Inhibitors of Kallikrein in Human Plasma

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A B S T R A C T Human plasma was fractionated by ammonium sulfate precipitation, DEAE-cellulose chromatography, and Sephadex G-200 gel filtration to determine which method would give the greatest number of clearly separable kallikrein inhibitory peaks. With G-200 gel filtration three peaks could be separated which were demonstrated to contain α_2-macroglobulin, $C\bar{1}$ inactivator, and α_1-antitrypsin. No other kallikrein inhibitors could be identified. The fractions containing $C\bar{1}$ inactivator and α_2-macroglobulin appeared to be more effective against kallikrein than that containing α_1-antitrypsin. A patient with hereditary angioneurotic edema was shown to have an abnormal $C\bar{1}$ inactivator protein capable of interfering with kallikrein's biologic, but not its esterolytic activity. Heat-treated human plasma, a commonly used source of kininogen for experiments with kallikrein, was shown to have kallikrein inhibitory activity.

INTRODUCTION

Plasma kallikrein is a proteolytic enzyme that generates vasoactive polypeptides from a substrate called kininogen (1). These polypeptides, termed kinins, are potent mediators of vasodilatation, increased vascular permeability, and pain, and because of these properties have been proposed as mediators of inflammation (2). The mode of kallikrein activation and the possibility of there being more than one kallikrein and more than one kininogen are currently the subjects of active investigation (3–18).

Three kallikrein inhibitors have been clearly identified in human plasma—$C\bar{1}$ inactivator (19), α_1-antitrypsin (20, 21), and α_2-macroglobulin (22). $C\bar{1}$ inactivator is an α_2-neuraminoglycoprotein with a sedimentation coefficient of 3.7 (23, 24). As an inhibitor it is effective against kallikrein, the first component of complement ($C\bar{1}$), the $C\bar{1}r$ subcomponent of $C\bar{1}$, plasmin, plasma thromboplastin antecedent, Hageman factor, and Pf/dil (permeability factor of dilution) (19, 25, 26). $C\bar{1}$ inactivator activity is absent in hereditary angioneurotic edema (HAE)[1] (27). However, specific antisera to $C\bar{1}$ inactivator can detect the protein antigen in some patients even though it is inactive (27). α_1-Antitrypsin is an α_1-globulin with a sedimentation coefficient of 3.4 and a molecular weight estimated to be 45,000 (28). As an inhibitor, it has been reported to be effective against kallikrein, trypsin, elastase, plasmin, and thrombin (20, 21, 29, 30). α_1-Antitrypsin activity is lacking in a hereditary form of emphysema (31). α_2-Macroglobulin is an α_2-globulin with a sedimentation coefficient of 19.6 and a molecular weight estimated to be 820,000 (28). As an inhibitor, it is effective against the proteolytic activities of kallikrein, trypsin, thrombin, and plasmin, but not the esterase activities of thrombin, plasmin, or $C\bar{1}$ (22, 32–34). It is more effective against kallikrein's kinin-releasing than against its esterase activity (22). To date, a systematic search for other inhibitors of kallikrein in human plasma has not been made.

Heated human plasma is a commonly used source of kininogen for experiments with plasma kallikrein. When kallikrein in varying dilutions is incubated with plasma heated to 60°C for 1 hr, the amount of kinin released increases for about 30 min, but then reaches a constant level and stops, the level attained varying with the concentration of kallikrein (35). This finding could be explained by the presence of an inhibitor of kallikrein in heated human plasma.

The present study had two major purposes—first, to define the nature of the kallikrein inhibitors in human plasma and, second, to determine whether heated human plasma contains kallikrein inhibitory capacity.

[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; ALMe, *N*-α-acetyl-L-lysine methyl ester; CM, carboxymethyl cellulose; HAE, hereditary angioneurotic edema; PTA, plasma thromboplastin antecedent; SBTI, soybean trypsin inhibitor; TAMe, *p*-tosyl-L-arginine-methyl ester.

METHODS

Synthetic bradykinin and soybean trypsin inhibitor (SBTI) 3 times crystallized were obtained from Calbiochem, Los Angeles, Calif. Rabbit anti-human α_1-antitrypsin, rabbit anti-human α_2-macroglobulin, rabbit anti-whole human serum, rabbit anti-human IgG (H-chain specific), goat anti-rabbit IgG, donkey anti-whole rabbit serum, and Partigen α_2-macroglobulin and α_1-antitrypsin radial immunodiffusion plates were obtained from Behring Diagnostics, Inc., Woodbury, N. Y. Goat anti-human CĪ inactivator was obtained from Miles Laboratories, Inc., Kankakee, Ill.

Siliconized glassware was prepared by an established method (36). Except where otherwise noted, plasma from normal persons and patients with HAE was obtained as follows: Blood was drawn with plastic syringes and disposable 20 gauge needles and placed into siliconized centrifuge tubes containing 0.2 ml of 20% trisodium citrate per 10 ml blood. Plasma was recovered after centrifugation at 2000 rpm for 20 min at +4°C, placed in 3.0 ml portions in siliconized glass tubes, stored at −60°C. Care was taken to avoid glass contact throughout the collection procedure.

The diagnosis of HAE was established by the estimation of CĪ inactivator levels with an esterase assay using N-α-acetyl-L-lysine methyl ester (ALMe) (37) (Cyclo Chemical Corp., Los Angeles, Calif.) and with an assay using Ouchterlony plates in which serial twofold dilutions of patient plasma and normal plasma were compared for their ability to react with specific antisera against CĪ inactivator. Plasma samples from two different patients with HAE were used in these studies. The diagnosis of HAE for the first patient was initially established in the laboratory of Dr. Fred Rosen. This patient's CĪ inactivator level was low (approximately 10% of normal) by both immunoassay and esterase assay. The second patient's CĪ inactivator level was normal by the immunoassay, but low by the esterase assay (approximately 15% of normal). This patient's plasma was also examined in the laboratory of Dr. Virginia Donaldson who confirmed that the patient had an antigenically normal, but functionally abnormal protein. Dr. Donaldson found that the patient's CĪ inactivator was low but measurable by her esterase assay (4 U/ml), was unable to protect C4̄ from inactivation, and had an electrophoretic mobility in an intermediate range when compared to partially functioning proteins from other HAE patients (38).

Protein fractionation. All fractionation procedures were performed at +4°C. Ion exchange chromatography was performed with diethylaminoethyl (DEAE) cellulose and carboxymethyl cellulose (CM) (Whatman DE52 and CM52 from H. Reeve Angel & Co., Inc., Clifton, N. J.) (39). Gel filtration was performed with Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (40). Details of individual runs are given under Results.

