Degradation of Human Fibrinogen by Plasma α_2-Macroglobulin-Enzyme Complexes

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ABSTRACT This study demonstrates that human plasma α_2-macroglobulin preparations possess an enzymic activity that degrades fibrinogen, resulting in the formation of products whose structure resembles that of circulating fibrinogen catabolites. The sequence of degradation is similar to that observed in plasmin-catalyzed digests, in that Aα-chain fragmentation precedes that of Bβ-chain. The addition of plasminogen activators to plasma induced an increase in the *N*-α-tosyl-L-arginine methyl ester HCl esterase and fibrinogenolytic activity associated with α_2-macroglobulin purified from this plasma, indicating that the enzymic activity of the complex was preserved and could be increased in the presence of other plasma enzyme inhibitors. Immunochemical studies demonstrated that an α_2-macroglobulin-plasmin complex had formed in urokinase-treated plasma. This α_2-macroglobulin preparation manifested an esterolytic profile like that of a complex prepared from plasmin and purified α_2-macroglobulin. After complex formation with α_2-macroglobulin in plasma, plasmin retained less than 0.1% of its fibrinogenolytic activity. That plasmin expressed its activity while bound to α_2-macroglobulin was suggested by immunoprecipitation of this activity with α_2-macroglobulin antibody and by the demonstration that pancreatic trypsin inhibitor did not effectively inhibit its fibrinogenolytic or esterolytic activity. These results raise the possibility that, in addition to its activity as a major plasma proteolytic enzyme inhibitor, α_2-macroglobulin may modulate enzyme-substrate interactions, such as those resulting in the formation of circulating fibrinogen catabolites, by providing a mechanism for the preservation and protection of a portion of the enzymic activity in the presence of other circulating inhibitors.

INTRODUCTION

The role that circulating enzyme inhibitors play in modulating the interactions involved in hemostatic and inflammatory processes is not well understood. One such inhibitor, plasma α_2-macroglobulin, apparently participates in the regulation of several different plasma enzyme systems, since it has been shown in vitro to form a complex with plasmin (1–3), thrombin (4–6), and kallikrein (7–9), resulting in inhibition of proteolytic activity. In contrast, the ability of these enzyme-inhibitor complexes to degrade synthetic amino acid ester substrates (esterolytic activity) is only slightly modified, demonstrating that the catalytic potential of the bound enzyme is retained.

Circulating human fibrinogen consists of a heterogenous family of coagulable species identifiable by solubility characteristics ranging in molecular weight from that of "native" fibrinogen, 325,000 (e.g., fraction I-4) to 261,000 (e.g., fraction I-9) (10–12). Turnover studies in rabbits have shown a classical precursor-product relationship between high and low molecular weight forms of isotopically labeled fibrinogen (13). Increased amounts of low molecular weight forms of fibrinogen have been shown in patients receiving urokinase therapy (13). These observations have provided conclusive support for the hypothesis (10) that such fibrinogens are catabolic intermediates. The demonstration that the amino terminal regions of these plasma fibrinogens were intact was unequivocal evidence that

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they had not been acted upon by thrombin (11, 12). Furthermore, the suggestion that the reduction in molecular size was the consequence of cleavage of peptide material from the COOH-terminal region of the core molecule (10, 11) has been confirmed (12) and extended by the demonstration that under physiologic circumstances such attack appears to be confined to the Aα-chains (12, 14). Similarities between the plasma catabolites and early fibrinogen derivatives resulting from plasmin-catalyzed hydrolysis (14–18) support the notion that it is plasmin or a plasmin-like enzyme that mediates this in vivo catabolism.

The studies to be reported were designed to examine the possibility that, in addition to its function as an inhibitor of plasmin, plasma α_2-macroglobulin might participate in fibrinogen catabolism by forming a biologically active complex with plasmin or plasmin-like enzymes. Our data indicate that (*a*) α_2-macroglobulin prepared from human plasma has an associated fibrinogenolytic activity; (*b*) both the fibrinogenolytic and esterolytic activities associated with α_2-macroglobulin preparations are increased by the addition of plasminogen activators, urokinase, or streptokinase to plasma before its purification; (*c*) the Aα-chain of fibrinogen is the chain most susceptible to degradation by α_2-macroglobulin-enzyme complexes, a sequence of proteolysis like that observed in plasmin-catalyzed digests; (*d*) α_2-macroglobulin prepared from urokinase-treated plasma is complexed with immunologically identifiable plasmin; (*e*) the enzymic activity complexed to α_2-macroglobulin preparations is resistant to inhibition by plasmin inhibitors.

METHODS

The chemicals used were reagent grade and were obtained from the following sources: *N*-α-tosyl-L-arginine methyl ester HCl (TAMe),[1] *N*-benzoyl-L-arginine methyl ester HCl (BAMe), *N*-α-acetyl-L-lysine methyl ester HCl (ALMe), and *N*-α-acetylglycyl-L-lysine methyl ester acetate (AGLMe) from Cyclo Chemical Co., Los Angeles, Calif.; Kunitz pancreatic trypsin inhibitor (grade PSIF) and soybean trypsin inhibitor (grade SI) from Worthington Biochemical Corp., Freehold, N. J.; ε-aminocaproic acid from Fluka AG, Basel, Switzerland; streptokinase, 10,000 U per vial, lot no. 10024, manufactured by A. B. Kabi, Stockholm, Sweden; polybrene (hexadimethrine bromide) and urokinase, 200,000 U/vial from Abbott Laboratories, North Chicago, Ill.; dithiothreitol (DTT) from Calbiochem, San Diego, Calif.; sodium dodecyl sulfate (SDS) from Sigma Chemical Co., St. Louis, Mo.

Human plasma α_2-macroglobulin was prepared from venous blood freshly collected from normal individuals into plastic containers to minimize activation of Hageman factor (Factor XII). The plasma was harvested after centrifugation at 2000 *g* for 15 min at 4°C. The anticoagulant used contained soybean trypsin inhibitor (0.5 mg/ml of 3.8% sodium citrate), unless subsequent incubation with plasminogen activators was planned. For these latter experiments plasma was incubated with urokinase at a final concentration of 100 U or 500 U/ml, or with streptokinase, 500 U/ml, for 1 h at 37°C, before the addition of soybean trypsin inhibitor to a final concentration of 0.1 mg/ml. Soybean trypsin inhibitor in this concentration was also added to buffers used in the chromatographic procedures. In some preparations, Polybrene (25 μg/ml, final concentration), an inhibitor of Hageman factor activation (19), or 0.1 M ε-aminocaproic acid in addition to soybean trypsin inhibitor was added to the anticoagulant and subsequent buffers.

