Inhibition by $C\bar{1}INH$ of Hageman Factor Fragment Activation of Coagulation, Fibrinolysis, and Kinin Generation

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ABSTRACT Highly purified inhibitor of the first component of complement ($C\bar{1}INH$) was shown to inhibit the capacity of active Hageman factor fragments to initiate kinin generation, fibrinolysis, and coagulation. The inhibition of prealbumin Hageman factor fragments observed was dependent upon the time of interaction of the fragments with $C\bar{1}INH$ and not to an effect upon kallikrein or plasmin generated. The inhibition of the coagulant activity of the intermediate sized Hageman factor fragment by $C\bar{1}INH$ was not due to an effect on PTA or other clotting factors. The inhibition by $C\bar{1}INH$ of both the prealbumin and intermediate sized Hageman factor fragments occurred in a dose response fashion. The $C\bar{1}INH$ did not appear to be consumed when the activity of the Hageman factor fragments was blocked, although the fragments themselves could no longer be recovered functionally or as a protein on alkaline disc gel electrophoretic analysis. These results suggest that the $C\bar{1}INH$ may have an enzymatic effect on the fragments or that an additional site on $C\bar{1}INH$ is involved in $C\bar{1}$ inactivation.

INTRODUCTION

The activation of Hageman factor results in the initiation of coagulation (1), kinin generation (2, 3), and fibrinolysis (4, 5) by activation of pre-plasma thromboplastin antecedent (pre-PTA)[1,2] (1), prekallikrein (6), and plasminogen proactivator (7, 8). These three plasma proenzyme substrates are activated both by intact activated Hageman factor and by fragments present in serum or derived from activated intact Hageman factor experimentally (6, 9). The inhibitor of the first component of complement ($C\bar{1}INH$) is recognized to inhibit kallikrein (10–12), plasmin (11), PTA (13), and the coagulant activity of activated Hageman factor (13). An additional critical site by which $C\bar{1}INH$ regulates these effector systems of tissue injury is shown to be its capacity to inhibit the action of the Hageman factor fragments upon each of these three plasma proenzymes.

METHODS

Antisera to α1 antitrypsin and α2 macroglobulin (α2M) (Behring Diagnostics Inc., Woodbury, N. Y.); hexadimethrine bromide (polybrene) (Aldrich Chemical Co., Inc., Milwaukee, Wis.); enzodiffusion fibrin plates and streptokinase (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) were obtained as indicated. Hageman factor-deficient plasma and PTA-deficient plasma were supplied by Sera-Tec Biologicals, New Brunswick, N. J. Concentration of various chromatographic fractions was performed by ultra filtration using UM-10 membranes (Amicon Corp., Lexington, Mass.) in either 500 ml, 50 ml, or 10 ml capacity Amicons as appropriate. All phosphate buffer used

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[1] In order to avoid ambiguity and confusion as to the state of activation of the molecule we have referred to the precursor of PTA as pre-PTA throughout the text. Other workers may consider PTA as the precursor form and refer to the activated molecule as activated PTA.

[2] *Abbreviations used in this paper:* α2M, α2 macroglobulin; $C\bar{1}INH$, inhibitor of the activated first component of complement; $EAC\bar{1}$, erythrocyte-bound $C\bar{1}$; PTA, plasma thromboplastin antecedent; PTT, partial thromboplastin time; QAE, Quartenary aminoethyl; SE Sephadex, Sulphoethyl Sephadex; SP Sephadex, Sulphopropyl Sephadex.

was 0.0035 M phosphate buffer pH 7.8–8.0 containing 110 mg $NaH_2PO_4 \cdot H_2O$ and 388 mg Na_2HPO_4 per liter. All phosphate-buffered saline was 0.0035 M phosphate buffer containing 0.15 M NaCl.

Plasma was prepared for the isolation of plasma proenzymes and C1INH in ethylenediaminetetraacetic acid (EDTA) and polybrene as described (6). The conversion of pre-PTA (1), prekallikrein (6), and plasminogen proactivator (7, 8) by the Hageman factor fragments was measured in terms of subsequent coagulation, bradykinin formation, and fibrinolysis. The coagulant activity of the Hageman factor fragment was determined by preparing two-fold falling dilutions of the preparation of Hageman factor fragment and relating its concentration to the partial thromboplastin time (PTT) achieved with Hageman factor-deficient plasma (Fig. 1).

