HUMAN PLASMA ALPHA 2-MACROGLOBULIN
AN INHIBITOR OF PLASMA KALLIKREIN*

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Activation of the human plasma kallikrein enzyme system results in the production of pharmacologically potent polypeptides known as kinins. These substances, in nanogram quantities, produce vasodilation, incite pain, increase vascular permeability, stimulate leukotaxis, and act on uterine and intestinal smooth muscle (1). The role of the kallikrein system in human disease is becoming more apparent (1-3). The plasma of patients with hereditary angioneurotic edema has been found to be deficient in Cl inactivator (5, 6) an α 2-neuraminoglycoprotein which in purified systems inhibits kallikrein, Cl esterase, PF/dil and plasmin (5, 7-9). In patients lacking this inhibitor, Cl activation has been demonstrated in vivo during attacks of angioedema (10, 11). The evidence for activation of the kallikrein system under similar circumstances is less convincing than that obtained for the complement system. As a consequence, it has been suggested that there may be other physiologically important inhibitors of plasma kallikrein (12).

In the present study, examination of the kinetics of kaolin-induced activation of plasma kallikrein esterolytic activity (13-15) in normal plasma and in plasma from patients with hereditary angioneurotic edema has indicated that a protein other than Cl inactivator is a major plasma inhibitor of kallikrein. This inhibitor has been identified as the plasma α 2-macroglobulin and has been shown to inhibit both the esterolytic and kinin-producing activities of plasma kallikrein.

Materials

Substrates.—N-acetyl-L-tyrosine ethyl ester (ATEe), N-α-acetyl-L-lysine methyl ester (ALMe), N-benzyol-L-arginine methyl ester (BAMe) and tosyl arginine methyl ester (TAMe)

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1 The complement terminology used in this report conforms to the nomenclature adopted in the World Health Organization Bulletin (4). In the new terminology, Cl inactivator replaces C1 esterase inhibitor. Cl designates the activated first component of complement and C1s the activated subcomponent of Cl.
2 Abbreviations used in this paper: ALMe, N-α-acetyl-L-lysine methyl ester; BAMe, N-benzyol-L-arginine methyl ester; BAPNA, benzoyl-DL-arginine-p-nitroanilide; HANE, hereditary angioneurotic edema; PEG, polyethylene glycol; PTA, plasma thromboplastin antecedent; SBTI, soybean trypsin inhibitor; TAMe, tosyl arginine methyl ester.

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were obtained from Cyclo Chemical Corp., Los Angeles, Calif. Benzoyl-\(\text{L}-\text{arginine-p-nitroanilide}\) (BAPNA) was obtained from Mann Research Laboratories, Inc. New York.

Soybean Trypsin Inhibitor.—Crude and purified soybean trypsin inhibitor preparations (SBTI) were obtained from Worthington Biochemical Corp., Freehold, N. J. The concentration of purified SBTI used in various experiments was calculated by determining the optical density of the SBTI solution at 280 m\(\mu\). This value was multiplied by 1.1 to convert it to the concentration of SBTI expressed in milligrams per milliliter (16).

Bradykinin triacetate (Calbiochem, Los Angeles, Calif.) was dissolved in 0.15 m sodium chloride and stored at -20°C.

Plasma.—Blood from normal individuals, from patients with hereditary angioneurotic edema, and from an individual with plasma thromboplastin antecedent (PTA) deficiency was collected in plastic syringes and placed in \(\frac{1}{4}\) volume of 3.8% sodium citrate in polypropylene test tubes. Plasma was obtained following centrifugation at 3000 rpm in an International refrigerated centrifuge model PR-2, and frozen in aliquots in polypropylene at -20°C. The plasma was thawed only once for use.

Antisera.—Rabbit anti whole human antiserum, anti \(\alpha_2\)-macroglobulin antiserum and antifibrinogen antiserum (Behring Diagnostics, Woodbury, N.Y.) were used in the various immunologic assays.

Methods

Kaolin activation of plasma arginine esterase was performed by a modification of the Colman technique (3). 10 mg of solid kaolin (Fisher Scientific Company, Fair Lawn, N. J.) was added per ml of plasma in plastic tubes at 25°C. 0.1 ml portions were removed at various intervals and pipetted into 2.2 ml of 0.1 m sodium phosphate buffer, pH 7.5, containing TAMe in a final concentration of 0.05 m in an ice bath. In experiments comparing the hydrolysis of BAMe and ALMe relative to TAMe, the final substrate concentration used was 0.015 m (15). In some experiments 0.6 mg SBTI was also included in the substrate tube. The hydrolysis of the substrate was measured by a previously described modification of the Siegelman technique (17, 18).

Esterolytic assays of column chromatography fractions of kallikrein preparations and of various incubation mixtures of kallikrein, \(\alpha_2\)-macroglobulin, SBTI and Cl inactivator were performed by a similar technique. The concentrations of the reactants used are detailed in the figure and table legends.

Acid-treated plasma was prepared as has been described (15, 19). One volume of \(\frac{1}{2}\) N HCl was added to plasma and allowed to incubate at 25°C for 10 min before neutralizing with 1 volume of \(\frac{1}{2}\) N NaOH and 1 volume of 0.1 m sodium phosphate buffer, pH 8.0. In kaolin activation experiments, 10 mg of solid kaolin per ml acid-treated plasma solution was added and 0.4 ml of solutions removed at various intervals for assay of TAMe esterase activity.

Cl inactivator concentrations in the plasma of individuals with hereditary angioneurotic edema were determined as previously described (18).

Benzoyl-\(\text{L}-\text{arginine-p-nitroanilide}\), (BAPNA) was used to test for trypsin-like activity associated with various kallikrein and \(\alpha_2\)-macroglobulin preparations (20).

Protein was measured by the Lowry procedure (21) using bovine serum albumin (Pentex, Inc., Kankakee, Ill.) as the reference standard.