Starch block electrophoresis was performed using a Buchler electrophoresis apparatus (Buchler Instruments, Inc., Fort Lee, N. J.) (41). Samples (3.0 ml) were introduced into a slit cut 5.5 cm from the cathodal end of blocks measuring 20 × 9.5 × 2.0 cm. Electrophoresis was carried out at 150 V for 13 hr. At the completion of a run, the starch was cut into 1 cm sections and each section eluted with 20 ml 0.01 M PO₄ ·0.15 M NaCl pH 7.5 (phosphate-saline). The eluates were concentrated approximately fivefold using CF50A Centriflo membrane cones (Amicon Corp., Lexington, Mass.) and then dialyzed against phosphate-saline before analysis for protein and inhibitor content. Concentration of protein solutions over 250 ml was performed with an Amicon model 2000 ultrafiltration cell with UM 10 membranes (Amicon Corp., Lexington, Mass.). Samples under 250 ml were concentrated by ultra-

filtration (42). Samples for immunoelectrophoresis were concentrated by lyophilization.

Immunoassays. Double diffusion in agar (43), immuno-electrophoresis (44), radial immunodiffusion (45), and precipitin reactions (46) were carried out by established methods.

C1 esterase (CĪs). Blood (4 parts) was taken into acid citrate dextrose (ACD) (1 part) in plastic bags (Fenwal Laboratories, Division of Travenol Laboratories, Inc., Morton Grove, Ill.) and centrifuged to separate the cells. The supernatant plasma was removed immediately and stored frozen at −60°C until used. This fresh frozen plasma was used as the starting material for the isolation of CĪs according to the method of Haines and Lepow (47). CĪs activity was assayed by its ability to hydrolyze ALMe (37).

Rabbit IgG fractions. For normal serum, blood was obtained from rabbits by cardiac puncture and allowed to clot in glass centifuge tubes at room temperature for 1 hr. The clot was separated by centifugation, and the serum drawn off and fractionated by DEAE-cellulose chromatography to obtain the IgG fraction (48). IgG fractions containing specific antibody against α_1-antitrypsin and α_2-macroglobulin were obtained by fractionating commercial antisera in a similar manner. The antibodies in these rabbit IgG fractions were judged to be monospecific as they produced single lines when tested against whole human serum fractionated by immuno-electrophoresis. All the rabbit IgG fractions, when examined by immunoelectrophoresis, gave a single line migrating in the gamma region when tested with both goat anti-rabbit IgG and donkey anti-whole rabbit serum.

Plasma kallikrein. A crude prekallikrein preparation was prepared from fresh frozen plasma by a DEAE-cellulose batch procedure (35). The dilute prekallikrein solution obtained was concentrated fivefold and dialyzed against phosphate-saline. This solution was activated with plasma-coated glass ballotini (120 μ diameter, class B glass beads, 3M Company, St. Paul, Minn. (35) and after activation had a specific activity of 0.493 μM TAMe hydrolyzed/min per mg protein. (For assay methods, see below.) Partially purified kallikrein was prepared from crude prekallikrein. This was dialyzed against 0.05 M Na₂HPO₄ and then adsorbed to 75 g of Celite (acid-washed, calcined diatomaceous earth, Celite Analytical Filter Aid L-665-A, Johns-Manville Products Corp., Celite Div., New York) previously washed with the same buffer. The Celite was washed first with 250-ml portions of this buffer, second with two 250-ml portions of 0.05 M·0.15 M NaCl, and finally with four 250-ml portions of Na₂HPO₄·2.0 M NaCl. The final four washes contained the prekallikrein. These were dialyzed against 0.01 M PO₄ pH 6.0 in preparation for CM chromatography carried out according to the method of Colman, Mattler, and Sherry (18). The CM chromatography yielded 130 ml of material that, when activated, had a specific activity of 3.17 μM TAMe hydrolyzed/min per mg protein. (For assay methods, see below.) Analysis of this material by immunoelectrophoresis and double diffusion in agar against rabbit anti-whole human serum and rabbit anti-human IgG demonstrated the major protein contaminant to be IgG. This material was also tested for plasma thromboplastin antecedent (PTA) activity by Mr. Michael Drillings in the laboratory of Dr. Hymie Nossel (49) and found to have a level of 5 U/ml (PTA level of normal plasma taken to be 100 U/ml). When 3.0 ml of both the crude and partially purified kallikrein preparations were further fractionated by Sephadex G-200 gel filtration at +4°C (2.5 × 90 cm column, phosphate saline used as eluting buffer, flow rate 15 ml/hr, 5.0 ml fractions collected), all the kallikrein activity eluted in the descending limb of the IgG protein peak. From these properties, it was concluded that the kallikrein isolated was identical

with the kallikrein I described by Colman et al. (18). None of the kallikrein preparations studied had any uterus-contracting activity when tested alone in the rat uterus bioassay (see below).

Kininogen. Heated human plasma was prepared by heating 1 liter of outdated ACD plasma for 1 hr at 61°C. The precipitate that formed was separated by centrifugation and the supernatant fluid dialyzed against phosphate saline. The substrate for kallikrein was prepared by adding an equal volume of a saturated solution of $(NH_4)_2SO_4$ in 0.2 M sodium phosphate pH 6.7 to this heated human plasma. The resulting precipitate was harvested by centrifugation at 2000 rpm at +4°C for 30 min and then made into a slurry with a small amount of demineralized water. On dialysis against phosphate-saline at +4°C, the precipitate dissolved. This material was then brought back to the original plasma volume with phosphate-saline and stored at −60°C until used. Before use, each batch of kininogen substrate was adjusted to a standard strength as outlined below under kallikrein assay. Neither the kininogen substrate nor the heated human plasma had uterus-contracting activity in the rat uterus bioassay (See below). In addition, when incubated for 1 hr with 20 ng of bradykinin (amount giving an intermediate response in the bioassay), neither preparation demonstrated kininase activity in the concentrations used in this study.

Protein assay. During fractionation procedures, protein concentration was determined from absorbance at 280 mμ. For determining specific activity of kallikrein preparations, the biuret method was used with bovine serum albumin as a standard (Mann Research Labs., Inc., New York) (50).

Rat uterus assay—kinins. Kinins were assayed on an isolated rat uterus suspended in a 5.0 ml organ bath in de Jalon's solution (51). Isometric muscle contractions were recorded on a Grass polygraph with a Grass force-transducer having no spring load (Grass Instrument Co., Quincy, Mass.). Test solutions were diluted in 10 ml of de Jalon's solution at 30°C and then gently injected into the uterus chamber so as to displace the bathing de Jalon's solution. The kinin concentration in an unknown solution was determined by comparing the contraction it produced with that produced by known amounts of bradykinin standard. Results were expressed in nanograms of bradykinin equivalent. Test solutions were applied at 5-min intervals and unknown solutions were alternated with standards.