Hageman factor prealbumin fragments were prepared as follows: plasma dialyzed against 0.0035 M phosphate buffer pH 8.0 containing 0.06 M NaCl was applied to Quartenary aminoethyl (QAE) Sephadex (5 × 100 cm) equilibrated with 0.0035 M phosphate buffer pH 8.0. The column was batch eluted with the dialysis buffer, concentrated to the starting volume, activated by stirring in a glass beaker for 24 h at 4°C, dialyzed against 0.0035 M phosphate buffer pH 8.0, and rechromatographed on QAE Sephadex with a linear salt gradient of 2,500 ml of dialysis buffer and 2,500 ml of 0.0035 M phosphate buffer pH 8.0 containing 0.3 M NaCl. The column was run at 50 ml/h and 12 ml fractions were collected. The Hageman factor fragments eluted between 0.23 and 0.28 M NaCl, were concentrated to 4 ml, and were fractionated at 30 ml/h on Sephadex G-100 (5 × 100 cm) equilibrated with 0.0035 M phosphate buffer pH 8.0 containing 0.15 M NaCl. The Hageman factor prealbumin fragments eluted at a mol wt of 32,500, were concentrated to approximately 20–25 μg/ml, aliquoted, and stored at −70°C for routine use. Disc gel electrophoresis (14) revealed prealbumin bands with only trace contamination with albumin.

An intermediate sized Hageman factor fragment was isolated from human plasma by applying plasma dialyzed against 0.0035 M phosphate buffer pH 8.0 containing 0.04 M NaCl to QAE Sephadex (5 × 100 cm) equilibrated with 0.0035 M phosphate buffer pH 8.0. The column was batch eluted with the dialysis buffer, concentrated to the starting volume, activated by stirring in a glass beaker for 24 h at 4°C, dialyzed against 0.0035 M phosphate buffer pH 8.0, and rechromatographed on QAE Sephadex with a linear salt gradient of 2,500 ml of dialysis buffer and 2,500 ml of 0.0035 M phosphate buffer pH 8.0 containing 0.3 M NaCl. 12 ml fractions were collected at 50 ml/h. The intermediate sized Hageman factor fragment eluted between 0.08 M and 0.10 M NaCl, was concentrated to 4 ml, and was fractionated at 30 ml/h on Sephadex G-100 (5 × 100 cm) equilibrated with 0.0035 M phosphate buffer pH 8.0 containing 0.15 M NaCl. The intermediate sized Hageman factor fragment eluted at a mol wt of 80,000 and was divided and stored at −70°C for further use. Assessment of the preparation by disc gel electrophoresis revealed two bands in the β-globulin region, one corresponding to the Hageman factor fragment and the other to a transferrin contaminant.

Plasminogen was prepared by affinity chromatography of 100 ml of plasma utilizing lysine-sepharose columns and ε-aminocaproic acid elution (8, 15). After dialysis against 0.0035 M phosphate buffer pH 8.0 containing 0.15 M NaCl, the preparation was fractionated by Sephadex G-100 gel filtration at 30 ml/h utilizing a 5 × 100 cm column equilibrated with the dialysis buffer. Fractions containing plasminogen as assessed by streptokinase activation followed by application to fibrin plates were pooled, concentrated, and stored at −70°C. Disc gel electrophoresis revealed a single broad band identified as plasminogen by functional analysis of an unstained sliced replicated disc gel. There were no contaminating proteins detected. Plasmin was prepared by activating 500 μl of plasminogen (100 μg/ml) with 50 μl (1,500 U) of streptokinase for 30 min at 30°C and assayed on human fibrin plates (8).

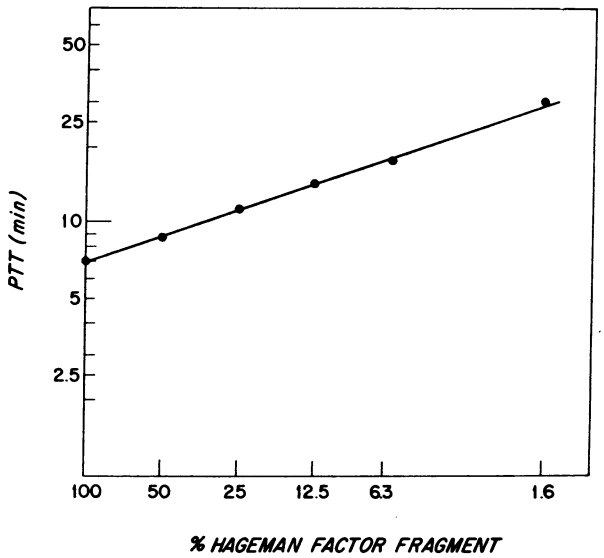

FIGURE 1 Correction of the partial thromboplastin time of Hageman factor-deficient plasma by the intermediate sized Hageman factor fragment.