Chromatography.—Diethylaminoethyl (DEAE) cellulose (DE-52, H. Reeve Angel and Co., Inc., Clifton, N. J.) was prepared according to the manufacturer’s instructions and equilibrated with buffers as indicated in the figure legends. Sephadex G-200, 40-120 \(\mu\) (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) was swollen and packed by gravity in citrate–sodium chloride buffer. Blue Dextran 2000 (Pharmacia) was used to determine the void volume of the G-200 column. Hydroxylapatite and nonionic cellulose were obtained from Bio-Rad
Laboratories, Richmond, Calif. All column chromatographic procedures were carried out at 4°C.

Immunoelectrophoresis (22), double diffusion analysis (23) and quantitative radial immunodiffusion (24) were performed by established techniques.

Acrylamide gel electrophoresis was performed as previously described (25), a 4% gel being used for the separation of purified α₂-macroglobulin.

Purification of Plasma α₂-Macroglobulin, Kallikrein, C1s and C1 Inactivator.—α₂-macroglobulin was purified from freshly obtained human plasma by modifications of previously described methods (26-28). The purification procedure consisted of fractional precipitation with polyethylene glycol and Rivanol followed by gel filtration chromatography. Whole blood was collected in plastic tubes in 1/6 volume 3.8% sodium citrate containing crude soybean inhibitor to achieve a final concentration of 0.2 mg per ml in whole blood. All subsequent procedures were carried out in polypropylene containers. Following centrifugation the plasma coagulation factors II, VII, IX and X were removed by consecutive adsorption with barium chloride and barium sulfate. Two volumes of 0.02 M phosphate buffer, pH 7.4, containing 0.1 M NaCl were added to the adsorbed plasma and 50% polyethylene glycol (PEG) (6000 molecular weight, General Biochemicals Div., Chagrin Falls, Ohio) (29, 30) was added to a final concentration of 4% at 0°C. The precipitate contained the bulk of the plasma fibrinogen. The concentration of PEG in the supernatant was increased to 12%. The second precipitate containing the plasma α₂-macroglobulin was resuspended in 1/5 the original plasma volume of 0.1 M sodium phosphate buffer, pH 7.6. 0.4 volume of 0.5% Rivanol (K&K Laboratories, Plainview, N. Y.) was added to the crude resuspended α₂-macroglobulin, the precipitate discarded, and an additional 0.6 volume 0.5% Rivanol was added. This precipitate was extracted with 1/20 the original plasma volume of 5% sodium chloride for 30 min, dialyzed against distilled water for 2 hr, then for 18 hr against 0.85% sodium chloride, 0.16% sodium citrate (citrate-saline buffer). The α₂-macroglobulin comprised 15-25% of the total protein of this Rivanol fraction.

Further purification was achieved by column chromatography on a 5 × 100 cm agarose column (Bio-Gel A-1.5m, 200-400 mesh, Bio-Rad Laboratories, Richmond, Calif.) in citrate-saline buffer. The fractions containing α₂-macroglobulin, as identified by double immunodiffusion analysis using α₂-macroglobulin antibody, were pooled, precipitated by adding 50% PEG to a final concentration of 15%, and resuspended in citrate-saline buffer in a concentration of 24 mg α₂-macroglobulin per ml and stored in plastic tubes at 4°C. The total yield was about 50% calculated on the basis of a plasma α₂-macroglobulin concentration of 200 mg/100 ml.

Immunoelectrophoresis of this product showed traces of gamma M macroglobulin and haptoglobin in addition to the main α₂-macroglobulin arc. No CI inactivator was identifiable in this preparation by double immunodiffusion analysis using CI inactivator antibody generously provided by Dr. Fred Rosen, or by a biochemical assay for this inhibitor (18). The identity of the major protein as the α₂-macroglobulin was further established by the ability of this preparation to protect a purified trypsin solution from inhibition by SBTI utilizing BAPNA as the substrate (26). No BAPNA hydrolyzing activity was detected in the α₂-macroglobulin preparation. These preparations had low levels of spontaneous TAME esterase activity averaging 0.07 μmoles of TAME hydrolyzed/min per mg α₂-macroglobulin. In contrast, α₂-macroglobulin prepared as described, but without SBTI added to the freshly collected whole blood, contained approximately ten times the TAME esterase activity of the inhibited plasma. In some instances the α₂-macroglobulin preparation obtained following gel filtration was further purified by preparative electrophoresis on Pevikon (Mercer Chem. Co., New York.) (31) and the α₂-macroglobulin fractions so obtained contained no other proteins identifiable by double diffusion analysis using polyvalent rabbit antihuman serum antibody.

Preparation of α₂-Macroglobulin from Kaolin-Activated Plasma.—α₂-macroglobulin was
purified from kaolin-activated plasma or from noncontact, inhibited plasma by methods similar to the method described. The plasma to be activated by kaolin was collected in polypropylene containers without the addition of SBTI. Following incubation with 10 mg of kaolin per ml plasma at room temperature, the α2-macroglobulin was purified as described. Alternately the inhibited plasma was collected in SBTI and Trasylol (compound A-128, proteinase inhibitor; FBA Medical Research Division of Metachem, Inc., New York) and all buffers used in the purification procedure contained 0.2 mg per ml SBTI and 200-250 units Trasylol (15, 32).

Plasma kallikrein was purified from outdated plasma by a method based on the finding that human plasma celite eluate was a source of crude kallikrein (33). Coagulation factors, II, VII, IX, and X were removed from the plasma as described in the purification procedure for α2-macroglobulin. The plasma was treated with acid and neutralized as previously detailed in order to destroy the plasma kallikrein inhibitors (15, 19, 34). This procedure increased the yield of arginine esterase, rat uterine-contracting and PTA-clotting activity in the celite eluate by 4-fold as compared to the celite eluate from untreated plasma. Celite, 20 mg per ml final concentration, (Celite Analytical Filter Aid, Johns-Manville Celite Division, Cleveland, Ohio) was added and mixed with the acid-treated plasma for 15 min. The celite was washed four times each with twice the plasma volume of 0.15 m sodium chloride and eluted with 2.5 times the original plasma volume of 10% sodium chloride in one of two buffers, either 0.01 M sodium phosphate buffer, pH 6.0, or 0.05 M sodium carbonate buffer, pH 10.0. The eluates were dialyzed against distilled water for 18 h, followed by 48 h of dialysis against large volumes of 0.05 M Tris-chloride, pH 8.0, containing 0.02 M sodium chloride. The recovery of TAME esterase activity, comparing the celite eluates to the esterase activity of the starting kaolin-activated plasma was 18% at pH 6.0, and 32% at pH 10.0. The specific activities of the two eluates were 3.8 μmoles of TAME/min per mg at pH 6.0, and 0.7 μmoles of TAME/min per mg at pH 10.0. Compared to the starting kaolin-activated plasma, this represented a 223-fold and 41-fold purification of TAME esterase activity.