Rat uterus assay—kallikrein. Kallikrein was assayed by a modification of a previously described method (35). Serial twofold dilutions of the solution to be tested were made up in phosphate-saline. Each dilution (0.3 ml) was mixed with substrate (0.3 ml) and the mixture incubated for 10 min at 37°C. Then 0.2 ml of the mixture was removed and tested for kinin content. When the logarithm of the dilution of kallikrein was plotted against the amount of bradykinin equivalent released, a straight line resulted at higher dilutions of the enzyme (Fig. 1). At lower dilutions, the amount of kinin released was so great that it could not be measured in this system. The slope of the dose-response curve obtained at the high dilutions varied with the strength of the kininogen, steeper slopes resulting with strong preparations and shallower slopes with weak ones. All kininogen preparations used in this study were adjusted to give dose-response curves similar to that shown in Fig. 1. For a given kallikrein preparation, the dilution that on the average released 20 ng of bradykinin could be determined from its dose-response curve. A "kallikrein dose" was defined as the amount of kallikrein in 0.1 ml that on the average released 20 ng of bradykinin equivalent from 0.1 ml of substrate when incubated with that substrate for 10 min at 37°C at a pH of 7.5 and ionic strength of 0.15.
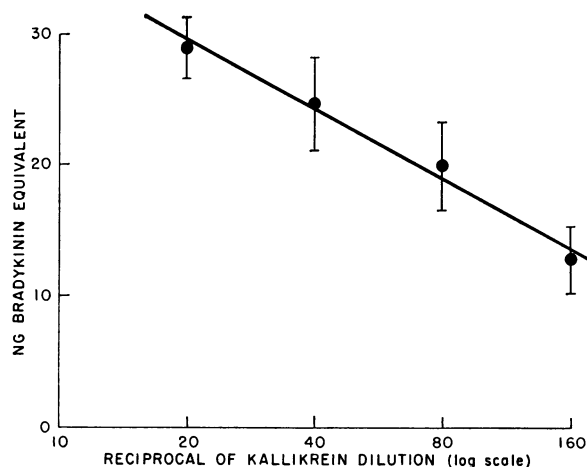

FIGURE 1 Bradykinin equivalent released from standard substrate by graded dilutions of a kallikrein preparation after incubation at 37°C for 10 min, pH 7.5, ionic strength 0.15. Each point represents the average of 13 separate trials on different days with different uterus preparations. Vertical lines represent standard deviations.

Throughout these studies, a standard deviation of approximately ±4 ng bradykinin equivalent was observed for the amount of kinin released by a single dilution of kallikrein.

Rat uterus assay—kallikrein inhibition. To measure kallikrein inhibition, an equal volume of inhibitor in phosphate-saline was added to each of serial twofold dilutions of kallikrein. Control solutions were set up and the two series incubated at 37°C for varying times. The activities of the control and inhibited series were then determined as outlined above. With the exception noted under Results, the inhibited series of kallikrein dilutions gave dose-response curves parallel to the controls. The potency ratio obtained by dividing a control kallikrein solution's activity by that of an inhibited solution gave an estimate of the inhibitor's strength. For the purpose of screening fractions from columns and electrophoretic runs, a less quantitative, but quicker assay was used. A solution of kallikrein that, when diluted 10-fold, on the average released 20 ng bradykinin equivalent was selected. This solution (0.1 ml) was then incubated with the fraction to be tested (0.9 ml) for 2 hr at 37°C and tested for kinin-releasing activity. A reduction of at least 5 ng in the amount of kinin released in two contiguous fractions was accepted as indicating the presence of an inhibitor. This screening assay, although useful for checking many individual fractions, tended to give false positive results. Therefore, adjacent fractions thought to contain inhibitor activity were pooled, concentrated, and tested by the quantitative assay outlined above. In addition, fractions thought not to contain inhibitor were also pooled, concentrated, and checked by the quantitative assay.

Esterase assay. Kallikrein was assayed for esterase activity with p-tosly-L-arginine-methyl ester as a substrate (TAMe, ICN Nutritional Biochemicals Corporation, Cleveland, Ohio) (52). Activity was expressed in μmoles TAMe hydrolyzed/ml per hour. Throughout these studies, a standard deviation of approximately ±40 μM/ml per hr was observed for the determination of a given kallikrein preparation's activity.

For inhibition studies, an equal volume of inhibitor and kallikrein were mixed and incubated at 37°C for varying times. A 0.2 ml sample was then removed and assayed for esterase

WHOLE PLASMA

(NH₄)₂SO₄   G-200
                    ↓
SUPERNATANT   PRECIPITATE   3 PEAKS
     ↓   ↓
INHIBITION   NO INHIBITION

DEAE   G-200   DEAE   G-200
  ↓   ↓   ↓   ↓
I PEAK 3 PEAKS I PEAK I PEAK
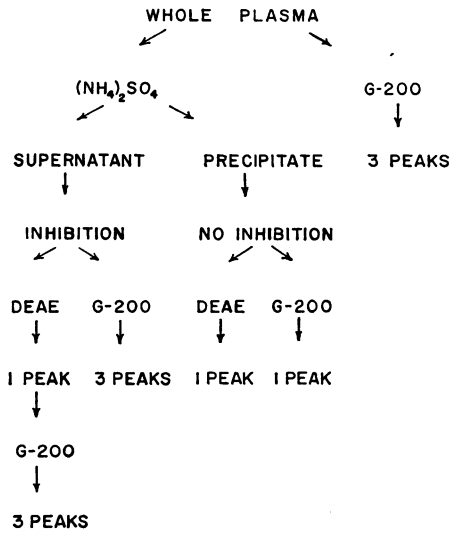 ↓
G-200
  ↓
3 PEAKS

FIGURE 2 Scheme used to determine which protein fractionation method would give the greatest number of separate kallikrein inhibitory peaks.

activity. Control preparations of inhibitor alone and kallikrein alone were run at the same time. The potency ratio obtained by dividing the control kallikrein solution's activity by that of the inhibited solution gave an estimate of the inhibitor's strength.

RESULTS

Except where otherwise noted, all experiments were performed with both crude and partially purified kallikrein. No difference was noted between the results obtained with these two preparations.