The plasminogen proactivator was isolated by applying 80 ml of dialyzed human plasma to QAE Sephadex (5 × 100 cm) equilibrated in 0.0035 M phosphate buffer pH 8.0. The column was batch eluted with the dialysis buffer, concentrated to 10 ml, dialyzed against 0.0035 M phosphate buffer pH 6.0, and applied to Sulphoethyl (SE) Sephadex (3.5 × 30 cm) equilibrated with the dialysis buffer. The plasminogen proactivator was eluted with a linear salt gradient of 2,000 ml of equilibrating buffer and 2,000 ml of equilibrating buffer containing 0.35 M NaCl. The column was run at 50 ml/h and 10 ml fractions were collected. The plasminogen proactivator eluted between 0.12 and 0.14 M NaCl and was pooled, concentrated to 25 ml, and fractionated on Sephadex G-150 (2.5 × 150 cm) equilibrated in 0.0035 M phosphate buffer pH 8.0 containing 0.15 M NaCl. Fractionation was performed by upward flow at 10 ml/h and 2.5 ml fractions were collected. The plasminogen proactivator eluted at a mol wt of approximately 100,000 and was pooled, concentrated to 10 ml and stored at −70°C. The preparation contained trace prekallikrein and IgG contamination when assessed by bioassay and Ouchterlony or alkaline disc gel electrophoretic analysis, respectively. The plasminogen proactivator did not contain plasminogen or PTA.

Purification of C1INH. 80 ml of plasma dialyzed against 0.0035 M phosphate buffer pH 7.8 were applied to a 5 × 100 cm column of QAE Sephadex equilibrated with the same buffer. The column was washed with 600 ml of equilibrating

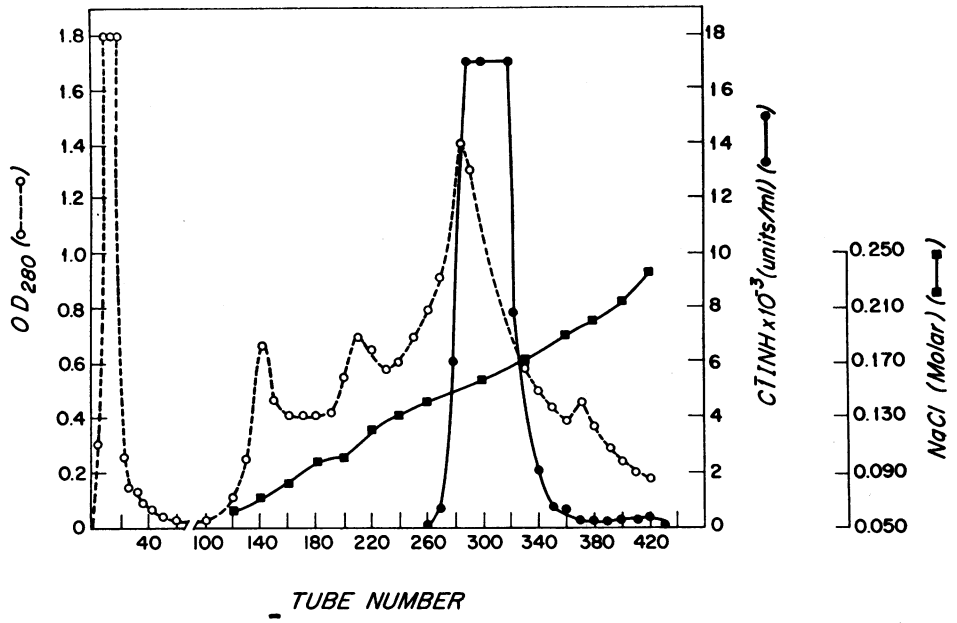

FIGURE 2 Isolation of C1INH by chromatography of human plasma on QAE Sephadex.

