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J Clin Invest. 1972;51(7):1813-1822. <https://doi.org/10.1172/JCI106983>.

Research Article

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Studies on the Interaction between Collagen and a Plasma Kallikrein-Like Activity

EVIDENCE FOR A SURFACE-ACTIVE ENZYME SYSTEM

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ABSTRACT This study has demonstrated that collagen particles, after exposure to platelet-poor human plasma and subsequent washing, generate a kinin-like agent when incubated with prekinin substrate. The binding of kinin-generating activity to collagen in the plasma collagen incubation mixture occurs rapidly, whereas the loss of this activity in the incubation mixture occurs relatively slowly. The Hageman factor appeared to be necessary for the surface-bound kinin-generating activity, as this activity was absent in collagen exposed to Hageman factor-deficient plasma. These studies have partially characterized the plasma-derived enzymatic activity bound to collagen. Incubation of collagen with plasma caused a concentration-dependent reduction in the kinin-producing activity which was generated by the addition of ellagic acid, a known activator of plasma kallikrein. The kinin-inducing activity bound to collagen is inhibited by soybean trypsin inhibitor, Trasylol, serum C $\bar{1}$ inactivator and the plasma α_2 -macroglobulin, but not by lima bean trypsin inhibitor. An eluate prepared from plasma-treated collagen, when compared with purified plasma kallikrein, shared a similar inhibitor profile. Selective chemical blockage of the free carboxyl groups on the collagen molecule, or heat denaturation, inactivated the ability of the collagen to generate kinin-like activity after incubation with plasma. Removal of the collagen telopeptides or blockage of the free amino groups failed to affect the collagen-plasma interaction. The binding of

partially purified plasma kallikrein to collagen was found to have similar structural and chemical requirements. These data indicate that there is a structural and chemical specificity for the activation and binding of plasma kallikrein-like activity by collagen. These studies suggest that a plasma kallikrein may function as a surface-bound enzyme system.

INTRODUCTION

Human plasma kallikreins are proteolytic enzymes which circulate in the form of inactive precursors. Their activation results in the release of kinins from a plasma α_2 -globulin substrate termed prekinin or kininogen (1). The kinins are a group of related polypeptides which are among the most potent vasoactive pharmacologic agents known. These substances, in nanogram quantities, produce hypotension, increase blood flow and microvascular permeability, influence smooth muscle contraction, incite pain, and stimulate *in vivo* leukotaxis (1, 2). The biologic activity of the kinins suggests that they may be primary chemical mediators of the inflammatory reaction (2).

Neither the mechanisms which trigger the activation of the plasma kallikrein-kinin enzyme system *in vivo* nor the way in which this system participates in pathophysiologic states are well understood. It is generally agreed that the activation of Hageman factor (factor XII) represents one pathway for the activation of plasma kallikrein activity (3, 4). A variety of substances, insoluble in plasma, have been reported to activate the kallikrein system. These agents include glass, celite, kaolin (5, 6), articular cartilage (7), and antigen-antibody complexes (8-10). Whereas the activation of plasma kallikrein-like activity by these substances in the fluid phase has been documented, the

This study was presented in part at the 44th Scientific Sessions of the American Heart Association, Anaheim, Calif., 12 November 1971.

Dr. Harpel is the recipient of a U. S. Public Health Service Research Career Development Award 1-KO4-HE 50, 285.

Received for publication 17 January 1972 and in revised form 24 February 1972.

possible contribution of surface-bound kallikrein to the activities observed has not been studied. Recent observations have established that collagen, a constituent of the subendothelium of the blood vessel wall and of connective tissue, activates Hageman factor (11-13), thereby initiating blood coagulation. The interaction between purified collagen and the plasma kallikrein system, however, has not been previously investigated.

The present study was designed to examine (a) whether particulate collagen, after incubation with plasma, would bind kinin-generating activity, (b) the effect of time on this interaction, (c) the relationship of the collagen-bound enzyme to a purified plasma kallikrein, (d) the structural requirements of the collagen molecule necessary for the activation and binding of plasma kinin-inducing activity, and (e) the interaction of purified plasma kallikrein with native or with chemically modified collagen.

METHODS

Plasma. Venous blood from normal individuals and from a patient with Hageman factor deficiency was collected in plastic syringes and placed in 0.1 vol 3.8% trisodium citrate. The plasma was harvested after centrifugation at 2000 *g* for 15 min at 4°C, and recentrifuged twice at 20,000 *g* for 15 min at 4°C. The platelet-poor plasma was stored in 1.0-ml portions in plastic at -20°C. Plastic or silicone-coated glass equipment was used throughout to prevent Hageman factor activation (6).