Inhibitors of kallikrein in plasma from normals and

patients with HAE. Normal plasma was fractionated according to the scheme shown in Fig. 2 to determine the method giving the greatest number of clearly separable inhibitory fractions. If whole human plasma was fractionated with 50% ammonium sulfate, all detectable inhibitory activity was found in the supernatant fraction (Table I). In fact, incubation of kallikrein with the precipitate fraction appeared to enhance its activity, probably because of the presence of kallikrein and kininogen in this fraction. If the supernatant material was further fractionated by DEAE-cellulose chromatography, the inhibitory activity eluted in one broad area (Fig. 3). However, on G-200 gel filtration, it eluted in three distinct areas (Fig. 4, upper). When the inhibitory fractions from the DEAE-cellulose chromatography were pooled, con-
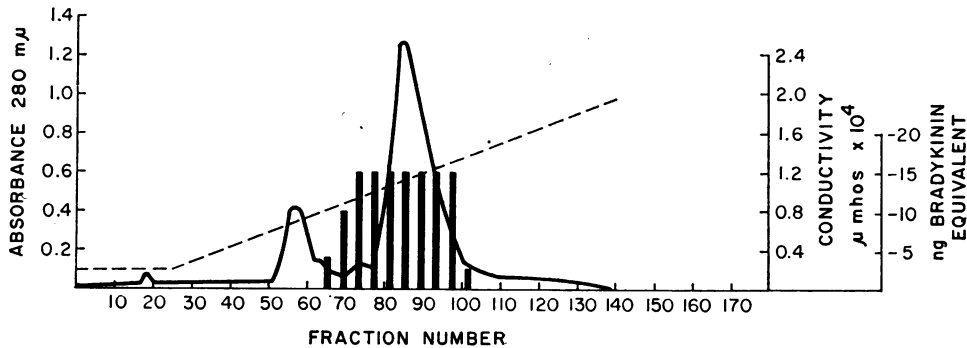

FRACTION NUMBER

FIGURE 3 DEAE-cellulose chromatography of the supernatant fluid from plasma brought to 50% saturation with (NH₄)₂SO₄. Supernatant fluid (31 ml) was dialyzed against 0.025 M Tris-HCl pH 7.6 and then applied to a 2.5 × 90 cm column of DEAE-cellulose that had been equilibrated with the same buffer. The protein was eluted by a salt gradient created with a device containing 800 ml of 0.025 M Tris HCl pH 7.6 in the first chamber and 800 ml of 0.05 M Tris HCl·0.3 M NaCl pH 7.6 in the second chamber. Each fraction contained 10 ml and the flow rate was 70 ml/hr. The pH remained relatively constant between 7.6 and 8.0. The effluent was monitored for: protein (————), conductivity (— — — — —), and kallikrein inhibitory activity expressed as the decrease in nanograms of bradykinin equivalents (bars).
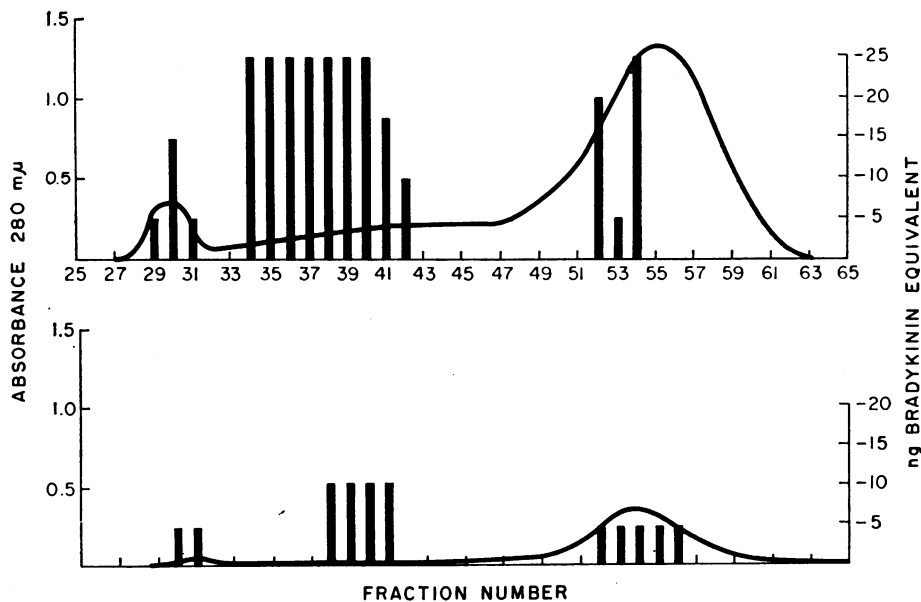
FIGURE 4 Gel filtration on Sephadex G-200 of: upper, supernatant fluid from plasma brought to 50% saturation with $(NH_4)_2SO_4$ and concentrated twofold; lower, inhibitory fraction from DEAE-cellulose chromatography (see Fig. 3) pooled and concentrated sixfold. Column 90 × 2.5 cm, 3.0-ml samples applied. Fow rate, 15 ml/hr. 5 ml fractions. Buffer-phosphate-saline. The effluent was monitored for protein (————) and kallikrein inhibitory activity expressed as the decrease in nanograms of bradykinin equivalents (bars).

centrated, and subjected to G-200 gel filtration, the inhibitory activity again eluted in three areas (Fig. 4, lower). To determine whether kallikrein and kininogen were masking the presence of an inhibitor in the $(NH_4)_2SO_4$ precipitate fraction, this material was also fractionated by DEAE-cellulose chromatography and G-200 gel filtration. Fractionation on G-200 revealed only one inhibitory peak eluting in the same position as the early eluting peak in Fig. 4. DEAE-cellulose chromatography revealed one peak eluting in the same position as the peak in Fig. 3.

When normal whole human plasma was fractionated on G-200 (Fig. 5, upper) three areas of inhibitory activity were obtained similar to those shown in Fig. 4. Thus, of the methods for fractionating plasma employed, gel filtration on Sephadex G-200 appeared to give the greatest number of clearly separable kallikrein inhibitory peaks. To determine whether any of the known inhibitors of kallikrein were present in these three peaks, each was tested with specific antisera to α_2-macroglobulin, C̄I inactivator, and α_1-antitrypsin by double diffusion in agar. The early eluting fractions contained protein reacting with antisera to α_2-macroglobulin; the intermediate, to C̄I inactivator; and the late, to α_1-antitrypsin. To determine whether other inhibitors of kallikrein might be present, it was necessary to study the G-200 fractions in the absence of the known inhibitors.

For the fractions containing C̄I inactivator, this goal was accomplished by fractionating on G-200 the plasma of the two patients with HAE. When plasma from the HAE patient with low levels of immunologically detectable C̄I inactivator was fractionated, only the early and late eluting peaks could be detected (Fig. 5, middle). This finding suggested that the middle peak might be caused by C̄I inactivator. Surprisingly, when plasma from the HAE patient with immunologically normal levels of C̄I inactivator was fractionated, an inhibitory peak eluting slightly earlier than the normal one was detected in a position similar to that noted by Rosen, Alper, Pensky, Klemperer, and Donaldson for other abnormal C̄I inactivator proteins (38).