buffer and eluted with a linear salt gradient of 2,500 ml of equilibrating buffer and 2,500 ml of 0.0035 M phosphate buffer pH 7.8 containing 0.3 M NaCl. The column was run at 50 ml/h and 10 ml fractions were collected. The CĪINH was assayed functionally in microtiter plates; the CĪINH peak eluted at 0.15 M NaCl (Fig. 2). Fractions containing CĪINH were pooled, concentrated to 20 ml, dialyzed for 5 h against 0.05 M sodium acetate buffer pH 5.0, and applied to a 3.5 × 30 cm column of Sulphopropyl (SP) Sephadex equilibrated with the same buffer. The column was washed with 200 ml of equilibrating buffer, and the CĪINH was batch eluted by washing the column with approximately 200 ml of 0.05 M sodium acetate buffer pH 5.0 containing 0.09 M NaCl. The column was run at 50 ml/h and fractions were collected in glass tubes containing 5 ml of 2 M Tris Cl buffer pH 8.0 to total 13 ml per tube, in order to raise the final pH above 7.2. The CĪINH pool was concentrated to 4 ml and applied to a 5 × 100 cm column of Sephadex G-200 equilibrated in 0.0035 M phosphate buffer pH 7.8 containing 0.15 M NaCl. Fractionation was performed by upward flow at 10 ml/h and 10 ml fractions were collected. CĪINH eluted along the ascending limb of the

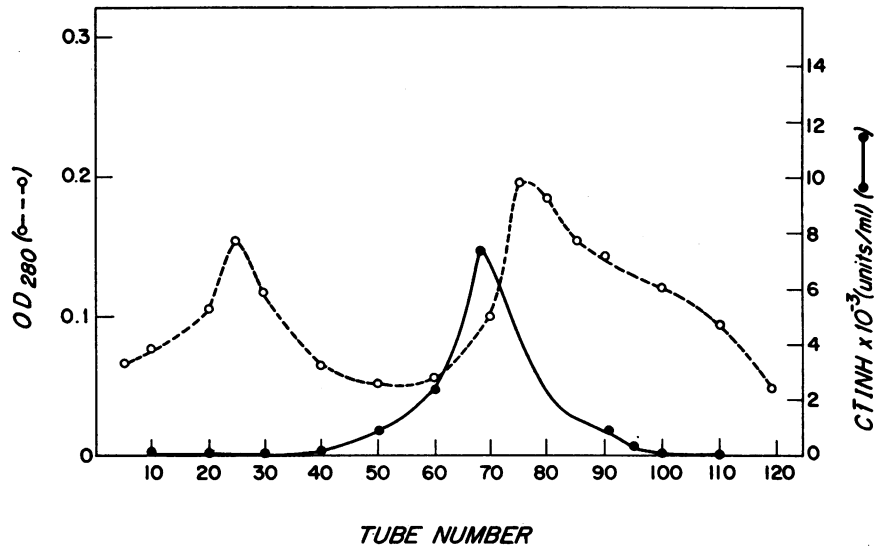

FIGURE 3 Isolation of CĪINH obtained by QAE and SP Sephadex chromatography by Sephadex G-200 gel filtration.

second protein peak as shown in Fig. 3. The fractions containing C1INH were concentrated to 2.0 ml and further fractionated by Sephadex G-150 (2.5 × 150 cm) gel filtration equilibrated in 0.0035 M phosphate buffer pH 7.8 containing 0.15 M NaCl. Fractionation was performed by upward flow at 10 ml/h and 2.5 ml fractions were collected. No optical density at 280 nm was observed; fractions containing C1INH eluted at 55% of bed volume and were pooled (total volume 27 ml) and stored at −70°C for further studies. This preparation had no detectable α1 antitrypsin or α2M as assessed by Ouchterlony analysis or electroimmunodiffusion utilizing monospecific antisera to each. This highly purified C1INH preparation (6,000 U/ml) gave a double band on alkaline disc gel electrophoresis in a region from which unstained gels yielded active C1INH upon elution (Fig. 4).

The C1INH was assessed functionally by its ability to inhibit the hemolytic activity of erythrocyte bound C1 (EAC1) by either microtiter plate or tube titrations (16) as indicated and was quantitated by immunodiffusion (17, 18). The titer of 1,613 U/μg of C1INH obtained is comparable to the activity in normal serum.

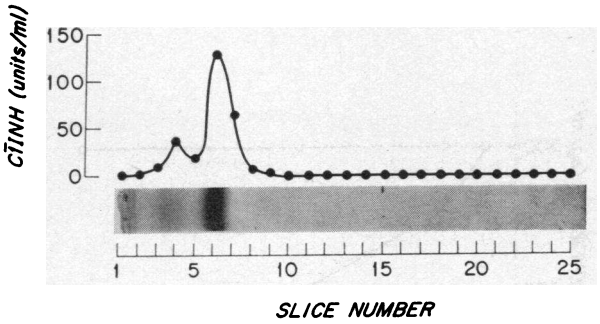

FIGURE 4 Disc gel electrophoresis of C1INH obtained by QAE Sephadex (Fig. 2), SP Sephadex, Sephadex G-200 (Fig. 3), and Sephadex G-150 chromatography.