The celite eluates obtained at pH 10.0 and 6.0, were further purified by DEAE-cellulose chromatography (Whatman DE 52 preswollen microgranular exchanger). The eluate was applied to a 2.5 × 30 cm column equilibrated with 0.05 M Tris-chloride buffer, pH 8.0, containing 0.02 M sodium chloride. The material not absorbed to the column was concentrated by pressure ultrafiltration. The specific activity of this material calculated as previously described (35), was 2.1 μmoles of TAME/min per mg protein for the pH 10.0 extracted eluate, representing a 200-fold purification over the starting plasma, and varied between 7.0 and 10.0 μmoles TAME/min per mg for several different preparations of the pH 6.0 celite eluate. The most highly purified preparations achieved a 500-fold purification of TAME esterase activity. This was comparable to the TAME esterase activity reported for purified plasma kallikrein (35).

The probable identity of the TAME esterase in these two preparations as plasma kallikrein was suggested by the finding that the ratio of hydrolysis of BAME and ALMe relative to TAME was similar to that described previously for plasma kallikrein (35). Both SBTI and Cl– inactivator in appropriate concentrations inhibited the esterolytic activity of the DEAE-cellulose purified celite eluates. These preparations were active in stimulating the contraction of the rat uterus in the presence of both unheated plasma and plasma heated to 61°C for 30 min, a test which distinguishes kallikrein from PF/dil (36-40). This activity was inhibited by SBTI and Cl– inactivator. That the uterine-contracting substance released by the action of the kallikrein preparation on heated plasma was a kinin-like material was established by the destruction of this activity by incubation with carboxypeptidase B (Worthington Biochemical Corp., Freehold, N. J.) (41). These preparations also induced vascular permeability.

Harpel, P., and A. Samarel, 1969. Unpublished observations.
changes in the skin of guinea pigs. This effect was abolished by preincubation with C1 inactivator, indicating that the active substance behaved as a kallikrein (42).

The kallikrein preparations contained no detectable plasminogen, plasmin (43), C1 (44), or clotting factors II, V, VII-X (45), or X activity (46). No factor VIII or IX activity was demonstrated using specifically deficient plasma (47). Factor XI activity was identified using specific PTA deficient plasma (48). A standard calibration curve was established relating arbitrary units of activated PTA to clotting time in which 1 ml of normal plasma contained 10 PTA units. Representative PTA concentrations for the DEAE-cellulose purified celite eluate extracted at pH 6.0 were 5.2 PTA units/TAMe unit, and for the pH 10.0 preparation, 7.3 PTA units/TAMe unit. A TAMe unit was defined as that volume which hydrolyzed 1 μ mole TAMe/min. Although assays could not be performed for Hageman factor due to the presence of activated PTA, it is unlikely that significant concentrations were present in the kallikrein preparations as human Hageman factor is absorbed to DEAE-cellulose (49, 50). A kallikrein preparation containing 20% of the PTA activity of the DEAE-cellulose purified material was prepared by hydroxylapatite column chromatography of the celite eluate. Chromatographic procedures were performed as described by Hjerten (51). The major TAMe esterase, kallikrein peak was absorbed to the column and contained 1.1 PTA unit per TAMe unit.

C1s, the activated subunit of the first component of complement, was prepared by a method modified from that of Haines and Lepow (18, 52).

C1 inactivator was purified from human serum according to the method of Pensky (7, 18) and its activity measured using acetyl tyrosine ethyl ester as the substrate for purified C1s as has been previously described (53, 54). No α2-macroglobulin was detectable by immunodiffusion in the C1 inactivator preparations, nor did these preparations display trypsin inhibitory activity in the BAPNA assay (20).

The evolution of kinin-like activity was assayed by measuring the contraction of a rat uterine horn as described in Table III (55). Uterine contractions were measured isotonically with a linear motion transducer, model ST-2, the transformer of which was supplied by an exciter-demodulator (Phipps and Bird Inc., Richmond, Va.) and recorded on a Bausch and Lomb VOM-5 recorder.

Vascular permeability was measured by a method similar to that described by Miles and Wilhelm (56) using depilated female albino guinea pigs weighing 450–600 g which had been injected intravenously with 0.5% Evans blue (Warner-Chilcott, Morris Plains, N. J.) in amounts of 2.0 ml/kg body weight. Test solutions were injected intradermally as detailed in Table IV.

RESULTS

The Effect of Soybean Trypsin Inhibitor on the Kaolin-Activated Arginine Esterase Activity of Normal Plasma and Plasma from Persons with Hereditary Angioneurotic Edema (HANE).—Incubation of normal plasma with kaolin (Fig. 1, left panel) resulted in the development of TAMe esterase activity similar to that described by Colman et al (15). Peak esterolytic activity occurred at 1 min of incubation, following which there was a reduction in activity to 50% of peak activity at 5 min. In a parallel experiment, SBTI was added to the substrate solution to study the effect of this inhibitor on the active enzyme(s). In the presence of SBTI, the 1 min peak activity was reduced by 86%, however, proportionally less inhibition by this inhibitor was demonstrated as the activation continued.
Plasma, treated with acid, a procedure known to inactivate plasma inhibitors of several proteolytic enzymes (19, 34) was incubated with kaolin and the TAMe esterase activity evolved measured with and without SBTI added to the substrate (Fig. 1, right panel). As has been described (15), no loss of TAMe esterase activity occurred with time, which suggests that the decay in esterase activity in nonacid-treated plasma was due to the presence of plasma inhibitors. In contrast to the findings in kaolin-activated plasma, SBTI added to the TAMe substrate, prior to the addition of the kaolin-activated acid-treated plasma, produced a profound reduction in esterolytic activity. Approximately 89% of the esterase activity of acid-treated kaolin-activated plasma was inhibited at 5 min by SBTI in comparison to the 63% inhibition in nonacid-treated plasma.