To investigate this unexpected finding further, the inhibitory fractions from five separate gel filtrations of this patient's plasma were pooled and subjected to further analysis. In the subsequent experiments, the early eluting fraction was concentrated 5-fold; the intermediate, 10-fold; and the late, 8-fold. These pools were compared with similar pools from a normal subject. The kallikrein inhibition produced by each pool was quantitated in both the rat uterus and the esterase assays by determining the potency ratio between a control and an inhibited preparation of kallikrein. The potency ratio in each system was calculated as

follows:

Rat uterus assay:

$$\text{Potency ratio} = \frac{\text{Kallikrein doses/ml (control)}}{\text{Kallikrein doses/ml (inhibited)}},$$

Esterase assay:

$$\text{Potency ratio} = \frac{\mu M/\text{ml per hr (control)}}{\mu M/\text{ml per hr (inhibited)}}.$$

A potency ratio of 1 indicated no inhibition; progressively higher numbers indicated progressively greater inhibition. The activities of varying concentrations of kallikrein measured in the rat uterus assay paralleled those measured in the esterase assay. Therefore, if an inhibitor were equally effective in reducing a kallikrein preparation's kinin-releasing and esterase activities, it would be expected to give identical potency ratios in both assays.

Fig. 6 shows the results of incubating each inhibitor fraction from the HAE patient with kallikrein for varying periods of time. With the fraction containing α_1-antitrypsin, inhibition measured with both assays was similar and progressed slowly over a 1 hr period (Fig. 6, upper). The fraction containing α_2-macroglobulin gave greater inhibition in the rat uterus than the esterase assay (Fig. 6, middle)—a finding also noted
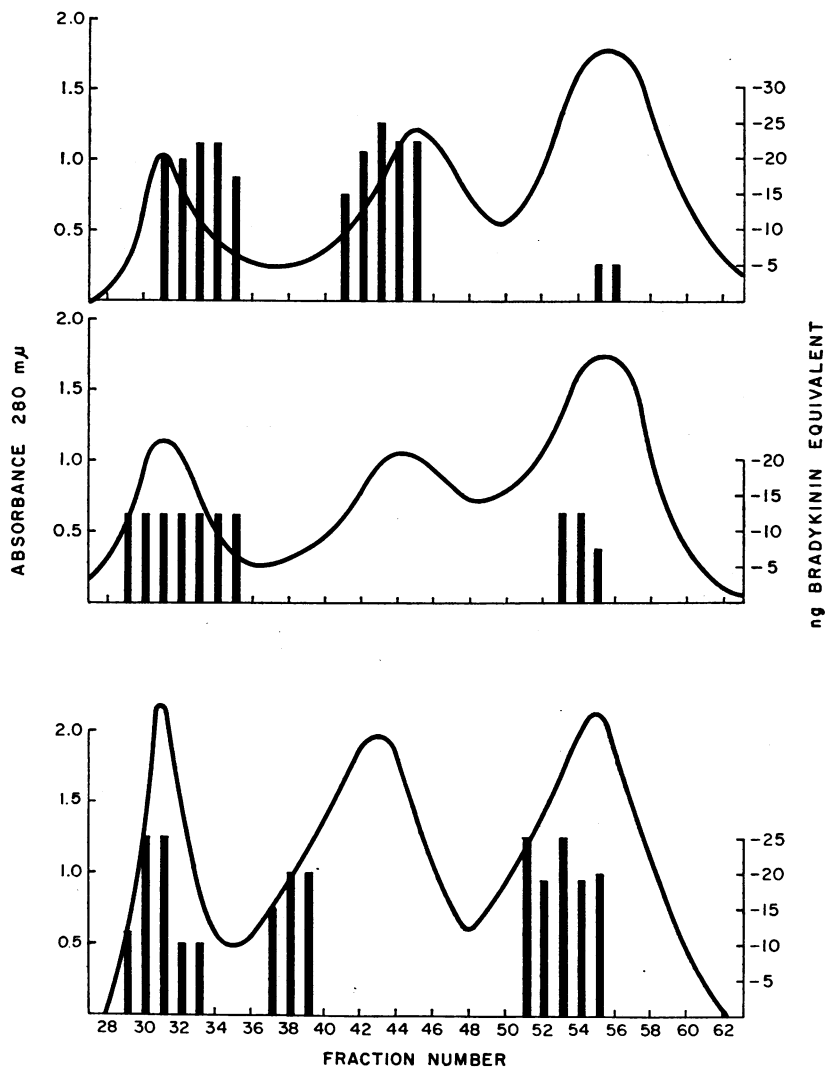

FIGURE 5   Gel filtration on Sephadex G-200 of: upper, normal whole human plasma, middle, HAE plasma lacking CĪ inactivator protein, lower, HAE plasma with abnormal CĪ inactivator protein. Conditions of these experiments were identical with those cited in Fig. 4.

by Harpel (22). The α_2-macroglobulin fraction had associated with it both esterase (0.94 μM/ml per min) and kinin-releasing activity (15 kallikrein doses/ml).
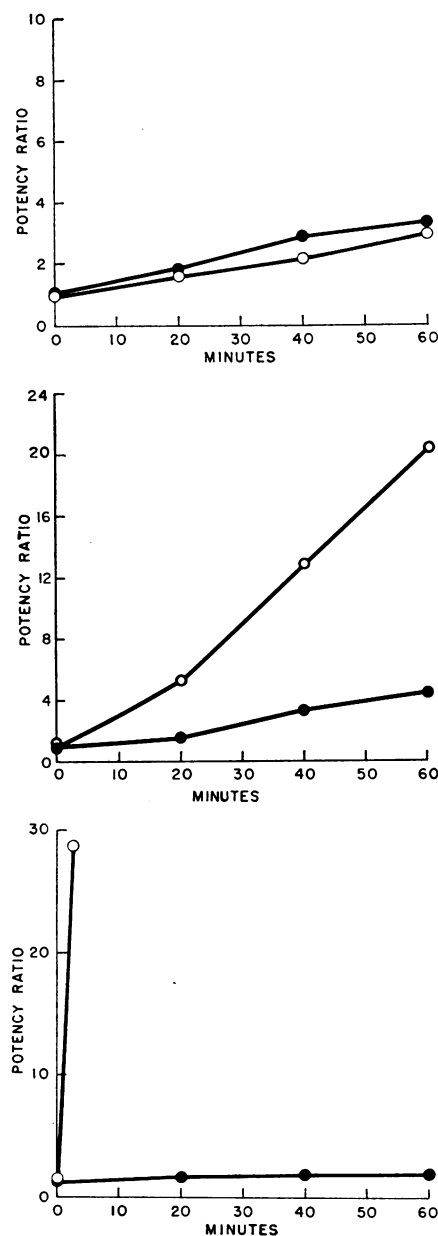