RESULTS

Inhibition of the action of Hageman factor fragments upon prekallikrein, plasminogen proactivator, and pre-PTA

Prekallikrein. 10 μl of Hageman factor fragments were incubated with increasing volumes of highly purified C1INH (1,000 U/ml) at 37°C for 30 min. Each mixture was brought to a final volume of 160 μl and incubated for 2 min at 37°C with fresh human plasma as a source of prekallikrein and kininogen, and the bradykinin generated was determined. As illustrated in Fig. 5, as the amount of C1INH was increased, the bradykinin generating activity of the Hageman factor fragments was progressively decreased. In order to distinguish inhibition by C1INH of the Hageman factor fragments from direct inhibition of kallikrein, the Hageman factor fragments were incubated with C1INH (1,000 U/ml) for varying time intervals before introduction into fresh plasma. No inhibition of bradykinin generation was observed when the Hageman factor fragments and C1INH were preincubated for up to 2 min, the time interval subsequently used to assess the bradykinin generating capacity of the Hageman factor fragments in fresh plasma. After 2 min of preincubation of the fragments with C1INH, a progressive decrease in the capacity of the fragments to generate bradykinin from fresh plasma was observed (Fig. 6). These kinetic studies reveal that the effect of C1INH is on the Hageman factor fragments and not upon kallikrein generated.

250 μl of Hageman factor fragments were incubated with either 250 μl of C1INH (10,000 U) or with buffer alone for 90 min at 37°C. In addition, 250 μl of C1INH were incubated with buffer under the same conditions. Replicate 225 μl samples from each mixture were then assessed by disc gel electrophoresis; one disc gel was

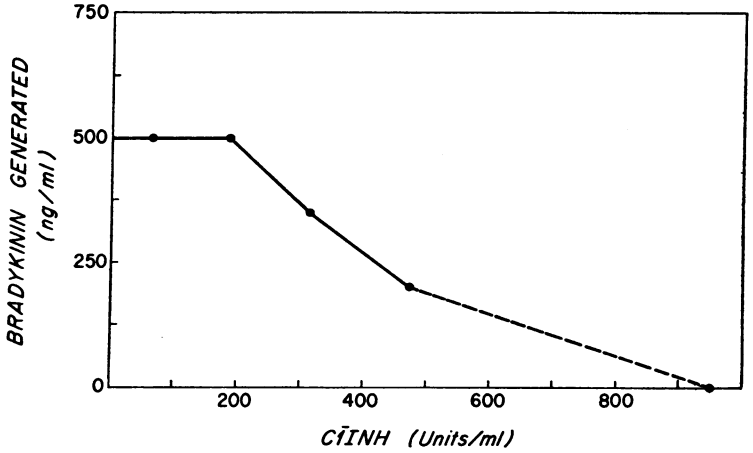

FIGURE 5 Inhibition by C1INH of the ability of the prealbumin Hageman factor fragments to generate bradykinin from fresh plasma.

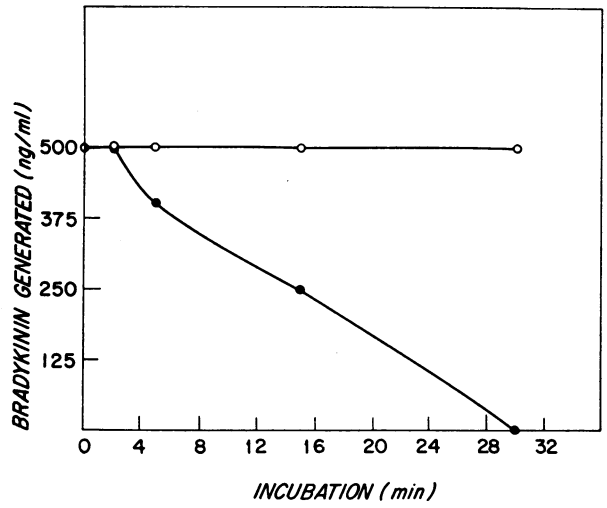

FIGURE 6 Kinetics of inhibition by CĪINH (closed circles) of the capacity of the prealbumin Hageman factor fragments to generate bradykinin from fresh plasma. Open circles refer to prealbumin Hageman factor fragments not treated with CĪINH.

stained with Coomassie blue stain (Colab Lab, Inc., Chicago Heights, Ill.) and the replicate gel sliced into 1 mm sections, crushed, eluted in 200 μl 0.15 M sodium chloride, and assayed for prekallikrein activating activity. Prekallikrein activating activity was found in slices 7–9 of the disc gel of Hageman factor fragments incubated in buffer alone. No prekallikrein activating activity was detected in the disc gel eluates from Hageman factor fragments preincubated with CĪINH (Fig. 7). In addition, the stained gel of the Hageman factor fragments incubated in buffer contained prealbumin bands at the position corresponding to slices 7–9, whereas no prealbumin bands were present in the disc gel of Hageman factor fragments preincubated with CĪINH.