α_2-Macroglobulin was prepared from these plasma samples by the following procedures, described previously in detail (7). After initial adsorption with barium chloride and barium sulfate, and precipitation with polyethylene glycol, the preparation was subjected to gel filtration chromatography and Pevikon block electrophoresis (Kema Nord, Stockholm, Sweden). The final product, by immunoelectrophoretic analysis, formed a single precipitin arc against rabbit whole antihuman serum (20) and was identified as α_2-macroglobulin using specific antiserum.

The human fibrinogen used had been rendered plasminogen-free by precipitation (21) and was further purified by DEAE-cellulose column chromatography (22). The final material was more than 99% coagulable and showed no signs of degradation after 48 h of incubation at 37°C in the presence of urokinase as assessed by SDS-polyacrylamide gel electrophoresis.

Plasmin, spontaneously activated in 50% glycerol, containing 77 caseinolytic U/ml (23), was obtained from the Michigan Department of Public Health, Lansing, Mich. Plasmin was also prepared by urokinase-induced activation of plasminogen prepared by affinity chromatography (24), and provided by Dr. Alan Johnson, New York University School of Medicine.

Antisera against human α_2-macroglobulin, and plasminogen, and serum prepared in rabbits were obtained from Behring Diagnostics, Inc., subsidiary of American Hoechst Corporation, Woodbury, N. Y. Immunochemical quantitation of α_2-macroglobulin preparations was performed by single diffusion analysis (25), using plates obtained from Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. The IgG fraction of rabbit antihuman α_2-macroglobulin containing 5.5 mg antibody/ml (Lot JA23032) was provided by Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.

Esterolytic assays using different amino acid methyl ester substrates at a final concentration of 0.015 M were carried out as previously described (26).

Fibrinogen and the various purified α_2-macroglobulin preparations were incubated at 37°C in the concentrations detailed in the figure legends. Portions of the incubation mixtures were removed at intervals and heated for 10 min at 56–61°C to precipitate the fibrinogen, which was then dissolved and reduced in a solution of 10 M urea, 1% SDS and 14 mM DTT for 45 min at 37°C. Alternatively, the incubation mixture was added to this solution directly. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (9% gels) as described by Weber and Osborn and were stained with coomassie brilliant blue (27). Comparison of fibrinogen chains prepared from the heat

[1] *Abbreviations used in this paper:* AGLMe, *N*-α-acetylglycyl-L-lysine methyl ester acetate; ALMe, *N*-α-acetyl-L-lysine methyl ester HCl; BAMe, *N*-benzoyl-L-arginine methyl ester HCl; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TAMe, *N*-α-tosyl-L-arginine methyl ester HCl.

precipitate or from the whole mixture showed no appreciable qualitative or quantitative differences. Although there was no overlap between the α_2-macroglobulin preparations and the intact Aα-, Bβ-, and γ-chain positions of fibrinogen, it was convenient for gel scanning and rendering fibrinogen subunit chains more prominent to separate the fibrinogen from most of the other materials in the mixture by heat precipitation. Densitometric scans of gels were carried out in a Gilford model 240 spectrophotometer, equipped with a gel scanning attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a Densicord recorder equipped with an integrator (Photovolt Corp., New York City).

Electroimmunoanalysis of the α_2-macroglobulin preparations was performed in agarose gels containing plasminogen antiserum (1% vol/vol) at 40 V/cm for 1 h as described by Laurell (28, 29) and as applied to the plasmin-rich α_2-macroglobulin preparations by Niléhn and Ganrot (30).

Quantitation of the fibrinogenolytic activity of α_2-macroglobulin preparations. Approximate quantitative estimates of the fibrinogenolytic activity of α_2-macroglobulin preparations were made from comparison of the degree of hydrolysis of fibrinogen rendered by known amounts of plasmin and of α_2-macroglobulin. The means selected for achieving this estimate was comparison of the band pattern of SDS-polyacrylamide gels of samples from fibrinogen digests. In these comparisons, the time required to reach a selected degradative pattern was noted (viz., time for plasmin versus time for α_2-macroglobulin). The results were then converted to caseinolytic units per milligram of α_2-macroglobulin by extrapolation. For example, if plasmin at a concentration of 1×10^{-2} caseinolytic U/ml required 15 min to achieve a given gel pattern of degradation, and α_2-macroglobulin required 24 h to achieve the same degree of degradation, the caseinolytic activity of the α_2-macroglobulin-enzyme complex was approximately 1×10^{-4} caseinolytic U/mg α_2-macroglobulin.

Immunoprecipitation studies were performed by adding 6 mg of the IgG fraction of α_2-macroglobulin antiserum to an equal amount of α_2-macroglobulin from urokinase-treated plasma (500 U/ml plasma), or adding 0.15 M NaCl to the α_2-macroglobulin control in place of the antibody preparation. After incubation for 30 min at 37°C and then overnight at 4°C, the precipitate was removed by centrifugation for 10 min at 3,000 rpm (9). Polyethylene glycol (molecular weight 6,000) was added to the supernate (final concentration 15%), and the precipitate was resuspended in 1.0 ml buffer. The immune-absorbed sample contained no α_2-macroglobulin as assayed by single diffusion analysis, whereas the control specimen contained 5.6 mg. These preparations were incubated with fibrinogen, and alterations in the subunit chains of fibrinogen were assessed by SDS-polyacrylamide gel electrophoresis.

RESULTS

The effect of urokinase and streptokinase on the TAMe esterase activity associated with human plasma α_2-macroglobulin (*Table I*). α_2-Macroglobulin, prepared from platelet-poor human plasma, processed entirely in plastic containers in the presence of soybean trypsin inhibitor, was associated with a low level of TAMe esterolytic activity, a finding previously reported (7) and extended in this study to include those processed in the presence of Polybrene or ϵ-aminocaproic acid. α_2-Macroglobulin purified from plasma

TABLE I

The TAMe Esterase Activity of Human Plasma α_2-Macroglobulin Prepared from Plasma Incubated with Urokinase (UK) and Streptokinase (SK)

α_2-Macroglobulin prepared from plasma incubated with:*	TAMe esterase activity‡	
	μmol hydrolyzed/mg α_2 M/h	
	No inhibitor	Pancreatic trypsin inhibitor
Buffer	0.5	0.5
100 U UK/ml plasma	7.3	7.5
500 U UK/ml plasma	20.0	21.1
500 U SK/ml plasma	19.6	20.6

* The α_2-macroglobulin was prepared as described in Methods.
‡ The TAMe esterase activity of the α_2-macroglobulin preparations was measured in the presence and absence of pancreatic trypsin inhibitor (100 μg/ml, final concentration). Plasmin (two preparations), under the conditions of this experiment, hydrolyzed 28.2 μmol TAMe/caseinolytic U/h; this activity was completely inhibited by pancreatic trypsin inhibitor.