FIGURE 6 Inhibition of kallikrein with time by inhibitor fractions from the HAE patient with abnormal C$\bar{1}$ inactivator. Inhibition was measured by determining the potency ratios between the activities of control and inhibited kallikrein preparations. Progressively higher numbers indicate progressively greater inhibition. Upper, results with fraction containing α_1-antitrypsin; middle, results with fraction containing α_2-macroglobulin; lower, results with fraction containing abnormal C$\bar{1}$ inactivator. Open circles, rat uterus assay; closed circles, esterase assay.
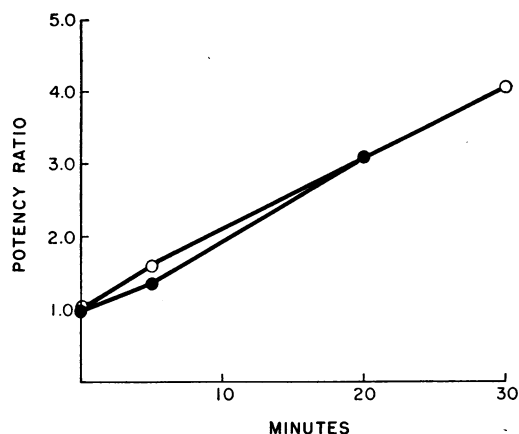

FIGURE 7 Inhibition of kallikrein with time by a fraction containing normal C$\bar{1}$ inactivator. Potency ratios were calculated as noted in Fig. 6 and the text. Open circles, rat uterus assay; closed circles, esterase assay.

Therefore, the esterase and kinin-releasing activities of α_2-macroglobulin-kallikrein mixtures had to be corrected for these activities before the potency ratios shown in Fig. 6 (middle) could be calculated. The esterase activity associated with the α_2-macroglobulin was totally protected from inhibition by 100 μg/ml of SBTI. The pools containing α_1-antitrypsin and α_2-macroglobulin from the normal subject gave results similar to those noted above.

The fraction containing abnormal C$\bar{1}$ inactivator gave much greater inhibition in the rat uterus assay than in the esterase assay (Fig. 6, lower). The normal C$\bar{1}$ inactivator pool gave similar inhibition in both assays (Fig. 7). This preparation was diluted to give less inhibition than the abnormal one in the bioassay, but produced more inhibition in the esterase assay.

With more dilute preparations of the abnormal C$\bar{1}$ inactivator a peculiar phenomenon was observed. Although inhibition of kallikrein could be demonstrated, it was not complete. Slight kallikrein activity persisted in the presence of inhibitor even at relatively high dilutions of kallikrein. An example of this phenomenon is shown in Fig. 8 (upper). The persisting activity at high kallikrein dilutions could not be attributed to activity carried by the abnormal protein as it had no kinin-releasing ability when tested alone. The results obtained with a normal preparation of C$\bar{1}$ inactivator are shown for comparison (Fig. 8, lower). With the normal C$\bar{1}$ inactivator, kallikrein activity was not detectable at dilutions above 1/32. Although the abnormal inhibitor apparently could bind kallikrein sufficiently to interfere with its reaction with kininogen, it could not protect kallikrein from SBTI 100 or 75 μg/ml and had no esterase activity associated with it when tested alone.
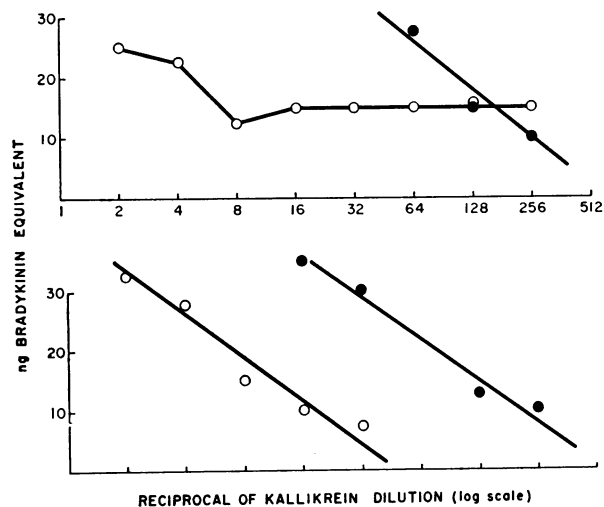
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FIGURE 8 Inhibition of graded dilutions of kallikrein by CĪ inactivator preparations. Upper, results obtained with a dilute preparation of the abnormal CĪ inactivator; lower, results obtained with a normal CĪ inactivator preparation. Open circles, inhibited preparation; closed circles, control preparation. Inhibited and control solutions preincubated for 30 min at 37°C before addition to kininogen substrate.

That CĪ inactivator was the protein responsible for the intermediate eluting inhibitor peak seemed likely as it was absent in the patient with low levels of CĪ inactivator and behaved atypically in the patient with the abnormal CĪ inactivator. The nature of this inhibitor activity was investigated further by attempting to neutralize it with CĪs. Forbes, Pensky, and Ratnoff have shown that CĪs can neutralize CĪ inactivator's effect on plasma thromboplastin antecedent and Hageman factor (26).

Preliminary experiments demonstrated that, when a CĪs preparation (21.0 U/ml) was tested in the standard fashion for kallikrein activity, none could be detected (0.3 ml of CĪs incubated with 0.3 ml of kininogen for 10 min at 37°C and then 0.2 ml of this mixture tested for uterus-contracting activity). However, when the incubation period of CĪs with kininogen was lengthened to 30 min, uterus-contracting activity was generated. When this CĪs preparation was incubated with a G-200 fraction containing normal esterase inhibitor, an apparent 5.6-fold reversal of kallikrein inhibition was observed. However, addition of the same amount of CĪs without inhibitor to the kallikrein preparation resulted in an apparent fourfold enhancement of its kinin-releasing activity. These results rendered it impossible to determine whether the preparation of CĪs studied was capable of reversing CĪ inactivator's action on kallikrein.

Starch block electrophoresis and immunoelectrophoresis provided further data on the behavior of

inhibitory pools from the HAE patient with the abnormal CĪ inactivator. On starch block electrophoresis, the patterns shown in Fig. 9 were obtained. The early and intermediate fractions migrated as α_2-globulins and continued to react with antisera against α_2-macroglobulin and CĪ inactivator, respectively (Fig. 9, upper and middle). The late eluting peak migrated as an α_1-globulin and reacted with antiserum against α_1-antitrypsin (Fig. 9, lower). No other inhibitor peaks were noted. Immunoelectrophoretic analysis of these fractions with specific antisera confirmed that they contained α_2-macroglobulin, α_1-antitrypsin, and a protein behaving immunologically and electrophoretically like CĪ inactivator. Analysis with anti-whole human serum revealed that they also contained several other proteins which were not identified.