Since partially purified preparations of PTA (factor XI) have been shown to possess arginine esterase activity (57), and since kaolin activates this enzyme in plasma (58), the possible contribution of this factor to the kaolin-activated plasma TAMe esterase activity was assessed. Confirming previous findings (59), PTA-deficient plasma showed a kaolin-activated TAMe esterase curve identical with that found in normal plasma. Furthermore, the esterase activity assayed with SBTI added to the substrate was similar to that found in normal plasma. The contribution of PTA to the plasma esterase activation curves was therefore probably insignificant.

The TAMe esterase activation curves of plasma from four persons belonging
to one kindred with a clinical history consistent with hereditary angioneurotic edema (HANE) and whose plasma were biochemically and immunologically deficient in C1 inactivator, were examined. A representative experiment in which a C1 inactivator deficient plasma was activated before or following acid treatment is presented (Fig. 2). Peak TAME esterase activity occurred at 1 min and was greater than in normal plasma (Fig. 2, left panel). At 5 min there was a reduction to 70% of peak activity. The TAME esterase activity of the four patients studied at peak activity (1 min) and at 5 min is presented in Table I.

![Graph](image_url)

**Fig. 2.** The effect of soybean trypsin inhibitor on the TAME esterase activity of kaolin-activated plasma and kaolin-activated acid-treated plasma from a patient with hereditary angioneurotic edema (HANE). Plasma obtained from a patient with no detectable C1 inactivator (see Methods) was activated with kaolin, or acid treated and activated. The time course of TAME esterase activation with and without SBTI added to the TAME substrate was followed as detailed in Fig. 1.

The mean decrease in esterolytic activity comparing the 5 and 1 min samples was 30.1%. SBTI added to the substrate TAME caused a 72% reduction in the 1 min peak activity. As demonstrated with normal plasma, SBTI caused less inhibition as the activation progressed. The TAME esterase kaolin activation curve of acid-treated HANE plasma (Fig. 2, right panel) paralleled that demonstrated for normal plasma. Acid treatment prevented the reduction in TAME esterase activity observed with time in untreated HANE plasma. The arginine esterase activity of the acid-treated HANE plasma was inhibited by 88% when SBTI was added to the substrate (Fig. 2, right panel).

The ratio of hydrolysis of ALMe and BAMe relative to TAME by normal and HANE plasma at peak activation by kaolin (1 min) and at 5 min of incubation,
when decay in activity had occurred, was compared and found to be similar. Kaolin-activated acid-treated normal and acid-treated HANE plasma hydrolyzed the substrates in a similar fashion. These results supported the concept that the same esterase, or closely related esterases were responsible for the enzymic activity in the preparations studied, and suggested that an acid-sensitive factor partially inhibited the esterolytic enzyme and protected it from inhibition by SBTI. Since human plasma α2-macroglobulin has been reported to possess these characteristics in relation to the enzymes plasmin, trypsin and thrombin (28, 60, 61), the effect of purified α2-macroglobulin on partially purified plasma kallikrein was studied.

The Effect of Increasing Concentrations of α2-Macroglobulin, Cl Inactivator and SBTI on a Constant Concentration of Purified Plasma Kallikrein TAME Esterase.—Kallikrein TAME esterase activity was progressively inhibited by increasing concentrations of α2-macroglobulin, Cl inactivator and SBTI (Fig. 3). Inhibition by α2-macroglobulin, in the experiment illustrated, was maximal at 53% and was not increased by adding a 4-fold increase in α2-macroglobulin concentration. Similar results were obtained utilizing an α2-macroglobulin preparation purified by Pevikon block electrophoresis which contained no other proteins identifiable by double diffusion analysis or by acrylamide gel electro-

### TABLE I

| Patient | C1 inactivator* | TAME esterase activity† | Reduction of esterase activity at 5 min compared to 1 min ‡ | Reduction of esterase activity at 5 min with SBTI added to substrate ||
|---------|-----------------|-------------------------|-----------------------------------------------------------|-----------------------------------------------------------|
|         | units/ml        | μmoles/ml/ hr           | %                                                         | %                                                         |
| H. D.   | 0               | 127.8                   | 90.3                                                      | 70.6                                                      | 29.3                                                | 21.8                        |
| B. D.   | 3.1             | 120.9                   | 91.3                                                      | 67.6                                                      | 24.5                                                | 26.0                        |
| C. K.   | 2.5             | 113.5                   | 72.5                                                      | 46.4                                                      | 36.1                                                | 36.0                        |
| F. D.   | 0               | 94.5                    | 65.9                                                      | 48.1                                                      | 30.3                                                | 27.0                        |

* The concentration of C1 inactivator in the patient's plasma was measured by a method described in the text (see Methods), the normal concentration being 41.5 ± 8.4 units/ml.
‡ 2.0 ml plasma from each of 4 persons with hereditary angioneurotic edema was incubated with 20 mg of solid kaolin, and the TAME esterase activity evolved with time was determined in duplicate (see Methods). 0.6 mg of SBTI was added to one set of tubes containing the TAME substrate.
§ The loss of esterase activity at 5 min of kaolin activation as compared to the 1 min sample is expressed as per cent reduction in esterase activity.
|| The reduction in esterase activity at 5 min with SBTI added to the substrate was compared to that found at 5 min without the inhibitor and is expressed as per cent reduction in activity.
phoresis. A kallikrein preparation was purified by hydroxylapatite column chromatography and contained 20% of the PTA clotting activity of the preparation used in Fig. 3 (see Methods). The α2-macroglobulin inhibited the TAMe esterase activity of this material in a manner identical with that shown. This suggested that the PTA found in the kallikrein preparations used played little, if any role, in the α2-macroglobulin, TAMe esterase interaction.