preincubated with urokinase possessed greatly enhanced TAMe esterase activity and the increase in activity was commensurate with the final concentration of urokinase. A plasma urokinase concentration of 100 U/ml resulted in a 15-fold increase in TAMe esterase activity, whereas α_2-macroglobulin obtained from plasma incubated with 500 U urokinase/ml demonstrated a 40-fold increase in activity. The possibility that the esterolytic activity associated with the α_2-macroglobulin from urokinase-treated plasma was due to an α_2-macroglobulin-urokinase complex was eliminated by the following control experiment. A 10-min incubation mixture of α_2-macroglobulin purified from untreated plasma (6.5 mg) and urokinase (500 U) was applied to a Sephadex G-75 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The TAMe esterase activity of the α_2-macroglobulin fractions was identical to the activity of the control α_2-macroglobulin preparation. The TAMe esterase peak associated with the urokinase preparation was completely separated from the α_2-macroglobulin fractions by this procedure. Streptokinase, which has no intrinsic esterase activity, produced an enhancement in α_2-macroglobulin-associated esterase activity similar to that obtained after the addition of urokinase to plasma. Though pancreatic trypsin inhibitor (100 μg/ml) completely inhibited the TAMe esterase activity of plasmin, it failed to inhibit the esterolytic activity of α_2-macroglobulin preparations studied. Soybean trypsin inhibitor was equally ineffective.

The hydrolysis of substituted arginine and lysine methyl esters by plasmin, by an α_2-macroglobulin-plas-

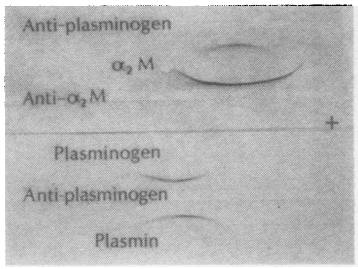

FIGURE 1 Immunoelectrophoretic pattern of α_2-macroglobulin (α_2-M) from urokinase-treated plasma (12 mg/ml, upper slide) diffused against human plasminogen or α_2-macroglobulin antibody. The immunoelectrophoretic pattern of human plasminogen or urokinase-activated plasmin is shown in the lower slide. The anode is to the right.

min complex, and by α_2-macroglobulin prepared from urokinase-treated plasma (Table II). Amino acid esters were employed as substrates to identify the enzyme associated with α_2-macroglobulin prepared from urokinase-treated plasma, because previous studies had suggested that proteolytic enzymes could be identified by the rates at which they catalyze the hydrolysis of different synthetic esters (31–33). α_2-Macroglobulin prepared from urokinase-treated plasma had a hydrolytic profile similar, but not identical, to that found for the α_2-macroglobulin-plasmin complex, suggesting that plasmin or plasmin-like activity was bound to this α_2-macroglobulin. On the other hand, though the observed ratio of the plasmin-catalyzed hydrolysis of BAMe, ALMe, and AGLMe relative to that of TAMe was characteristic for the free enzyme (32), the profile differed markedly from that of the α_2-macroglobulin-plasmin complex. This was clearly apparent in the impaired ability of the complex to hydrolyze BAMe, ALMe, and AGLMe, and on this basis, permits the conclusion that plasmin bound to α_2-macroglobulin has an altered substrate specificity.

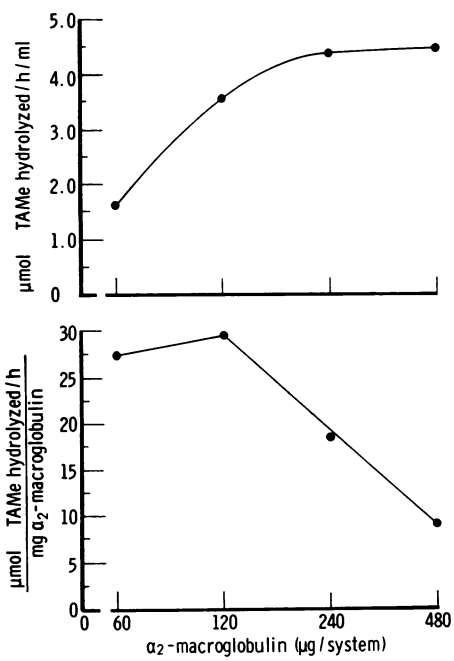

FIGURE 2 The binding of plasmin to α_2-macroglobulin as determined by the ability of increasing concentrations of α_2-macroglobulin to protect the TAMe esterase activity of a constant concentration of plasmin from inhibition by soybean trypsin inhibitor. Dilutions of α_2-macroglobulin were added to human plasmin (0.2 caseinolytic U). After 1 min preincubation, 100 μg soybean trypsin inhibitor was added, and the mixture was incubated with TAMe. The data in the upper graph are plotted to indicate the capacity of α_2-macroglobulin to protect bound plasmin against inhibition by soybean trypsin inhibitor. The data in the lower graph are plotted to indicate the specific activity of α_2-macroglobulin.

Immunologic identification of a plasmin-related antigen complexed to α_2-macroglobulin prepared from urokinase-treated plasma (Fig 1). Precipitin arcs with similar electrophoretic mobilities formed after immunoelectrophoresis (20) of α_2-macroglobulin from urokinase-treated plasma against antisera to human plasminogen or α_2-macroglobulin (Fig. 1, top slide). Purified human

TABLE II

The Hydrolysis of Substituted Arginine and Lysine Methyl Esters by Plasmin, by an α_2-Macroglobulin-Plasmin Complex, and by the α_2-Macroglobulin Prepared from Urokinase-Treated Plasma

Substrate*	Plasmin‡		α_2M-plasmin complex§		α_2M from UK-treated plasma‖	
	μmol/ml/h	*ratio*	*μmol/ml/h*	*ratio*	*μmol/ml/h*	*ratio*
TAMe	3.34	1.0	3.94	1.0	6.28	1.0
BAMe	5.43	1.6	2.64	0.67	3.92	0.62
ALMe	10.28	3.1	5.10	1.29	6.16	0.98
AGLMe	18.32	5.5	15.30	3.88	16.26	2.60

* The incubation mixture contained one of the following substrates in a final concentration of 0.015 M: TAMe, BAMe, ALMe, and AGLMe. Hydrolysis rates shown are the means of duplicate analyses. The ratios of these rates relative to that of TAMe are listed.

‡ 0.1 ml plasmin (2.0 caseinolytic U/ml) was added to 2.2 ml 0.1 M phosphate buffer, pH 7.6, containing 0.015 M of each of the substrates and the hydrolysis rates measured.