In order to determine whether other inhibitors might be present in the G-200 pools containing α_1-antitrypsin and α_2-macroglobulin, these pools were treated with specific antibody and then examined for inhibitory

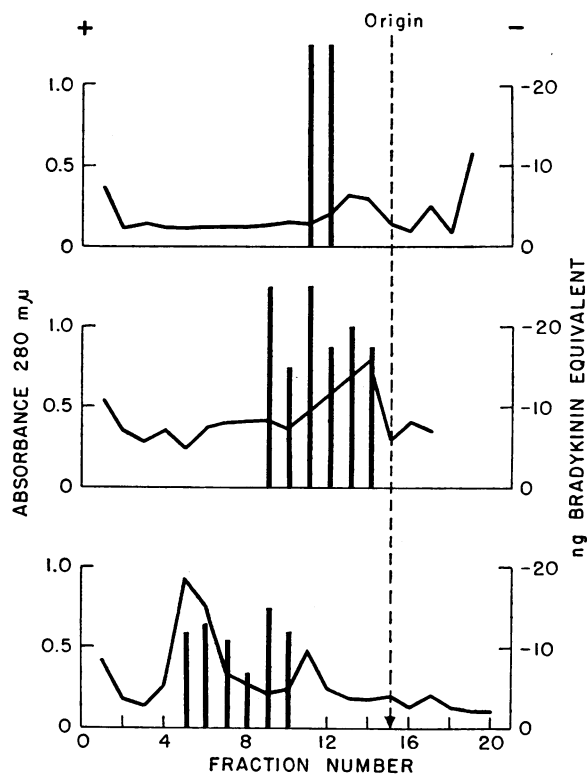FIGURE 9 Starch block electrophoresis of: upper, early; middle, intermediate; and lower, late eluting peaks of inhibitory activity from Sephadex G-200 gel filtration of plasma from the HAE patient with an abnormal CĪ inactivator protein. Conditions of the electrophoresis are given under Methods. Fractions were tested for: protein (———), kallikrein inhibitory activity (bars), and ability to react with specific antisera (see text).

TABLE II

Effect of Antibody against α_1-Antitrypsin on Kallikrein Inhibition by G-200 Fraction with this Protein

	Solutions		
	Kallikrein + α_1-antitrypsin + antibody	Kallikrein + phosphate-saline	Kallikrein + α_1-antitrypsin
α_1-Antitrypsin* (mg/100 ml)	14	undetectable	41
TAMe assay ($\mu M/ml$ per hr)‡			
observed	334	443	208
predicted	363	—	—
Rat uterus assay‡ (ng bradykinin equivalent released by a single kallikrein dilution)			
observed	12.5	15	7.5
predicted	13.5	—	—

* Final concentration of α_1-antitrypsin after being mixed with kallikrein.

‡ For both assays, the α_1-antitrypsin preparation was incubated for 2 hr with kallikrein before testing. The "observed" values are those obtained experimentally and the "predicted" values are those that would be expected if all the inhibitory activity were caused by α_1-antitrypsin that had been partially removed by precipitation with specific antibody.

capacity. The IgG fractions containing specific antibody were isolated as outlined under Methods. The α_2-macroglobulin-containing fraction studied was concentrated to 30 mg/100 ml α_2-macroglobulin and the α_1-antitrypsin-containing fraction to 82 mg/100 ml α_1-antitrypsin as determined by radial immunodiffusion. The amount of antibody necessary to precipitate all the α_2-macroglobulin or α_1-antitrypsin in each fraction was determined (46). Each antibody preparation was then dialyzed against 0.05 M NH$_4$HCO$_3$, lyophilized, and resuspended in the respective inhibitor fraction. In the case of α_2-macroglobulin, sufficient antibody to precipitate all the inhibitor was used; in the case of α_1-antitrypsin, the inhibitor remained in excess. The mixtures were incubated at 37°C for 30 min and then overnight at +4°C. The resulting precipitate was removed by centrifugation for 10 min at 3000 rpm and the supernatant fluid tested for kallikrein inhibitory activity in the rat uterus and esterase assays and

antigen content by radial immunodiffusion. The results are shown in Tables II and III. The agreement between the observed values and those that would be predicted, if all the inhibitory activity in a given fraction were caused by α_2-macroglobulin or α_1-antitrypsin was reasonably good and fell within the standard deviation observed throughout this study for both the esterase assay (± 40 $\mu M/ml$ per hr) and the bioassay (± 4 ng bradykinin equivalent). Normal rabbit IgG was unable to reduce the inhibitory capacity of either the α_2-macroglobulin- or the α_1-antitrypsin-containing fractions. None of the IgG fractions studied had detectable kallikrein activity. Presumably, any kallikrein originally present in these fractions was lost because of prolonged storage at +4°C or lyophilization.

Inhibition of kallikrein by heated human plasma. The following experiment was carried out to determine whether heated human plasma contained kallikrein inhibitory activity. A crude kallikrein preparation

TABLE III

Effect of Antibody against α_2-Macroglobulin on Kallikrein Inhibition by G-200 Fractions with this Protein

	Solutions		
	Kallikrein + α_2-macroglobulin + antibody	Kallikrein + phosphate-saline	Kallikrein + α_2-macroglobulin
α_2-Macroglobulin* (mg/100 ml)	undetectable	undetectable	15
TAMe assay‡ ($\mu M/ml$ per hr)	423	406	348
Rat uterus assay‡ (ng bradykinin equivalent released by a single kallikrein dilution)	37.5	35	<5

* Final concentration of α_2-macroglobulin after being mixed with kallikrein.

‡ For both assays, the α_2-macroglobulin preparation was incubated for 1 hr with kallikrein before testing. As noted in the text sufficient antibody was used to precipitate all the α_2-macroglobulin in the G-200 fraction.

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(1.5 ml) was mixed with an equal volume of heated human plasma. Controls were set up with kallikrein alone and heated human plasma alone with phosphate-saline. The mixtures and controls were incubated at 37°C for 1 hr and then dialyzed overnight against phosphate-saline at +4°C to rid the solution of any free kinin generated. None of the solutions, when tested alone, had any uterus-contracting activity. They were then assayed for kallikrein content in the standard fashion as outlined under Methods. The control kallikrein solution had 5560 kallikrein doses/ml, while the mixture of kallikrein plus heated human plasma had only 1925 kallikrein doses/ml—a 2.9-fold reduction in activity. The decrease in kinin production noted in the inhibited preparation could not be attributed to kininase activity in the heated human plasma as this preparation had no detectable kininase activity when incubated with bradykinin for periods up to 1 hr (see Methods). The incubation period during which kinin activity was generated by the inhibited and control preparations of kallikrein was only 10 min. When heated human plasma was brought to 50% saturation with $(NH_4)_2SO_4$ as described in the preparation of kininogen under Methods, neither the resulting precipitate nor the supernatant fraction could be shown to contain inhibitory activity by this method. It was apparently lost or obscured in the fractionation procedure.