In contrast to α2-macroglobulin, both CI inactivator and SBTI produced a 90% inhibition of the TAMe esterase activity of the preparations examined.

This was consistent with the previously reported ability of these inhibitors to inactivate kallikrein arginine esterase activity (35, 62, 63). The α2-macroglobulin failed to inhibit the ATEe hydrolyzing activity of a purified preparation of C1s, the activated subunit of the first component of complement.

DEAE-Cellulose Column Chromatography of Plasma Kallikrein TAMe Esterase and Kallikrein, α2-Macroglobulin Incubation Mixtures.—Purified plasma kallikrein TAMe esterase activity was eluted from DEAE-cellulose with 0.05 M Tris-chloride buffer, pH 8.0, containing 0.02 M NaCl (Fig. 4, top panel, peak A). No further protein or TAMe esterase activity was eluted by a stepwise increase in NaCl concentration to 0.15 M. After incubation of the same concentration of kallikrein with α2-macroglobulin, one protein peak was identified following
DEAE-cellulose chromatography (Fig. 4, middle panel, peak B) and corresponded to the α2-macroglobulin peak (bottom panel). No protein or esterolytic activity was demonstrable in a location corresponding to the kallikrein protein peak, whereas peak B, eluted by 0.15 M NaCl, hydrolyzed TAME. Comparison of the total esterase activity in peak B of the kallikrein α2-macroglobulin mixture, following subtraction of the spontaneous activity associated with the purified α2-macroglobulin peak to the TAME activity of peak A, kallikrein alone, showed a 47% loss of activity. The ratio of esterolytic activity of the two

![Graph showing DEAE-cellulose chromatography results](image)
peaks against BAMe and ALMe using TAME as the reference substrate, was similar, and compared to that previously reported for purified plasma kallikrein (35).

Gel Filtration Chromatography of Plasma Kallikrein and Kallikrein, $\alpha_2$-Macroglobulin

Fig. 5. Sephadex G-200 column chromatography of human plasma kallikrein, kallikrein plus $\alpha_2$-macroglobulin, and $\alpha_2$-macroglobulin. 0.8 ml of kallikrein preparation as described in Fig. 4, was applied to a 2.5 X 30 cm column of Sephadex G-200 equilibrated with 0.85% sodium chloride, 0.16% sodium citrate buffer (top panel). 4 ml fractions were collected at a rate of 10 ml per hour. 0.8 ml of kallikrein plus 1.0 ml of $\alpha_2$-macroglobulin (24 mg per ml) in citrate-saline buffer or buffer plus $\alpha_2$-macroglobulin were incubated 5 min at 37°C and chromatographed under similar conditions (middle and bottom panels). Blue Dextran 2000 was used to indicate the void volume. The hydrolysis of TAME was measured as described in the Methods.

$\alpha_2$-macroglobulin Incubation Mixtures.—TAME esterase activity of a kallikrein preparation was eluted following Sephadex G-200 chromatography (Fig. 5, top panel) at a position consistent with its reported molecular weight below 200,000 (35). Chromatography of the same concentration of kallikrein incubated with purified
α2-macroglobulin produced one peak of TAMe esterase activity (Fig. 5, middle panel) eluted just following the void volume. This peak corresponded to the esterase activity associated with the α2-macroglobulin eluted from Sephadex G-200 (Fig. 5, bottom panel). Following incubation of kallikrein and α2-macroglobulin, no esterolytic activity could be demonstrated in Sephadex G-200 fractions in the location of kallikrein alone. The ratio of esterolytic activity of the two peaks against BAMe and ALMe using TAMe as the reference substrate was similar. The Sephadex G-200 peaks containing TAMe esterase activity from chromatography of kallikrein, or a kallikrein, α2-macroglobulin mixture were preincubated with SBTI or Cl− inactivator prior to assay (Table II). Both inhibitors caused 90% or greater inhibition of hydrolysis of TAMe by the kallikrein peak whereas the α2-macroglobulin, kallikrein peak was not inhibited by Cl− inactivator and inhibited 19% by SBTI.

The Effect of a Mixture of α2-Macroglobulin and Cl− Inactivator on the TAMe Esterase Activity of Kallikrein.—Cl− inactivator inhibited 95.2% of the TAMe esterase activity of kallikrein following 5 min incubation whereas α2-macroglobulin inhibited 53.3% of the initial activity under similar conditions. A mixture of the two inhibitors identical in final concentration with the above experiments, produced 66.2% inhibition. This inhibitory effect was found to be time dependent (Fig. 6). Inhibition of TAMe esterase activity increased sharply in mixtures containing kallikrein with Cl− inactivator or with α2-macroglobulin during the first 3 min of incubation. The combination of the two inhibitors produced an inhibitory curve which was intermediate in position as compared to each inhibitor alone.

### Table II

| Fraction* | TAMe hydrolyzed | Inhibition | µmoles/ml/hr | % |
|-----------|----------------|------------|--------------|---|
| Kallikrein peak | 0.80 | — | 0.03 | 96.3 |
| " plus 0.6 mg SBTI | 0.08 | 90.0 |
| " plus 4.0 units Cl− inactivator | 3.7 | — | 3.0 | 18.9 |
| α2-macroglobulin, kallikrein peak | 3.7 | 0 |
| " plus 0.6 mg SBTI | 3.7 | 0 |
| " plus 4.0 units Cl− inactivator | 3.7 | 0 |

*The fractions with peak TAMe esterase activity obtained following Sephadex G-200 column chromatography (Fig. 5) were assayed using TAMe as the substrate in the presence of SBTI or Cl− inactivator. 0.5 ml of the fractions were incubated 5 min at 37°C with buffer, 0.6 mg of SBTI, or 4.0 units of Cl− inactivator. The incubation mixtures were then assayed in a final volume of 2.3 ml for residual esterolytic activity using TAMe in a final concentration of 0.015 M (see Methods).
Comparison of the TAMe Esterase Activity Associated with the α2-Macroglobulin Prepared from Non-contact, Inhibited Plasma or Kaolin-Activated Plasma —

