressed between 3 and 10 h.

The processes were studied by a combination of gel electrophoresis, gel filtration and immunochemical techniques to achieve a partial separation of the components under conditions that would not denature the components, radically change the environment or strongly displace any equilibria. A low-molecular-weight compound (benzoylarginine ethyl ester) and a protein (casein) were used as substrates for the assay of enzyme activity, to disclose any dissociation of esterase and protease activity. This is known to occur for the plasmin- α_2 macroglobulin complex, which loses almost all proteolytic activity, but maintains almost the same esterase activity as free plasmin (Ganrot, 1967).

The components were characterized by their elution characteristics after gel filtration and electrophoretic migration in agarose gel, and by their enzymic properties.

The pattern by crossed immunoelectrophoresis and the esterase and proteolytic activity of the isolated 19S gel-filtration fraction and α_2 gel-electrophoresis fraction from activated plasma, indicated that the 19S- α_2 component was plasmin in a complex with α_2

macroglobulin. The pattern of the plasminogen+plasmin and α_2 macroglobulin immunoprecipitates (Plates 4 and 5) suggested that the complex had a slightly lower mobility than α_2 macroglobulin itself and that the larger excess of α_2 macroglobulin in the border region limited or prevented the formation of immunoprecipitates of plasmin.

The appearance of the immunoprecipitates and the enzymic property of the 7S₂- β_2 components of the unactivated and activated samples showed that they were identical with plasminogen. Spontaneous activity was observed in the gel liquid of these fractions especially from the clotted and lysed sample. Some plasmin may have been present in plasma or developed during the isolation and assay procedures.

Two further components were observed in activated plasma samples after gel filtration with lysine buffer. They were both eluted by gel filtration with the 7S₂ proteins almost as plasminogen, but by gel electrophoresis a component with γ mobility and one with β_1 mobility were observed. The 7S₂- β_1 component appeared to be a modified and possibly partly denatured plasminogen. This component may be responsible for the plasminogen activity of the β_1 section from preparative electrophoresis of sample Bcl (Table 6). The γ fraction isolated by gel electrophoresis contained both plasmin and plasminogen. Both may have been present, or plasminogen may have been partly activated by traces of urokinase during the isolation and assay. The 7S₂- γ components constituted about one-third of the plasminogen in the unactivated sample, isolated and treated identically. The effect of the many isolation procedures on the enzymic properties of the components should be considered, however.

The 7S₁-post β_1 component was inactive against both substrates and could not be activated by an excess of urokinase. Acid treatment of the isolated component did not release any enzyme activity. The component may be denatured plasmin released by dissociation of the plasmin- α_2 macroglobulin complex by gel filtration as originally suggested by Niléhn & Ganrot (1967). However, no dissociation of the isolated complex was observed by gel filtration or gel electrophoresis (Müllertz, 1972a) or in the present work (Plate 5). The early elution of the 7S₁-post β_1 component by gel filtration as compared with plasminogen indicated an increase in relative molecular mass (M_r) or a change in conformation of a transformed plasminogen or plasmin molecule. These data and the lack of enzyme or proenzyme activity suggest the following possibilities: plasmin in a complex with a strong (unknown) inhibitor, a changed and denatured plasminogen or plasmin molecule, or a polymerization phenomenon.

Multiple molecular forms of plasminogen have been demonstrated by ion-exchange chromatographic, electrophoretic and isoelectric-focusing

methods (Wallén & Wiman, 1970, 1972; Summaria *et al.*, 1972). One main form has pI about 6.0–6.6, NH₂-terminal glutamic acid (Rickli & Cuendet, 1971), and M_r approx. 93000 (Wiman & Wallén, 1973). Another main form has pI about 6.7–8.3, NH₂-terminal lysine and M_r approx. 81000 (Summaria *et al.*, 1973), or NH₂-terminal valine or methionine, and M_r approx. 86000 (Wiman & Wallén, 1973). It is now beyond doubt that the first form is the native plasminogen of plasma with β_2 mobility by gel electrophoresis at pH 8.6. The second form or forms are produced by proteolytic degradation of the first form by plasmin or by specific activators of plasminogen. The degradation produces conformational changes and a swelling of the molecule leading to an apparent M_r of 105000 as determined by gel filtration on Sephadex G-200 (Wallén & Wiman, 1972).

The activation of purified plasminogen by urokinase appears to involve the cleavage of at least two peptide bonds. One event is the formation of the intermediate form of plasminogen by the release of a peptide of 64 amino acid residues from the *N*-terminal end of the native molecule (Rickli & Otavsky, 1973; Wiman & Wallén, 1973). Another event is the cleavage of an arginine–valine bond and the formation of a molecule with two chains connected by disulphide bridges (Summaria *et al.*, 1967). All degraded forms of plasminogen have higher pI and lower anodic mobilities than native plasminogen (Wallén & Wiman, 1972). Plasmin has pI 7.2–7.7 and almost the same electrophoretic mobility as the more basic forms of plasminogen (Summaria *et al.*, 1972).

The 7S₂- γ fraction of the urokinase-activated plasma may well contain the intermediate, degraded form of plasminogen, and possibly free plasmin.

The α_2 macroglobulin-bound plasmin is probably the same as purified plasmin, which complexes easily with α_2 macroglobulin (Ganrot, 1967).

The 7S₂- β_1 and 7S₁-post β_1 components, however, have no immediate correlates among those observed in studies on purified plasminogen and plasmin.