DISCUSSION

The above results demonstrate that there are three inhibitors of kallikrein in normal human plasma which can best be separated by G-200 gel filtration. On the basis of the work presented here and that reported by others, the early eluting inhibitor can be identified as α_2-macroglobulin, the intermediate as C$\bar{1}$ inactivator, and the late as α_1-antitrypsin. The evidence for the identity of each is as follows.

The early inhibitor eluted just after the void volume on G-200 gel filtration, migrated as an α_2-globulin on electrophoresis, could be shown to contain α_2-macroglobulin on Ouchterlony plate analysis and immunoelectrophoresis, and could be neutralized by specific antibody against α_2-macroglobulin. It was more potent in the rat uterus than the esterase assay and protected the esterase activity associated with it from SBTI. The results reported here agree with those reported by Harpel for α_2-macroglobulin (22). The α_2-macroglobulin isolated in this study had considerably more esterase activity bound to it than did Harpel's. However, in the present study SBTI was not added to plasma during collection to avoid confusing it with a normal plasma inhibitor. Harpel also found more esterase activity bound to α_2-macroglobulin isolated in the absence of SBTI. Kallikrein was probably not the

sole TAMe esterase bound to the α_2-macroglobulin as plasmin and thrombin also bind to this protein without losing esterase activity (30). Our assay system detected kinin-releasing activity associated with α_2-macroglobulin, while Harpel's did not—a finding possibly attributable to a difference in the sensitivities of the two assays.

The intermediate inhibitor fraction eluted slightly before the IgG peak on G-200 gel filtration and could be shown to contain C$\bar{1}$ inactivator on double diffusion in agar and immunoelectrophoresis. That the intermediate eluting inhibitor must be C$\bar{1}$ inactivator is evident since it was missing from the HAE patient's plasma who lacked this protein antigen. The inhibitor isolated from normals was equally potent in the esterase and rat uterus assays, but that from the HAE patient with abnormal C$\bar{1}$ inactivator was much more effective in the rat uterus than the esterase assay. In this respect, the abnormal inhibitor seemed similar to α_2-macroglobulin. However, unlike α_2-macroglobulin it did not protect kallikrein esterase activity from SBTI. In addition, as it was isolated from the G-200 column it had no esterase activity bound to it as did α_2-macroglobulin. It seems likely that the abnormal C$\bar{1}$ inactivator from this patient binds kallikrein weakly and is relatively ineffective as an inhibitor compared to α_2-macroglobulin. Weak binding of kallikrein may account for its peculiar behavior on dilution. C$\bar{1}$ inactivator is an α_2-neuraminoglycoprotein (23) and the abnormal inhibitor migrated with an α_2-mobility on starch block electrophoresis. As C$\bar{1}$ inactivator has been reported to have a sedimentation coefficient of 3.7 (24), it was surprising to find both the normal and abnormal C$\bar{1}$ inactivators eluting before IgG on G-200 gel filtration. This phenomenon has also been noted by others and probably can be attributed to the inhibitor's properties as a glycoprotein (38, 53). Rosen et al. have studied a number of abnormal C$\bar{1}$ inactivator proteins and found that they varied considerably in their ability to inhibit C$\bar{1}$ esterase activity although none were effective in preventing C4 inactivation (38).

The late inhibitor eluted slightly before the albumin peak on G-200 gel filtration, migrated as an α_1-globulin on starch block electrophoresis, could be shown to contain α_1-antitrypsin on Ouchterlony plate analysis and immunoelectrophoresis, and could be neutralized by specific antibody against α_1-antitrypsin. It was equally potent in both the esterase and rat uterus assays but reacted slowly over a 1 hr period. In all these respects, the results reported here agree with those reported by Habermann for α_1-antitrypsin (20).

Have the results presented excluded the presence of other major kallikrein inhibitors in normal plasma? Of the techniques employed, G-200 gel filtration gave the greatest number of clearly separable inhibitory

fractions. From the data obtained with the HAE plasmas, it seems unlikely that there are other inhibitors in the intermediate area of the G-200 chromatogram. Although it is possible that other unidentified inhibitors were removed in the experiments with specific antibody against α_1-antitrypsin and α_2-macroglobulin, it seems unlikely. If so they would have to be quite similar to them in size, shape, and charge. In addition, they would have to behave remarkably like the known purified proteins in their action on kallikrein.

Could kininogen or kallikrein have masked an inhibitor present in one of the noninhibitory fractions from the gel filtration of whole plasma? If a significant amount of kininogen had been present when this fraction was tested for inhibitory activity, the slope of the kallikrein dose-response curve obtained should have been steeper than that of the control. This finding was not observed with any of the fractions tested. In addition, fractionation of plasma by other methods followed by G-200 gel filtration failed to uncover any inhibitory areas other than those found on fractionation of whole plasma. It is unlikely that kallikrein masked the presence of an inhibitor in the DEAE-cellulose chromatography fractions as it is known that the major portion of kallikrein activity elutes in the first protein peak and a previous study has shown that this peak contains no kallikrein inhibitory activity (35). Thus, although possible, it seems unlikely that major kallikrein inhibitors exist in human plasma other than those discussed in this paper.

The primary purpose of this work was to identify the inhibitors of plasma kallikrein in human plasma. However, the data also permit a preliminary analysis of the relative importance of each of the inhibitors studied—α_2-macroglobulin, α_1-antitrypsin, and the normal and abnormal $C\bar{1}$ inactivators. Kinin release is the most likely biologic function of kallikrein (2) and ability to inhibit this function is of prime importance in assessing each inhibitor. Of the inhibitory pools isolated from the HAE patient, the one containing α_1-antitrypsin, when 8-fold concentrated, was much less potent against kinin release than either the one with α_2-macroglobulin (5-fold concentrated) or the one with abnormal $C\bar{1}$ inactivator (10-fold concentrated). Both the α_2-macroglobulin and the $C\bar{1}$ inactivator pools from the normal subject were effective against kinin release. Harpel has shown that dilution affects the potency of these two inhibitors differently (22). Which is most effective cannot be determined until methods are developed for studying them at their normal in vivo concentrations. The results obtained with the abnormal $C\bar{1}$ inactivator preparation suggest that ability to bind tightly to kallikrein and inhibit its esterase activity are also important functions determining inhibitor potency.

As kallikrein is thought to play a role in the inflammatory response, ability to pass through vessel walls should also be considered in assessing the relative importance of each inhibitor. In this regard, one would expect α_1-antitrypsin and $C\bar{1}$ inactivator to be more effective than α_2-macroglobulin because of their smaller size. Ganrot, Laurell, and Ohlsson have shown that the ratio of α_1-antitrypsin to α_2-macroglobulin in lymph is twice that of plasma (54).

From the results reported, it is clear that heated human plasma contains kallikrein inhibitory activity which can be removed from the kininogen substrate by ammonium sulfate precipitation. Jahrreiss and Habermann have also recently noted the presence of kallikrein inhibitory activity in heated human plasma (55).

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