The α2-macroglobulin fraction obtained from inhibitor-treated plasma as described in the methods, had a specific activity of 0.005 μ moles TAMe/min per mg, whereas the fraction obtained from kaolin-activated plasma had a specific activity of 1.24 μ moles/min per mg α2-macroglobulin, a 240-fold difference in activity. Neither Trasylol, SBTI, or purified Cl inactivator significantly in-

![Graph](image_url)

**Fig. 6.** The effect of time on the inhibition of the TAMe esterase activity of kallikrein by Cl inactivator, by α2-macroglobulin, and by a mixture of the two inhibitors. 1.6 ml of a plasma kallikrein preparation containing 1.1 mg of protein/ml and a specific activity of 2.2 μmoles of TAMe hydrolyzed/min per mg, were added in duplicate to plastic tubes containing 0.8 ml of α2-macroglobulin (8 mg per ml) and 0.8 ml of Cl inactivator (40 units per ml), or similar concentrations of α2-macroglobulin or Cl inactivator plus phosphate buffer. The mixtures were incubated at 37°C and 0.4 ml portions were assayed at varying time intervals for residual TAMe esterase activity. The per cent inhibition was calculated by comparing the residual esterase activity in the inhibited incubation mixtures to the uninhibited kalli-

krene preparation.

inhibited this TAMe esterase activity. The relationship of the TAMe esterase activity of the α2-macroglobulin to kallikrein was suggested by the finding that the ratio of hydrolysis of ALMe and BAMe, relative to TAMe, resembled that documented for purified plasma kallikrein. Further purification of these frac-
tions by agarose column chromatography, followed by Pevikon preparative electrophoresis, showed that all the esterolytic activity was associated with the α2-macroglobulin.

**Effect of α2-Macroglobulin on Rat Uterus Contracting Activity of Plasma Kalli-
krein.** — Increasing concentration of α2-macroglobulin in a preincubation mixture, progressively inhibited the ability of a constant concentration of plasma
kallikrein to release rat uterine contracting substances from plasma previously heated to 61°C for 30 min (Table III). The addition of 0.1 mg SBTI to a preincubation mixture of kallikrein and buffer inhibited the uterine contraction. That the biologically active substance responsible for uterine contractions was most likely a kinin was suggested by the finding that the addition of carboxypeptidase B to a preincubation mixture of kallikrein and heated plasma substrate prevented uterine contractions, α₂-macroglobulin added to purified bradykinin in a preincubation mixture did not affect the amplitude of uterine contractions as compared to bradykinin alone.

**The Effect of α₂-Macroglobulin on the Vascular Permeability Induced by Kallikrein.**—The vascular permeability-inducing effect of purified plasma kallikrein was completely inhibited by incubation with α₂-macroglobulin (Table IV). That the permeability-inducing effect was due to the enzymic activity of kallikrein was suggested by its inhibition by purified C₁ inactivator. C₁ inactivator

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### Table III

| Initial incubation mixture* | Uterine contraction |
|-----------------------------|---------------------|
| α₂-macroglobulin | Kallikrein | Citrate-saline buffer |
| ml | mg/ml | ml | ml |
| 0.20 | 2.4 | + | 0.2 | + | 0.10 | 0 |
| 0.10 | + | + | 0.2 | + | 0.20 | 0 |
| 0.15 | 1.2 | + | + | 0.2 | + | 0.15 | 29 |
| 0.10 | + | + | 0.25 | + | 0.20 | 60 |
| 0.05 | + | + | 0.25 | + | 0.30 | 76 |
| 0 | + | + | 0.30 | + | 0.20 | 108 |

0.2 ml α₂-macroglobulin, 1.2 mg/ml, added to uterine bath prior to addition of 0.2 ml kallikrein

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* Plasma kallikrein containing 0.08 mg of protein/ml and a specific activity of 4.2 μmoles of TAME/min per mg, was preincubated 5 min at 25°C in plastic tubes with varying concentrations of α₂-macroglobulin as indicated. The final volume of the mixture was 0.5 ml. 0.4 ml of the incubation mixture was added to a 25°C bath containing 20 ml of de Jalon’s solution in which was suspended a rat uterine horn segment obtained from 250–300 gm virgin rats in estrus. Prior to the addition of the incubation mixture, 0.1 ml of plasma substrate previously heated to 61°C for 30 min had been added. Uterine contractions were recorded as detailed in Methods. The height of the amplified contractions in millimeters is recorded in the table. In the absence of plasma substrate neither α₂-macroglobulin nor kallikrein caused uterine contractions. Bradykinin was added to the uterine bath without the plasma substrate as a control.
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has been shown to inhibit kallikrein but not to inhibit the kinins generated by the enzyme (8).

The Uterine-Contracting and Vascular Permeability-Inducing Activities of Fractions Obtained Following DEAE-Cellulose Column Chromatography of Plasma Kallikrein and Kallikrein, α2-Macroglobulin Incubation Mixtures.—Chromatography of three incubation mixtures of kallikrein, kallikrein plus α2-macroglobulin and α2-macroglobulin was performed as described in Fig. 4. The fractions obtained were tested for uterine-contracting and vascular permeability-inducing activities. These activities were found in association with the TAMe esterase peak of the kallikrein preparation. No fraction obtained from chromatography of the kallikrein, α2-macroglobulin mixture possessed biologic activity.

DISCUSSION

These studies have shown that the α2-macroglobulin is an inhibitor of partially purified human plasma kallikrein. The inhibition of the arginine esterase activity, rat uterine-contracting and vascular permeability-inducing effects of kallikrein is accompanied by the formation of a high molecular weight complex between the enzyme and the α2-macroglobulin. These results support Becker's speculation that there may be another plasma inhibitor of kallikrein in addition to CI inactivator (12). This thesis was suggested by the study of individuals with hereditary angioneurotic edema who have a deficiency of a specific heat- and acid-labile α2-neuraminoglycoprotein designated CI inactivator (5-7, 42,
64). Although Cl inactivator effectively inhibits both kallikrein, PF/dil and Cl in purified systems (9, 54), the experimental evidence now available suggests that activation of kallikrein and the permeability globulin system, if it occurs in hereditary angioneurotic edema, is not proportionately as great as is the activation of the complement system (12).