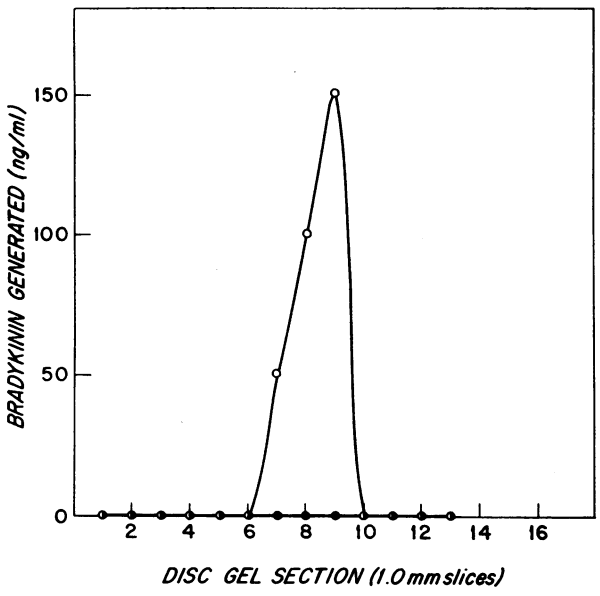

FIGURE 7 Elution of prekallikrein activating activity after disc gel electrophoresis of prealbumin Hageman factor fragments treated with CĪINH (closed circles) or buffer (open circles).

Plasminogen proactivator. 10 μl of Hageman factor fragments were incubated with 200 μl of CĪINH (1,000 U/ml) in phosphate-buffered saline (pH 7.8) or with buffer alone for 30 min at 37°C. 5 μl were removed from each incubation mixture and added to 50 μl of plasminogen proactivator; the mixture was placed at 4°C for 15 h to permit conversion of plasminogen proactivator to plasminogen activator. 20 μl of this mixture were then incubated with 20 μl of highly purified plasminogen (200 μg/ml) for 1 h at 37°C. The Hageman factor fragments incubated in buffer alone generated 10.5 μg/ml of plasmin, while the Hageman factor fragments preincubated with CĪINH yielded only 2.5 μg/ml of plasmin. This result is attributed to inhibition of the ability of the Hageman factor fragments to generate plasminogen activator from the plasminogen proactivator. At the concentration of CĪINH utilized, no inhibition of plasmin (50 μg/ml) obtained by interaction of plasminogen with either plasminogen activator or streptokinase was demonstrable.

Pre-PTA. For these experiments a higher molecular weight Hageman factor fragment (mol wt = 80,000) possessing greater pre-PTA activating activity than the

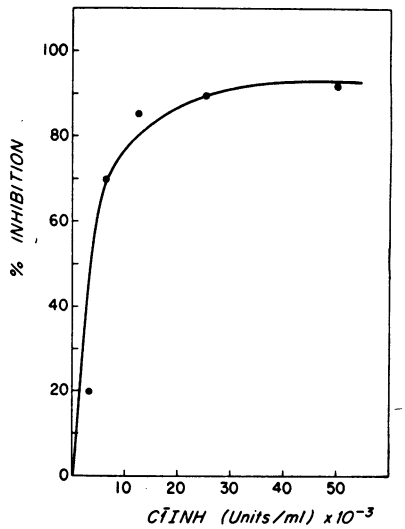

FIGURE 8 Inhibition by CĪINH of the ability of the intermediate size Hageman factor fragment to correct the clotting deficiency of Hageman factor-deficient plasma.