§ 0.1 mg purified α_2-macroglobulin from untreated plasma in a volume of 0.01 ml was preincubated for 1 min at 37°C with 0.01 ml plasmin (20 caseinolytic U/ml). 100 μg soybean trypsin inhibitor in 0.1 ml phosphate buffer was then added to inhibit residual plasmin not bound to α_2-macroglobulin. The mixture was made to a final volume of 2.3 ml as above, and then was assayed for esterolytic activity.

‖ 0.1 ml α_2-macroglobulin (0.3 mg) from plasma incubated with 500 U urokinase/ml was added to 2.2 ml phosphate buffer containing the ester substrates.

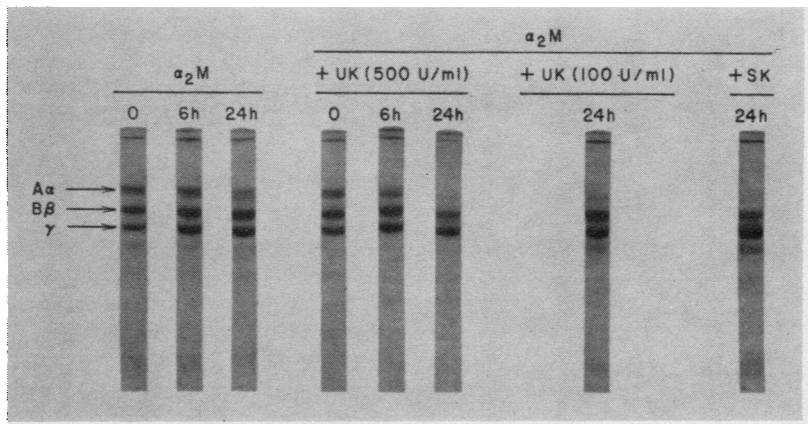

FIGURE 3 SDS-polyacrylamide gel electrophoresis of subunit chains of heat-precipitated fibrinogen after incubation with α_2-macroglobulin (α_2-M) preparations. α_2-Macroglobulin (4.8 mg/ml), prepared from untreated plasma or from plasma incubated with 100 or 500 U urokinase/ml or 500 U streptokinase/ml, was incubated at 37°C with fibrinogen (1.6 mg/ml) in 0.025 M Tris-HCl buffer, pH 7.5, containing 0.16% sodium citrate and 0.85% sodium chloride. Samples were removed at 0, 6, 24, and 48 h and the fibrinogen precipitated by heating. The precipitate was then redissolved in an SDS urea solution and reduced by treatment with DTT before acrylamide gel electrophoresis. From left to right, fibrinogen incubated with α_2-macroglobulin from untreated plasma, gels 1, 2, and 3; from plasma treated with 500 U urokinase/ml, gels 4, 5, and 6; from plasma incubated with 100 U urokinase/ml or with 500 U streptokinase/ml, gels 7 and 8, respectively.

plasminogen or urokinase-activated plasmin produced precipitin arcs similar to those described previously (34) (Fig. 1, lower slide) but differing in their electrophoretic mobilities from the α_2-macroglobulin associated plasminogen-related antigen. Since the antigenic properties of plasmin and plasminogen are the same (34), these findings suggest that in the samples tested, plasmin is complexed with α_2-macroglobulin and is the enzyme responsible for the esterolytic activity demonstrated in Table I.

Electroimmunoassay utilizing agarose gel containing plasminogen antibody (28–30) confirmed the presence of a plasmin-related antigen in α_2-macroglobulin from urokinase-treated plasma and permitted a semi-quantitative analysis of the lower limit of detectability of such an antigen by this technique. The lowest concentration of this α_2-macroglobulin sample that formed an immunoprecipitate was 0.6 mg/ml (20-fold dilution of the sample shown in Fig. 1). Though α_2-macroglobulin from untreated plasma failed to show immunoreactivity in this system at a protein concentration as high as 18 mg/ml, the 40-fold difference in the TAMe esterase activity (Table I) suggested that the enzymic activity in the untreated α_2-macroglobulin sample, even if attributable to plasmin, was too low to have been identified by this immunochemical method.

The binding capacity of α_2-macroglobulin for human plasmin (Fig. 2). The residual TAMe esterase activity of a mixture of α_2-macroglobulin and plasmin, after the addition of plasmin inhibitors (e.g., pancreatic or soybean trypsin inhibitor) reflects the plasmin that has been bound to α_2-macroglobulin and thereby protected from inhibition (3). Since the addition of α_2-macroglobulin to plasmin does not inhibit its TAMe esterase activity (Table II), such activity in the presence of a plasmin inhibitor can be used to measure α_2-macroglobulin-bound plasmin directly.

Based upon these considerations, the following experimental design was employed. After an incubation period of 1 min to allow for complex formation between α_2-macroglobulin and plasmin, soybean trypsin inhibitor was added. By comparison of specific activity (Fig. 2, lower graph) and esterase activity (Fig. 2, upper graph), it was evident that the lower concentrations of α_2-macroglobulin bound plasmin maximally. Since 1 caseinolytic U of plasmin catalyzed the hydrolysis of 28.2 μmol TAMe/h (Table I), it can be calculated that, under these conditions, 1 mg of the α_2-macroglobulin preparation bound approximately 1 caseinolytic U of plasmin. If we assume that the TAMe esterase activity of α_2-macroglobulin preparations from urokinase- or streptokinase-treated plasma was due to plasmin or a plasmin-like enzyme, similar calculations may be made. Therefore α_2-macroglobulin from plasma incubated with 100 U urokinase/ml bound approximately 0.2 caseinolytic U/mg α_2-macroglobulin, whereas α_2-macroglobulin prepared from plasma incubated with 500 U urokinase or streptokinase/ml bound 0.7 caseinolytic U/mg.