The chemicals used were reagent grade and were obtained from the following sources: ellagic acid from K & K Laboratories, Inc., Plainview, N. Y.; dinitrofluorobenzene (DNFB)¹ and bradykinin triacetate from Calbiochem, San Diego, Calif.; Trasylol (compound A-128, proteinase inhibitor) from FBA Medical Research, Division of Metachem Laboratories, Inc., Highland, Ind.; crystallized soybean trypsin inhibitor (SBTI), lima bean trypsin inhibitor (LBTI), pepsin, collagenase, and carboxypeptidase B from Worthington Biochemical Corp., Freehold, N. J.; elastase from Sigma Chemical Co., St. Louis, Mo., and *o*-phenanthroline from Fisher Scientific Co., Springfield, N. J. The following buffers were used: 0.05 M Tris-HCl, pH 8.0, containing 0.02 M NaCl (tris-saline buffer); 0.025 M sodium barbital, pH 7.4 containing 0.125 M sodium chloride (barbital-saline buffer); 0.05 M glycine-HCl buffer, pH 2.5 (13); and 1.7 M sodium chloride buffered with 0.05 M tris-HCl buffer, pH 8.0.

Calf skin collagen powder was kindly provided by Doctors Albert L. Rubin and Kurt H. Stenzel of the Rogosin Laboratories, Departments of Surgery and Biochemistry, The New York Hospital-Cornell Medical Center. It was prepared as previously described (14). Calf skin was placed in 10% sodium chloride solution for 12 hr at 4°C and then washed with tap water. Hair was removed with esterase in borax buffer pH 8.0, at 25°C. The skin was frozen with dry ice and powdered by a mechanical crusher. The powder was washed successively with 0.01 M acetic acid, 0.1 M disodium phosphate, distilled water, and then air dried. This

¹ *Abbreviations used in this paper:* DNFB, dinitrofluorobenzene; LBTI, lima bean trypsin inhibitor; SBTI, soybean trypsin inhibitor; TAME, tosyl arginine methyl ester.

preparation was utilized in the present study and is referred to as collagen or insoluble collagen. This preparation was assayed for tissue thromboplastin activity by Dr. Yale Nemerson (15) and was found to contain none.

Heat-denatured collagen was prepared by a modification of the method described by Wilner, Nossel, and LeRoy (13). 100 mg collagen was suspended in 100 ml glycine-HCl buffer, pH 2.5, and placed in a boiling water bath for 1 hr rather than at 35°C as utilized by Wilner. The solution was then dialyzed at 4°C for 24 hr against barbital-saline buffer followed by 48 hr of dialysis against distilled water. The dialyzed precipitate was lyophilized and stored at -20°C.

Acidified methanol-treated collagen, nitrous acid-treated collagen, DNFB-treated collagen, and pepsin-treated collagen were prepared as described by Wilner (13) using insoluble calf skin collagen powder.

Collagenase and elastase-treated collagen. 20 mg collagen was suspended in 3.0 ml tris-saline buffer containing 0.0025 M CaCl₂. 1 mg collagenase (240 U) or 0.5 mg elastase (75 U/mg, lot 110C-3200) were added and the mixtures incubated 48 hr at 37°C (13).

The clot-promoting activity of the various collagen preparations was assayed as has been described (13). 0.1 ml of the collagen suspension was preincubated with 0.1 ml platelet-poor plasma for 10 min at 37°C in silicone-coated 12 × 75-mm glass tubes with mixing every 30 sec. After incubation, 0.1 ml cephalin (Platelin, Warner-Chilcott Laboratories, Morris Plains, N. J.) and 0.1 ml 0.025 M calcium chloride were added. Clot formation was followed by frequent tilting of the tube.

The evolution of kinin-like activity was measured by the ability of test mixtures to induce contractions of an isolated segment of rat uterine horn suspended in a 10 ml bath containing de Jalon's solution (16) modified as described by Pettinger and Young (17). The buffer was maintained at 29-31°C and bubbled with 5% CO₂, 95% O₂. The uterine horns were obtained from virgin albino rats of the Sprague-Dawley strain weighing 200-250 g determined to be in estrus by the method of vaginal smears. Uterine contractions were measured isotonicly with a linear motion transducer (model ST-2, Phipps & Bird, Inc., Richmond, Va.), the transformer of which was supplied by an exciter-demodulator (Phipps & Bird, Inc.) and recorded on a Bausch & Lomb VOM-5 recorder (Bausch & Lomb Inc., Rochester, N. Y.). The sensitivity of the uterine muscles varied considerably, therefore each experiment was performed on a single muscle, and a reference bradykinin standard (2.5-15 ng) was utilized for each muscle. Frequent bradykinin standards were added to the muscle bath to check for alterations in the sensitivity of the preparation. Studies with the various collagen preparations were carried out as outlined in Fig. 1 by incubating the collagen with platelet-poor, non-contact plasma for varying time periods at 37°C with frequent mixing. The collagen was separated from the plasma by centrifugation and was resuspended in tris-saline buffer, recentrifuged, and dispersed again in buffer. The plasma-treated collagen preparation was then incubated with heated plasma, a source of prekinin, at 37°C for 15 min with frequent mixing. SBTI was added after incubation and the collagen removed by centrifugation and discarded. The supernate (labeled B in Fig. 1) was assayed for its rat uterine-contracting activity. Varying concentrations of the supernate from the collagen, platelet-poor plasma incubation (labeled supernate A in Fig. 1), was incubated with buffer, or with heated plasma substrate for 15 min at 37°C after which SBTI was added to prevent subsequent activation of