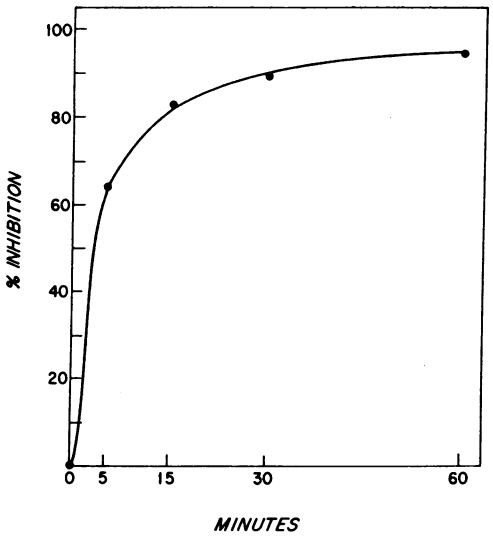

FIGURE 9 Kinetics of inhibition by C1INH of the capacity of the intermediate size Hageman factor fragment to correct the clotting deficiency of Hageman factor-deficient plasma.

prealbumin fragments (mol wt = 32,500) was utilized. 25 μl of twofold falling dilutions of C1INH (50,000 U/ml) were incubated for 80 min at 37°C with 25 μl of Hageman factor fragment and the correction of the PTT of Hageman factor-deficient plasma determined. As shown in Fig. 8, as the concentration of the C1INH increased, there was progressive inhibition of the coagulant activity of the Hageman factor fragment.

A time-course of inhibition of the coagulant activity of the Hageman factor fragment by C1INH was studied as follows. 125 μl of Hageman factor fragment were incubated with either 125 μl of C1INH at a concentration of 50,000 U/ml in phosphate-buffered saline pH 7.8 or in buffer alone at 37°C. 50 μl samples of each mixture were then removed at designated intervals, and the ability to correct the PTT of Hageman factor-deficient plasma determined. Addition of kaolin did not lead to further activation or a decrease in PTT, as the material was completely active. No inhibition of the Hageman factor fragments occurred at 0 time, while inhibition was marked by 5 min and tended to reach a plateau after 15 min of interaction of C1INH with Hageman factor fragment (Fig. 9). This experiment indicates that the effect of C1INH is on the Hageman factor fragment and not upon the PTA generated or later clotting factors.

C1INH consumption

In order to ascertain whether the inhibition of the Hageman factor fragments by C1INH results in consumption of C1INH, residual C1INH was measured by its ability to inhibit C1. 75 μl of an 80,000 mol wt Hageman factor fragment preparation undiluted and diluted 1:2 with phosphate-buffered saline pH 7.8 were incubated with either 75 μl of C1INH (5,000 U/ml) or buffer alone at 37°C for 30 min. These mixtures were then examined for their prekallikrein and pre-PTA activating ability using fresh plasma and Hageman factor-deficient plasma, respectively, as described above. The C1INH inhibited 100% of the kinin generating activity and 50% of the coagulant activity in the undiluted preparation. In addition, 75 μl of C1INH were incubated with 75 μl of a partially purified preparation of kallikrein, and 10 μl of the mixture were examined for their ability to generate bradykinin from 200 μl of heat-inactivated plasma, using a 2 min incubation; the kallikrein was 90% inhibited. As shown in Fig. 10, incubation of C1INH with either the undiluted Hageman factor fragment or Hageman factor fragment diluted 1:2 did not change the C1INH titer even though the kinin generating and coagulant ability of the fragments was markedly reduced. The partially purified kallikrein preparation produced a decrease in the C1INH titer from 4,700 to 600 U/ml.

The effect of increasing the ratio of Hageman factor fragments to C1INH was examined by incubating 10, 20, or 30 μl of the 80,000 mol wt Hageman factor fragment preparation with 10 μl of the C1INH preparation (5,000 U/ml) for 30 min at 37°C in a total volume of 40 μl for each reaction mixture. 10 μl of C1INH were also incubated with the partially purified kallikrein preparation under the same conditions. As in the previous experiment, inhibition of kallikrein (100%) was associated with C1INH consumption. 100% inhibition

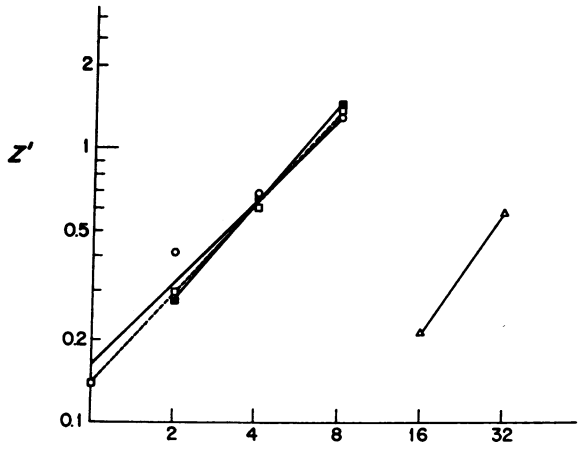

FIGURE 10 The effect of Hageman factor fragment (open and closed boxes) and kallikrein (open triangles) on the recovery of C1INH as measured by an effective molecule titration. C1INH incubated in buffer alone is indicated by the open circles.