Degradation of fibrinogen by α_2-macroglobulin preparations (Figs. 3 and 4). The ability of α_2-macroglo-

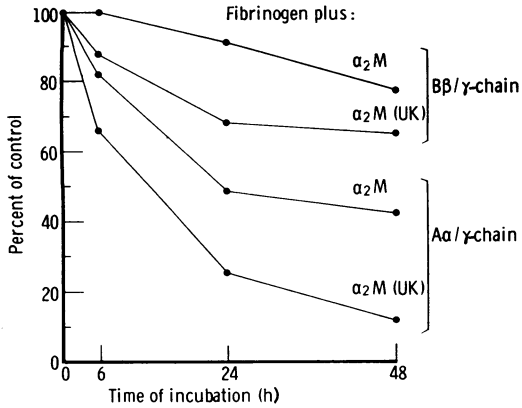

FIGURE 4 Sequential changes in the Aα- and Bβ-chains of fibrinogen induced by α_2-macroglobulin preparations as assessed by densitometric scans. The data are derived from scans of gels 1–6 of Fig. 3 and the corresponding 48-h samples. The ratios of the areas under the Aα- and Bβ-position, respectively, to that of the γ-chain position is expressed as percent of the ratio found at time 0.

bulin preparations to degrade fibrinogen was assessed by periodic sampling of a mixture of these two and determination of the resulting subunit structure of the fibrinogen. All α_2-macroglobulin preparations, including those purified in the presence of Polybrene and soybean trypsin inhibitor, or ε-aminocaproic acid and soybean inhibitor, induced progressive changes apparent from

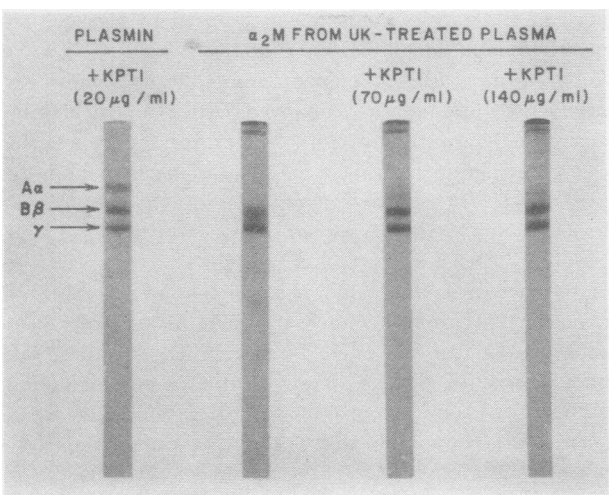

FIGURE 5 SDS-polyacrylamide gel electrophoresis of subunit chains of heat-precipitated fibrinogen after incubation with α_2-macroglobulin prepared from plasma treated with 500 U urokinase/ml. Fibrinogen (1.6 mg/ml) in the presence or absence of Kunitz pancreatic trypsin inhibitor (KPTI) was incubated at 37°C with human plasmin (0.07 caseinolytic U), or with α_2-macroglobulin (4.8 mg) prepared from urokinase-treated plasma. Samples were taken at 24 h. The gels referred to in the text are numbered from left to right.

inspection of the gels, as illustrated in Fig. 3, as well as from densitometric scans (Fig. 4), in which the depletion of intact Aα- or Bβ-chains was quantified by comparison with γ-chains. The γ-chains were assumed to be constant at this stage of digestion, since recent structural studies of circulating fibrinogen catabolites and of derivatives from plasmin-catalyzed digests (12, 35) indicate that γ-chains are relatively resistant to hydrolysis (12, 14–18), and that only small amounts of chains of non-γ origin occupy the γ-position in this system (12, 35).

The earliest and ultimately most extensive change was depletion of the intact Aα-chain position. Accompanying this, but at a slower rate (clearly evident in the densitometric scans) was a depletion of the intact Bβ-chain position and the appearance of a Bβ-chain derivative, termed /β-1 (36), which appeared in more extensively degraded samples (e.g., Fig. 3, gels 6–8). The /β-1 chain migrates in a position intermediate to that of intact Bβ- and γ-chains (12, 15, 37). Since this chain was often indistinct or not well resolved by the densitometric scan, its scan area was included in computation of the "intact" Bβ-chain position. Thus, the calculation of depletion of the Bβ-position in 24- and 48-h samples of urokinase- or streptokinase-treated α_2-macroglobulin was somewhat underestimated.

Compared with the proteolytic activity of α_2-macroglobulin from plasma, a far more extensive degradation of fibrinogen structure occurred in α_2-macroglobulin obtained from urokinase- or streptokinase-treated plasma. The fibrinogenolytic activity of the α_2-macroglobulin from urokinase-treated plasma (100 or 500 U/ml) was commensurate with the amount of urokinase added. Fibrinogen incubated with buffer or with urokinase alone showed no appreciable change in its subunit structure under the same experimental conditions, indicating that the structural changes that did occur were attributable solely to the proteolytic activity associated with the α_2-macroglobulin. Incubation of the α_2-macroglobulin preparations with urokinase failed to enhance their fibrinogenolytic activity, demonstrating the absence of detectable plasminogen.

To express the fibrinogenolytic potential of α_2-macroglobulin preparations in quantitative terms, a comparison was made between a selected degree of fibrinogen degradation catalyzed by known amounts of free plasmin and by α_2-macroglobulin. The time required to achieve that degree of degradation (viz., time for plasmin compared with time for α_2-macroglobulin) was converted to caseinolytic units by direct extrapolation. The results indicated that each milligram of α_2-macroglobulin obtained from untreated plasma had the fibrinogenolytic activity of approximately 10^{-5} caseinolytic U. Compared to this preparation, α_2-macroglobulin from urokinase-

treated plasma had an activity 3–5 times greater. In comparison with the amount of plasmin estimated to be bound to this preparation (i.e., 0.7 caseinolytic U/mg α_2-macroglobulin), the fibrinogenolytic potential of bound plasmin had been reduced to less than 0.1% of that of an equivalent amount of the free enzyme.

The effect of pancreatic trypsin inhibitor and ε-aminocaproic acid on the proteolysis of fibrinogen by plasmin or by α_2-macroglobulin-enzyme complexes (Fig. 5). Incubation of plasmin (0.07 caseinolytic U/ml) with fibrinogen resulted in extensive degradation of the subunit chains of fibrinogen within 10 min at 37°C (not shown). Pancreatic trypsin inhibitor (20 μg/ml) added to fibrinogen effectively inhibited this reaction as assessed by the 24-h sample (Fig. 5, gel 1). In sharp contrast, α_2-macroglobulin from urokinase-treated plasma caused extensive degradation of fibrinogen chains even in the presence of pancreatic trypsin inhibitor (gels 2, 3, and 4), though the preparations containing the inhibitor were somewhat less degraded. Thus, pancreatic trypsin inhibitor had only a slight effect in modifying the proteolytic activity of α_2-macroglobulin complexes. Parallel studies with ε-aminocaproic acid at concentrations of 0.1 M confirmed previous observations (38) that this agent is not an effective inhibitor of plasmin. Consistent with this, α_2-macroglobulin-mediated degradation of fibrinogen was also not effectively inhibited by ε-aminocaproic acid.