These findings may be explained by the results of the present investigation which indicate that plasma deficient in Cl inactivator inhibits plasma kallikrein. This was established by studying the kaolin-induced TAME esterase activity of normal plasma and of plasma obtained from individuals with hereditary angioneurotic edema. Recent data of Colman, Mattler, and Sherry (15, 35) have indicated that the kaolin-activated plasma arginine esterase is identical with plasma kallikrein. They demonstrated that maximal activation of TAME esterase activity by kaolin occurred at 1 min in both normal plasma and in hereditary angioneurotic edema plasma. Our studies have shown that this activity falls to 50% of peak activity at 5 min of kaolin activation in normal plasma and to 70% of peak activity in the plasma of four patients with hereditary angioneurotic edema. That Cl inactivator was not responsible for this inhibitory effect was indicated by the absence of this inhibitor in the plasma of these individuals as measured by double diffusion immunoassay using a monospecific antibody preparation, and by the finding of from 0 to 10% of normal inhibitor levels as determined by a sensitive biochemical assay.

The kaolin-activated TAME esterase in normal plasma and in hereditary angioneurotic edema plasma was less susceptible to inhibition by SBTI at 5 min of activation than at the 1 min peak activity. This decrease in susceptibility was quantitatively similar in normal and in Cl inactivator-deficient plasma. In contrast, the kallikrein TAME esterase activated by kaolin in acid-treated normal and hereditary angioneurotic edema plasma did not lose its activity with time and retained its susceptibility to inhibition by SBTI. Acid treatment therefore, apparently destroyed an inhibitor in normal and in Cl inactivator-deficient plasma which caused a reduction in esterolytic activity and which also protected the enzyme from the inhibitory effect of SBTI. This conclusion was supported by data which made it unlikely that an enzyme other than kallikrein was responsible for the resistance to SBTI observed in the 5 min sample of the kaolin-activated plasma. The ratio of hydrolysis of two synthetic ester substrates relative to TAME by the arginine esterase at 1 and at 5 min of kaolin activation in normal plasma, in hereditary angioneurotic edema plasma, and in acid treated normal and hereditary angioneurotic edema plasma was similar. This strongly suggests that the same enzyme, kallikrein, was responsible for the esterase activity in each sample. This was a particularly important observation since it has been demonstrated that activation of Hageman factor in hereditary angioneurotic edema plasma resulted in the conversion of the first component of complement into the active esterase, Cl (65). Cl, a TAME esterase which is
not inhibited by SBTI (44), has been found to hydrolyze acetyl-L-lysine methyl ester (ALMe) (66). This substrate is more susceptible to the action of CI than is TAMe. Since the ratio of hydrolysis of ALMe relative to TAMe remained unchanged in the hereditary angioneurotic edema plasma, significant activation of CI during the time period of the kaolin activation did not occur.

The α2-macroglobulin purified from human plasma has been shown to form a complex with the proteases thrombin (61), plasmin (60, 67), and trypsin (28), to partially inhibit their esterolytic activity, and to almost totally inhibit their proteolytic activity. After complex formation, the esterolytic activity of the bound trypsin and plasmin was no longer inhibited by SBTI (28, 67, 68). Acidification of purified α2-macroglobulin preparations to pH 3.0, resulted in complete loss of trypsin binding activity (26). The present investigation has shown that the α2-macroglobulin inhibits the esterolytic and proteolytic activity of purified plasma kallikrein in a manner analogous to that reported for thrombin, plasmin, and trypsin. The kallikrein preparations used had esterolytic and biologic activities as previously described for plasma kallikrein and corresponded to the major plasma kallikrein not absorbed to DEAE-cellulose (35, 62, 69). Incubation of kallikrein and α2-macroglobulin resulted in a concentration and time-dependent inhibition of kallikrein TAMe esterase activity to approximately 50%. The presence of significant concentrations of plasma thromboplastin antecedent (PTA) clotting activity in the purified plasma kallikrein preparation was shown to have no significant effect on the α2-macroglobulin, kallikrein inhibition curve. Results of anion exchange and gel filtration chromatographic experiments indicated that the purified α2-macroglobulin formed a complex with plasma kallikrein similar to that formed between the inhibitor and trypsin. The esterolytic activity of this complex was resistant to inhibition by both SBTI and purified CI inactivator, whereas these inhibitors blocked 95% or more of the TAMe esterase activity of the kallikrein peak.

A mixture of CI inactivator and α2-macroglobulin, in concentrations equivalent to the concentration measured in normal plasma, interacted with the kallikrein TAMe esterase in purified systems. As previously reported, the inhibition of kallikrein TAMe esterase activity of CI inactivator was progressive with time (63). The α2-macroglobulin induced inhibition of kallikrein esterase activity was found to be similarly time dependent. The inhibition curve produced by both inhibitors together was intermediate between that found for CI inactivator and α2-macroglobulin alone and suggested that the two inhibitors competed for kallikrein. Thus the α2-macroglobulin partially protected the esterolytic activity of kallikrein from inhibition by CI inactivator. These results may explain the differences in the kaolin activation curves obtained with normal and with hereditary angioneurotic edema plasma and provide support for the thesis that the α2-macroglobulin is the acid-labile plasma factor which partially inhibited and protected the kaolin-activated plasma arginine esterase
from inhibition by SBTI. The greater inhibition of kaolin-activated normal plasma esterase at 5 min as compared to the hereditary angioneurotic edema plasma esterase (50 versus 30%) may reflect, in the case of normal plasma, the interaction between kallikrein, Cl inactivator, and α2-macroglobulin. In the plasma deficient in Cl inactivator, the decay in TAMe esterase activity may be entirely due to interaction with α2-macroglobulin. This reduction in esterase activity cannot, therefore, be used as a measurement for Cl inhibitor levels as has recently been proposed (3).