The plasma protease inhibitors studied in the present paper are all known to inhibit plasmin (Heimburger *et al.*, 1971). Apart from α_2 macroglobulin, however, the gel-electrophoresis and gel-filtration studies showed that a complex between plasmin and these inhibitors, if any, was completely dissociated either by gel filtration or by gel electrophoresis.

The presence of small amounts of urokinase in plasma apparently transformed some plasminogen to a 7S₁-post β_1 component, but without any effect on fibrinogen. The presence of fibrin strongly accelerated the processes, the plasminogen concentration decreased markedly, α_2 macroglobulin-bound plasmin appeared and the fibrin was quickly lysed. At a higher concentration of urokinase similar transformations and lysis of fibrinogen took place in the absence of

fibrin, but at a much slower rate. The lysis of fibrin or fibrinogen was correlated to the appearance of the plasmin- α_2 macroglobulin complex.

It is well known that fibrin, when formed, is readily dissolved in blood otherwise showing little or no degradation of fibrinogen or other indications of free plasmin. The enhancing effect of fibrin on the fibrinolytic processes was reported by Ratnoff (1953) and Müllertz (1953). The mechanism has been explained by the assumption of a plasmin–inhibitor or an activator–inhibitor complex dissociated on the surface of fibrin because of higher affinities of the enzymes for fibrin than for the inhibitor (Müllertz, 1956).

Further studies are needed to decide the nature and structure of the different components developing during the activation process and their significance for the mechanism of fibrinolysis in blood.

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References

- Blombäck, B. & Blombäck, M. (1956) *Ark. Kemi* **10**, 415–443
- Chase, T., Jr. & Shaw, E. (1969) *Biochemistry* **8**, 2212–2234
- Christensen, U. & Müllertz, S. (1974) *Biochim. Biophys. Acta* **334**, 187–198
- Clausen, J. (1969) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.), vol. 1, part 3, pp. 517–518, North-Holland Publishing Co., Amsterdam and London
- Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170**, 1095–1096
- Ganrot, P. O. (1967) *Acta Univ. Lund. Sect. 2.M.M.* **2**, 1–36
- Ganrot, P. O. (1972) *Scand. J. Clin. Lab. Invest. Suppl.* **124**, 39–41.
- Heimburger, N., Haupt, H. & Schwick, H. G. (1971) in *Proc. Int. Res. Conf. Proteinase Inhibitors 1st* (Fritz, H. & Tschesche, H., eds.), pp. 1–21, De Gruyter, Berlin
- Johansson, B. G. (1972) *Scand. J. Clin. Lab. Invest. Suppl.* **124**, 7–19
- Kok, P. & Astrup, T. (1969) *Biochemistry* **8**, 79–86
- Laurell, C.-B. (1972) *Scand. J. Clin. Lab. Invest. Suppl.* **124**, 21–37
- Lauritsen, O. S. (1968) *Scand. J. Clin. Lab. Invest.* **22**, 239–246
- Müllertz, S. (1953) *Acta Physiol. Scand.* **28**, 29–40
- Müllertz, S. (1955) *Biochem. J.* **61**, 424–434
- Müllertz, S. (1956) *Acta Physiol. Scand. Suppl.* **130**, 53–55
- Müllertz, S. (1972a) *Scand. J. Clin. Lab. Invest.* **30**, 369–379
- Müllertz, S. (1972b) *Scand. J. Clin. Lab. Invest. Suppl.* **126**, 4.10
- Nilén, J. E. & Ganrot, P. O. (1967) *Scand. J. Clin. Lab. Invest.* **20**, 113–121
- Rabiner, S. F., Goldfine, I. D., Hart, A., Summaria, L. & Robbins, K. C. (1969) *J. Lab. Clin. Med.* **74**, 265–273
- Ratnoff, O. D. (1953) *J. Clin. Invest.* **32**, 473–479

- Rickli, E. E. & Cuendet, P. A. (1971) *Biochim. Biophys. Acta* **250**, 447–451
- Rickli, E. E. & Otavsky, W. I. (1973) *Biochim. Biophys. Acta* **295**, 381–384
- Sasahara, A. A., Hyers, T. M., Cole, C. M., Ederer, F., Murray, J. A., Wegner, N. K., Sherry, S. & Stengle, J. M. (1973) *Circulation Suppl.* **2**, 1–108.
- Summaria, L., Hsieh, B. & Robbins, K. C. (1967) *J. Biol. Chem.* **242**, 4279–4283
- Summaria, L., Arzadon, L., Bernabe, P. & Robbins, K. C. (1972) *J. Biol. Chem.* **247**, 4691–4702
- Summaria, L., Arzadon, L., Bernabe, P., Robbins, K. C. & Barlow, G. H. (1973) *J. Biol. Chem.* **248**, 2984–2991
- Vogel, R., Trautschold, I. & Werle, E. (1968) *Natural Proteinase Inhibitors*, 1st edn., pp. 2–8, Academic Press, London and New York
- Wallén, P. & Wiman, B. (1970) *Biochim. Biophys. Acta* **221**, 20–30
- Wallén, P. & Wiman, B. (1972) *Biochim. Biophys. Acta* **257**, 122–134
- White, W. F. & Barlow, G. H. (1970) *Methods Enzymol.* **19**, 665–672
- Wiman, B. & Wallén, P. (1973) *Eur. J. Biochem.* **36**, 25–31
- Worning, H. & Müllertz, S. (1966) *Scand. J. Gastroent.* **1**, 268–283