The effect of immunoprecipitation upon the fibrinogenolytic activity associated with α_2-macroglobulin preparations (Fig. 6). To provide even stronger evidence that all detectable fibrinogenolytic activity was closely associated with α_2-macroglobulin, the relationship of α_2-macroglobulin to its proteolytic activity was further explored by immunoprecipitation. An IgG fraction, itself possessing no antiplasmin activity as demonstrated by a fibrinolytic assay (39), having antibody specific for α_2-macroglobulin, was added in optimal amounts (40) to the α_2-macroglobulin prepared from urokinase-treated plasma. After removal of the precipitate which formed after an 18-h incubation at 4°C, the supernatant fluid contained no detectable α_2-macroglobulin as determined by electroimmunoanalysis (28). In contrast to the extensive fragmentation of fibrinogen rendered by the α_2-macroglobulin starting material, the supernatant fluid, depleted of α_2-macroglobulin, had lost its fibrinogenolytic potential as well.

DISCUSSION

The present study has demonstrated that human plasma α_2-macroglobulin preparations induce a time-dependent degradation in the subunit chains of fibrinogen. During this degradation certain species form whose structure resembles that of circulating fibrinogen catabolites; the sequence of degradation is similar to that observed in plasmin-catalyzed digests, in that Aα-chain fragmentation precedes that of Bβ-chain (12, 14–18). All α_2-macroglobulin preparations were capable of degrading fibrinogen, although those prepared from urokinase- or streptokinase-treated plasma had several times the fibrinogenolytic activity of the untreated control. The increase in the fibrinogenolytic activity associated with α_2-macroglobulin from urokinase- or streptokinase-treated plasma compared with the increase in TAMe esterase activity (viz., 3–5-fold vs. 40-fold, respectively), illustrated that only a small proportion of bound enzyme was expressed as proteolytic activity. Quantitation of the inhibition of the fibrinogenolytic activity of plasmin after complex formation with α_2-macroglobulin in plasma was possible, because mixing experiments established that α_2-macroglobulin failed to inhibit the TAMe esterase activity of plasmin. Therefore the TAMe esterase activity of α_2-macroglobulin preparations from plasma treated with urokinase was a direct reflection of the amount of plasmin bound to the inhibitor. By converting the TAMe activity to caseinolytic units plasmin, it could be calculated that 0.7 caseinolytic U of plasmin were bound/mg α_2-macro-

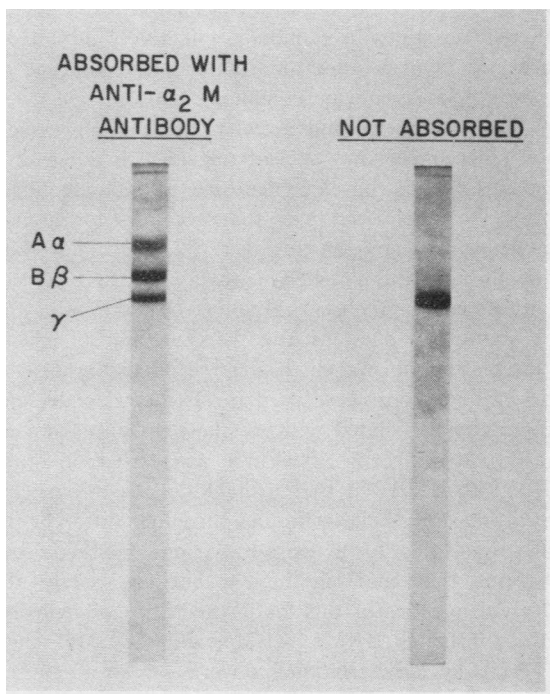

FIGURE 6 SDS-polyacrylamide gel electrophoresis of subunit chains of heat-precipitated fibrinogen after incubation with an antibody-absorbed α_2-macroglobulin preparation prepared from plasma treated with 500 U urokinase/ml, or with this preparation in the absence of immunoprecipitation. The electrophoretic patterns of samples taken at 48 h are shown in this figure.

globulin from urokinase-treated plasma (500 U urokinase/ml plasma). Less than 0.1% of this plasmin was expressed as fibinogenolytic activity. These data provide in quantitative terms a confirmation of the conclusion drawn by several previous investigators, that α_2-macroglobulin is a more effective inhibitor of proteolytic than of esterolytic activity (3, 4, 41). Thus, these studies suggest that in addition to the major role of α_2-macroglobulin as an inhibitor of plasmin, this protein also may function as a preserver of a portion of the proteolytic activity of the bound enzyme.

Our finding that α_2-macroglobulin preparations possess both fibrinogenolytic and esterolytic activities implies that these activities exist in plasma. It seems less likely that a substantial proportion of the observed activity was generated after blood collection, since the procedures of purification were performed under conditions designed to minimize proteolytic enzyme activation. Furthermore, the addition of plasminogen activators to plasma induces an increase in the TAMe esterase and in the fibrinogenolytic activity complexed to α_2-macroglobulin subsequently purified from this plasma. This indicates that a portion of the activity of the complex is preserved and can be increased in the presence of other plasma proteolytic enzyme inhibitors. The additional fact that the enzymic activities associated with α_2-macroglobulin cannot be effectively inhibited by pancreatic trypsin inhibitor supports the concept that α_2-macroglobulin-enzyme complexes provide one mechanism by which enzymic activity can be expressed in vivo. This concept has special relevance in view of the demonstration of the existence of a physiologic fibrinogenolytic catabolic pathway that accounts for a major proportion of fibrinogen turnover (12, 13). The relative contribution of this potential pathway for the production of fibrinogen catabolites as compared with other possible pathways, such as the direct action of plasmin on circulating fibrinogen, has not been explored.

Several methods were used to characterize the enzymic activity associated with α_2-macroglobulin from urokinase-treated plasma. The first was based on studies (31–33) that showed that proteolytic enzymes such as trypsin, plasmin, kallikrein, and thrombin differ in their relative abilities to hydrolyze various synthetic ester substrates, thus enabling them to be identified by their hydrolytic profile. In this study the ratios of hydrolysis of the substrates BAMe, ALMe, and AGLMe relative to TAMe by α_2-macroglobulin were similar to those of an α_2-macroglobulin-plasmin complex prepared in vitro, but dissimilar to those of free plasmin. This finding for plasmin is analogous to the reduction in the capacity to hydrolyze low molecular weight substrates observed when α_2-macroglobulin is added to trypsin (41–43), kallikrein (7), or cationic aspartate aminotransferase (44). The modification of enzymic hydrolysis of synthetic substrates resulting from the presence of α_2-macroglobulin underscores the difficulty in identifying proteolytic enzymes by their hydrolytic profile in complex biological fluids. Such identifications are based upon the assumption, shown here to be invalid for plasmin, that the substrate specificity of the enzyme is retained in a plasma or serum environment.