Additional indirect evidence for the ability of the α2-macroglobulin to form a complex with kallikrein in plasma was obtained by purifying α2-macroglobulin from plasma to which inhibitors of kallikrein and of its activation were added. The purified α2-macroglobulin obtained from kaolin-activated plasma contained 240 times the TAMe esterase activity of the α2-macroglobulin derived from the inhibited plasma. The ratio of its activity toward synthetic basic amino acid esters compared to TAMe suggested its relationship to purified kallikrein. These results extend the findings of Howard (26) who found an α2-macroglobulin-associated TAMe esterase which could be separated from esterase-inactive α2-macroglobulin by low ionic strength precipitation. Further investigations by Dyce et al. (70), reported in abstract form, have shown that an esterolytic enzyme with properties of a kallikrein can be dissociated from this purified α2-macroglobulin.

The α2-macroglobulin was found to inhibit the rat uterine-contracting ability of a kallikrein preparation as well as its vascular permeability-producing effect (71). Fractions of incubation mixtures of kallikrein, kallikrein and α2-macroglobulin, and α2-macroglobulin obtained by DEAE-cellulose chromatography were assayed for these two biologic activities. Following incubation with α2-macroglobulin, the kallikrein TAMe esterase, uterine-contracting and vascular permeability-inducing activities were no longer eluted in fractions where kalli-krein activity was found. This loss of biologic activity paralleled the loss of TAMe esterase activity and provided additional evidence both for the identity of kallikrein as the TAMe esterase studied, and for the binding of biologically active kallikrein by the α2-macroglobulin.

The finding that the human plasma α2-macroglobulin complexes with the major plasma kallikrein to partially inhibit its esterolytic activity and to completely inhibit its uterine-contracting and vascular permeability-inducing activities has potential relevance to various human disease states. The two plasma inhibitors, Cl inactivator and α2-macroglobulin, have been shown in this study to compete for the enzyme kallikrein in purified systems. While the biologic function of the α2-macroglobulin is unclear, it has been established that the enzymes thrombin and plasmin are inhibited in vitro by this plasma glycoprotein. Thus the α2-macroglobulin may serve as an inhibitor of three different plasma enzyme systems: the kallikrein, the coagulation, and the fibrinolytic
enzyme system. Ganrot (72) has shown that thrombin and plasmin may be bound to the same binding group of the α2-macroglobulin and that competition between these enzymes for this site can be demonstrated in vitro. Ganrot has postulated that such competition, if it exists in vivo might be an important mechanism controlling the equilibrium between intravascular coagulation and fibrinolysis in pathologic states. No information is yet available on the binding site of α2-macroglobulin for kallikrein or its possible interaction with the thrombin and plasmin binding sites. The formation of a kallikrein, α2-macroglobulin complex with the esterolytic activity of the enzyme protected from Cl inactivator raises the question of the physiologic significance of such a complex. James, Taylor, and Fudenberg (73) have suggested that the interaction of α2-macroglobulin with plasmin might provide a reservoir of enzyme free from the action of circulating inhibitors.

Evidence suggesting that in vivo interactions may occur between several components of the coagulation, fibrinolytic, kallikrein, and complement systems has been provided by Donaldson. She has shown that activation of Hageman factor or of the fibrinolytic enzyme system in the plasma of patients with hereditary angioneurotic edema was accompanied by conversion of the first component of complement into its esterase, Cl (65, 74). The pathway by which Hageman factor activates Cl is obscure, however, both the fibrinolytic and kallikrein systems may be involved. Several investigations (75, 76) have supported the concept that plasma fibrinolytic activity can be induced by Hageman factor activation. Kallikrein, which is also activated by Hageman factor (32), has been shown to convert plasminogen into the active enzyme plasmin (77). Plasmin has been shown to activate the first component of complement (44, 78) and kallikrein may also directly activate Cl (63, 65). In addition, plasmin destroys the inhibitory activity of Cl inactivator and alters it immunoelectrophoretic characteristics (25).

The ability of the α2-macroglobulin, a known inhibitor of thrombin and plasmin, to inhibit plasma kallikrein provides another example of an interrelation-ship between the coagulation, fibrinolytic, and kallikrein pathways. This inhibitor may play an important role in modulating the relative participation of these complex enzyme systems in human pathophysiologic states.

**SUMMARY**

Activation of plasma kallikrein arginine esterase activity by kaolin resulted in peak activity at 1 min of incubation and a 50% reduction in activity at 5 min in normal plasma, and 30% reduction in the plasma of patients with hereditary angioneurotic edema who lacked the Cl inactivator. The peak esterolytic activity was inhibited by soybean trypsin inhibitor whereas the 5 min activity was resistant to this inhibitor. Acid treatment of normal and hereditary angioneurotic edema plasma destroyed the factor responsible for the fall in esterase
activity at 5 min and the factor which rendered the esterase resistant to soybean trypsin inhibitor.

Purified α₂-macroglobulin inhibited approximately 50% of the TAMe esterase activity of purified plasma kallikrein without changing its activity toward basic amino acid esters. The interaction between the α₂-macroglobulin and kallikrein resulted in alterations in the gel filtration chromatographic pattern of the TAMe esterase and biologic activity of kallikrein, indicating that kallikrein was bound to the α₂-macroglobulin. The TAMe esterase activity of this complex, isolated by column chromatography, was resistant to CI inactivator and SBTI. Studies of incubation mixtures of kallikrein, α₂-macroglobulin and CI inactivator suggested that these inhibitors compete for the enzyme, and that the α₂-macroglobulin partially protects the esterase activity of kallikrein from CI inactivator. The α₂-macroglobulin isolated from kaolin-activated plasma possessed 240 times the esterolytic activity of the α₂-macroglobulin purified from plasma treated with inhibitors of kallikrein and of its activation. The α₂-macroglobulin blocked the uterine-containing activity and vascular permeability-inducing effects of plasma kallikrein. These studies suggest that the α₂-macroglobulin is a major plasma inhibitor of kallikrein and provide a new example of an interrelationship between the coagulation, fibrinolytic, and kallikrein enzyme systems.

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