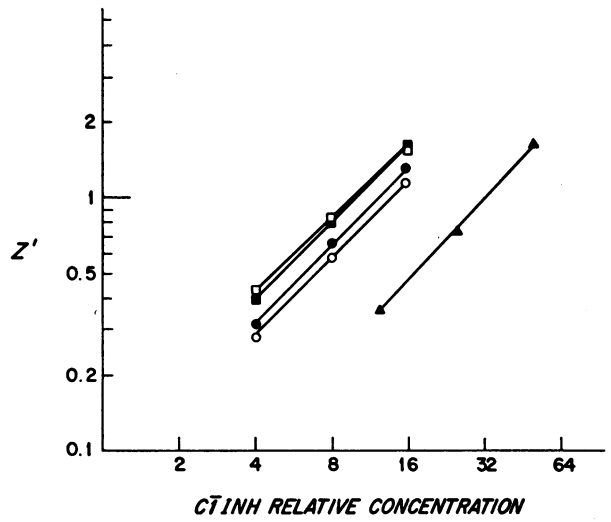

FIGURE 11 The effect of an increased ratio of Hageman factor fragment to C1INH (closed squares and open and closed circles) and kallikrein (closed triangles) on the recovery of C1INH as measured by an effective molecule titration. C1INH incubated in buffer alone is indicated by the open squares.

of the Hageman factor fragments in terms of kinin generation was not associated with C1INH consumption (Fig. 11.)

DISCUSSION

Highly purified C1INH (Fig. 4) was shown to inhibit the capacity of active Hageman factor fragments to initiate kinin generation, fibrinolysis, and coagulation. As C1INH had been previously recognized to inhibit kallikrein (10–12), plasmin (11), and PTA (13), it was essential to distinguish an action at these points from the effect of the C1INH on the Hageman factor fragments. The C1INH inhibited the kinin generating activity of the Hageman factor fragments in whole plasma in a dose response fashion (Fig. 5). The experimental conditions were such that the inhibition observed was dependent upon the time of interaction of the fragments with C1INH (Fig. 6), and not to an effect on the kallikrein generated. Similarly, a concentration of C1INH having no effect upon plasmin or the plasminogen activator was capable of protecting highly purified plasminogen proactivator from activation by the prealbumin Hageman factor fragments. Utilizing an intermediate sized Hageman factor fragment, it was possible to show that the C1INH yielded a dose-dependent inhibition of the capacity to correct the coagulation defect of Hageman factor-deficient plasma (Fig. 8). The inhibition observed was dependent upon the time of interaction of the fragment with C1INH and not to an effect upon PTA or the other clotting components generated (Fig. 9). Thus, the C1INH inhibited the action of intermediate and small Hageman factor fragments upon three of its naturally occurring substrates, prekallikrein, plasminogen proactivator, and pre-PTA, at concentrations of C1INH comparable to those present in normal plasma and under experimental conditions in which its effect on other enzymes in the reaction sequences was not a factor.

Recent studies have also revealed that partially purified C1INH suppresses the esterase activity of 37,000 mol wt Hageman factor fragments on benzoyl arginine ethyl ester (19). Forbes, Pensky, and Ratnoff have demonstrated that incubation of C1INH with $C\bar{1}$ esterase reverses the ability of C1INH to inhibit either activated Hageman factor or activated PTA in clotting assays and suggest a common site on C1INH for Hageman factor, PTA, and $C\bar{1}$ esterase (13). However, destruction by $C\bar{1}$ esterase as has been suggested by Loos, Wolf, and Opferkuch (20) might also account for this observation.

The interaction of C1INH with kallikrein was previously observed to deplete both functions from the reaction mixture (12) when kallikrein was measured as an esterase and C1INH assessed by an effective molecule titration. In contrast, in the present experiments inhibition of the 80,000 mol wt Hageman factor fragment by C1INH was not associated with consumption of the C1INH (Figs. 10 and 11). The functional integrity of the inhibitor after interaction with the 80,000 mol wt Hageman factor fragment was established by its action in interfering with the hemolytic titration of $C\bar{1}$. Even though the C1INH did not appear to be consumed when the prealbumin fragments were blocked, the fragments themselves could no longer be recovered either functionally or as a protein on disc gel electrophoretic analysis of the reaction mixture (Fig. 7). These results suggest the C1INH may have an enzymatic effect on the fragments, but might also be explained by an additional site on C1INH involved in $C\bar{1}$ inactivation.

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