Immunoelectrophoretic analysis demonstrated that a plasmin-related antigen was complexed to α_2-macroglobulin from urokinase-treated plasma. In contrast, plasmin-related antigen could not be identified in α_2-macroglobulin from untreated plasma. The TAMe esterase activity of this preparation was so low, relative to that of α_2-macroglobulin from urokinase-treated plasma, that even if the activity were due to plasmin, it could not have been detected by this method.

Binding of plasmin by α_2-macroglobulin in vivo has been suggested by the observation of a decrease in plasma α_2-macroglobulin concentration after streptokinase (30, 45, 46) and urokinase (46, 47) infusion. Niléhn and Ganrot (30) have shown the formation of an α_2-macroglobulin-plasmin complex after streptokinase infusion by immunochemical techniques. These results have been extended in the present study by the demonstration that the addition of urokinase or streptokinase to plasma resulted in enhanced fibrinogenolytic and esterolytic activity associated with the α_2-macroglobulin ultimately purified from this incubation mixture, and by the subsequent identification, as outlined, of the enzyme responsible for this activity as plasmin.

The data from this study strongly suggest that α_2-macroglobulin-enzyme complexes possess fibrinogenolytic activity that cannot be due to free enzyme. This proteolytic activity is closely associated with the high molecular weight complex itself, as evidenced by the observation that pancreatic trypsin inhibitor, when added to fibrinogen, completely inhibited the proteolytic activity of fee plasmin but failed to prevent the degradation of fibrinogen by the α_2-macroglobulin preparations. Furthermore, immunoprecipitation of α_2-macroglobulin by specific antiserum removed the α_2-macroglobulin and its fibrinogenolytic activity. Several other investigators have concluded that α_2-macroglobulin-enzyme complexes produced their proteolytic effect by a dissociation of the enzyme from the complex (30, 48–50). These experiments, however, were not designed to test whether the observed activity was due to dissociation, or as has been demonstrated in this study, due to the complex itself, which protects the bound enzyme from plasmin inhibitors.

The identification of α_2-macroglobulin as an enzyme-binding protein with the potential for mediating fibrinogen catabolism suggests that this protein may regulate

other proteolytic enzyme systems as well. Support for this idea has been provided by McConnell (9) and by Dyce, Wong, Adham, Mehl, and Haverback (51), who have identified an α_2-macroglobulin fraction possessing kallikrein-like activity, and by Rinderknecht and Geokas (52), who have suggested that an α_2-macroglobulin fraction prepared from serum possessed clotting activity. Thus, α_2-macroglobulin may modulate enzyme-substrate interactions not only by its well-established function as an inhibitor of proteolytic activity, but also by providing a mechanism for the preservation and protection of a portion of the biologic activity of an enzyme in the presence of circulating inhibitors.

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REFERENCES

1. Schultze, H. E., N. Heimburger, K. Heide, H. Haupt, K. Störiko, and H. G. Schwick. 1963. Preparation and characterization of α_1-trypsin inhibitor and α_2-plasmin inhibitor of human serum. Proceedings 9th Congress European Society Haematology, Lisbon. S. Karger AG., Basel, Switzerland. 1315 (Abstr.).
2. Steinbuch, M., M. Quentin, and L. Pejaudier. 1964. Technique d'isolement et étude de l'α_2-macroglobuline. *In* Protides of the Biological Fluids. H. Peeters, editor. Elsevier, N. V. Uitgeuerij. Mij. Amsterdam. 375.
3. Ganrot, P. O. 1967. Inhibition of plasmin activity by α_2-macroglobulin. *Clin. Chim. Acta.* **16**: 328.
4. Lanchantin, G. F., M. L. Plesset, J. A. Friedmann, and D. W. Hart. 1966. Dissociation of esterolytic and clotting activities of thrombin by trypsin-binding macroglobulin. *Proc. Soc. Exp. Biol. Med.* **121**: 444.
5. Steinbuch, M., C. Blatrix, and F. L. Josso. 1968. Action anti-protéase de l'α_2 macroglobuline. II. Son rôle d'anti-thrombine progressive. *Rev. Fr. Étud. Clin. Biol.* **13**: 179.
6. Shapiro, S. S., and J. Cooper. 1968. Biological activation of radioactive human prothrombin. *Fed. Proc.* **27**: 628, 2321 (Abstr.).
7. Harpel, P. C. 1970. Human plasma alpha 2-macroglobulin. An inhibitor of plasma kallikrein. *J. Exp. Med.* **132**: 329.
8. Harpel, P. C. 1971. Separation of plasma thromboplastin antecedent from kallikrein by the plasma α_2-macroglobulin, kallikrein inhibitor. *J. Clin. Invest.* **50**: 2084.
9. McConnell, D. J. 1972. Inhibitors of kallikrein in human plasma. *J. Clin. Invest.* **51**: 1611.
10. Mosesson, M. W., and S. Sherry. 1966. The preparation and properties of human fibrinogen of relatively high solubility. *Biochemistry.* **5**: 2829.
11. Mosesson, M. W., N. Alkjaersig, B. Sweet, and S. Sherry. 1967. Human fibrinogen of relatively high solubility. Comparative biophysical, biochemical, and biological studies with fibrinogen of lower solubility. *Biochemistry.* **6**: 3279.
12. Mosesson, M. W., J. S. Finlayson, R. A. Umfleet, and D. Galanakis. 1972. Human fibrinogen heterogeneities. I. Structural and related studies of plasma fibrinogens which are high solubility catabolic intermediates. *J. Biol. Chem.* **247**: 5210.
13. Sherman, L. A., A. P. Fletcher, and S. Sherry. 1969. In vivo transformation between fibrinogens of varying ethanol solubilities: a pathway of fibrinogen catabolism. *J. Lab. Clin. Med.* **73**: 574.
14. Mills, D., and S. Karpatkin. 1970. Heterogeneity of human fibrinogen: possible relation to proteolysis by thrombin and plasmin as studied by SDS-polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* **40**: 206.
15. Pizzo, S. V., M. L. Schwartz, R. L. Hill, and P. A. McKee. 1972. The effect of plasmin on the subunit structure of human fibrinogen. *J. Biol. Chem.* **247**: 636.
16. Sherman, L. A., M. W. Mosesson, and S. Sherry. 1969. Isolation and characterization of the clottable low molecular weight fibrinogen derived by limited plasmin hydrolysis of human fraction I-4. *Biochemistry.* **8**: 1515.
17. Gaffney, P. J., and P. Dobos. 1971. A structural aspect of human fibrinogen suggested by its plasmin degradation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **15**: 13.
18. Furlan, M., and E. A. Beck. 1972. Plasmin degradation of human fibrinogen. I. Structural characterization of degradation products. *Biochem. Biophys. Acta.* **263**: 631.
19. Nossel, H. L., H. Rubin, M. Drillings, and R. Hsieh. 1968. Inhibition of Hageman factor activation. *J. Clin. Invest.* **47**: 1172.
20. Scheidegger, J. J. 1955. Une micro-méthode de l'immuno-électrophorèse. *Int. Arch. Allergy Appl. Immunol.* **7**: 103.
21. Mosesson, M. W. 1962. The preparation of human fibrinogen free of plasminogen. *Biochim. Biophys. Acta.* **57**: 204.
22. Mosesson, M. W., and J. S. Finlayson. 1963. Subfractions of human fibrinogen. Preparation and analysis. *J. Lab. Clin. Med.* **62**: 663.
23. Sgouris, J. T., J. K. Inman, K. B. McCall, L. A. Hyndman, and H. D. Anderson. 1960. The preparation of human fibrinolysin (plasmin). *Vox Sang.* **5**: 357.
24. Collen, D., E. B. Ong, and A. J. Johnson. 1972. Native human plasminogen. *Fed. Proc.* **31**: 2, 108. (Abstr.)
25. Mancini, G., J. F. Vaerman, A. O. Carbonara, and J. F. Heremans. 1963. A single-radial diffusion method for immunological quantitation of proteins. *In* Protides of the Biological Fluids. H. Peeters, editor. Elsevier, N. V. Uitgeuerij. Mij., Amsterdam. 370.
26. Harpel, P. C. 1970. A sensitive, colorimetric method for the measurement of serum $C\bar{1}$ inactivator using the substrate *N*-α-acetyl-L-lysine methyl ester. *J. Immunol.* **104**: 1024.
27. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406.
28. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel-containing antibodies. *Anal. Biochem.* **15**: 45.
29. Laurell, C. B. 1972. Electroimmunoassay. *Scand. J. Clin. Lab. Invest.* **29**:(Suppl. 124) 21.

30. Niléhn, J. E., and P. O. Ganrot. 1967. Plasmin, plasmin inhibitors, and degradation products of fibrinogen in human serum during and after intravenous infusion of streptokinase. *Scand. J. Clin. Lab. Invest.* **20**: 113.
31. Sherry, S., N. Alkjaersig, and A. P. Fletcher. 1965. Comparative activity of thrombin on substituted arginine and lysine esters. *Am. J. Physiol.* **209**: 577.
32. Sherry, S., N. Alkjaersig, and A. P. Fletcher. 1966. Activity of plasmin and streptokinase-activator on substituted arginine and lysine esters. *Thromb. Diath. Haemorrh.* **16**: 18.
33. Colman, R. W., L. Mattler, and S. Sherry. 1969. Studies on the prekallikrein (kallikreinogen)-kallikrein enzyme system of human plasma. I. Isolation and purification of plasma kallikreins. *J. Clin. Invest.* **48**: 11.
34. Robbins, K. C., and L. Summaria. 1966. An immunochemical study of human plasminogen and plasmin. *Immunochemistry.* **3**: 29.
35. Finlayson, J. S., M. W. Mosesson, T. J. Bronzert, and J. J. Pisano. 1972. Human fibrinogen heterogeneities. II. Cross-linking capacity of high solubility catabolic intermediates. *J. Biol. Chem.* **247**: 5220.
36. Mosesson, M. W., J. S. Finlayson, and D. Galanakis. 1972. Human fibrinogen structure: characteristics of circulating catabolic intermediates and of derivatives produced in vitro by plasmin digestion. Proceedings 3rd Congress International Society Thrombosis and Haemostasis, Washington, D. C. Abstracts. 128.
37. Mills, D., and S. Karpatkin. 1972. The initial macromolecular derivatives of human fibrinogen produced by plasmin. *Biochim. Biophys. Acta.* **271**: 163.
38. Alkjaersig, N., A. P. Fletcher, and S. Sherry. 1959. ε-Aminocaproic acid: an inhibitor of plasminogen activation. *J. Biol. Chem.* **234**: 832.
39. Harpel, P. C. 1972. Studies on the interaction between collagen and a plasma kallikrein-like activity. Evidence for a surface-active enzyme system. *J. Clin. Invest.* **51**: 1813.
40. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. Charles C Thomas Publisher. Springfield, Ill. 2nd edition. 69.
41. Mehl, J. W., W. O'Connell, and J. DeGroot. 1964. Macroglobulin from human plasma which forms an enzymatically active compound with trypsin. *Science (Wash. D. C.).* **145**: 821.
42. Howard, S. M. 1966. Studies of trypsin-binding α_2-macroglobulin of human plasma. Dissertation. University of Southern California.
43. Nagasawa, S., B. H. Han, H. Sugihara, and T. Suzuki. 1970. Studies on α_2-macroglobulin in bovine plasma. II. Interaction of α_2-macroglobulin and trypsin. *J. Biochem.* **67**: 821.
44. Boyde, T. R. C. 1969. The interaction between α_2-macroglobulin and cationic aspartate aminotransferase. *Biochem. J.* **111**: 59.
45. Spöttl, F., and F. Holzknecht. 1970. The influence of inhibitors of plasmin and plasminogen activation on the streptokinase-induced fibrinolytic state. *Thromb. Diath. Haemorrh.* **24**: 100.
46. Fischer, M. 1971. Influence of thrombolytic therapy on human antiplasmins. Thrombolytic Therapy: Transactions of the 19th Annual Symposium on Blood. Friedrich-Karl Schattauer-Verlag. Stuttgart, Germany. 153.
47. Arnesen, H., and M. K. Fagerhol. 1972. α_2-Macroglobulin, α_1-antitrypsin, and antithrombin III in plasma and serum during fibrinolytic therapy with urokinase. *Scand. J. Clin. Lab. Invest.* **29**: 259.
48. Norman, P. 1958. Studies on the plasmin system. II. Inhibition of plasmin by serum or plasma. *J. Exp. Med.* **108**: 53.
49. Norman, P. S., and B. M. Hill. 1958. Studies of the plasmin system. III. Physical properties of the two plasmin inhibitors in plasma. *J. Exp. Med.* **108**: 639.
50. Ambrus, C. M., and G. Markus. 1960. Plasmin-antiplasmin complex as a reservoir of fibrinolytic enzyme. *Am. J. Physiol.* **199**: 491.
51. Dyce, B., T. Wong, N. F. Adham, J. Mehl, and B. J. Haverback. 1967. Human plasma kallikrein esterase associated with the alpha-2-macroglobulin-binding protein. *Clin. Res.* **15**: 101. (Abstr.)
52. Rinderknecht, H., and M. C. Geokas. 1972. Role for α_2-macroglobulin in haemostatic balance. *Nat. New Biol.* **239**: 116.