the normal plasma by dilution and glass contact when added to the muscle bath. Portions of these incubation mixtures were added to the rat uterine muscle bath to test for kinin-like activity. Kinin-like activity was defined as that activity generated in the heated plasma substrate which stimulated contractions of a rat uterine muscle obtained at estrus, and which was destroyed by carboxypeptidase B.

The effect of carboxypeptidase B on the rat uterine-contracting activity of the supernate of the plasma-treated collagen, heated plasma substrate incubation mixture was studied. 1.0 ml of the active supernate was incubated 15 min at 37°C with 0.1 ml tris-saline buffer, or with 50 µg carboxypeptidase B (batch COB-6078, 100 U/mg, Worthington Biochemical Corp.) in 0.1 ml tris buffer. The enzyme was also added to the plasma supernate immediately before assay in the uterine muscle bath to control for the effect of carboxypeptidase B on the muscle.

Heated plasma, the source of prekinin substrate, was prepared by heating outdated plasma to 62°C for 2 hr, followed by centrifugation to remove the denatured protein, and dialysis for 48 hr against 0.15 M sodium chloride at 4°C. This reagent, in the dilutions used, was free of kinin-forming enzymes, kinin-like activity, CI inactivator, and served as a source of prekinin (18-22). Kinin-generating activity was defined as that property of a test substance which, when incubated with the heated plasma substrate, generated rat uterine muscle-contracting activity.

The effect of collagen on ellagic acid-induced plasma kinin-generating activity was examined. 1.5 ml 2×10^{-4} M ellagic acid, a soluble activator of Hageman factor and of the kallikrein system (23-25) or 1.5 ml of tris-saline buffer were added to 0.5 ml platelet-poor plasma and 1.5 ml tris-saline buffer in polypropylene tubes. After 15 min preincubation at 37°C, 10 mg collagen was added and incubated with mixing for 15 sec. After centrifugation, the collagen was washed in 8.0 ml buffer, recentrifuged, and suspended in 2.0 ml buffer. The collagen suspensions were incubated 30 min at 37°C with 1.0 ml heated plasma after which 0.2 ml SBTI was added. The mixtures were assayed for their ability to stimulate rat uterine muscle contractions.

The effect of collagen in adsorbing ellagic acid activatable kinin-generating activity was studied. 5-20 mg collagen were preincubated with 0.9 ml tris-saline buffer and 0.1 ml plasma in polypropylene tubes for 15 sec at 37°C. After centrifugation, 0.5 ml of the supernate was added to 0.2 ml 0.01 M *o*-phenanthroline and 0.3 ml 2×10^{-4} M ellagic acid, and incubated 5 min at 37°C. The chelating agent, *o*-phenanthroline, was added to inhibit the destruction of kinins by plasma kininases (26). After incubation, 0.4 ml SBTI (4 mg/ml) was added to stop the generation of kinins and portions were tested for uterine-contracting activity.

Human plasma kallikrein, CI inactivator, and α_2 -macroglobulin were prepared by previously described methods (27). The partially purified kallikrein preparation contained 4.1 mg protein/ml, and hydrolyzed 162 µmoles tosyl arginine methyl ester (TAMe)/mg per hr under the conditions previously detailed (27). CI inactivator preparations contained 50 inhibitor U/ml, 1 U of CI inactivator being defined as that amount which neutralized the esterolytic activity of 10 U of CI. The α_2 -macroglobulin preparation contained 5 mg of α_2 -macroglobulin/ml and possessed insignificant TAMe esterase activity.

An eluate of plasma-treated collagen was prepared by incubating 100 mg insoluble calf skin collagen in 15 ml tris-saline buffer with 5 ml noncontact human plasma for 15 min at 37°C with frequent mixing. The collagen was har-

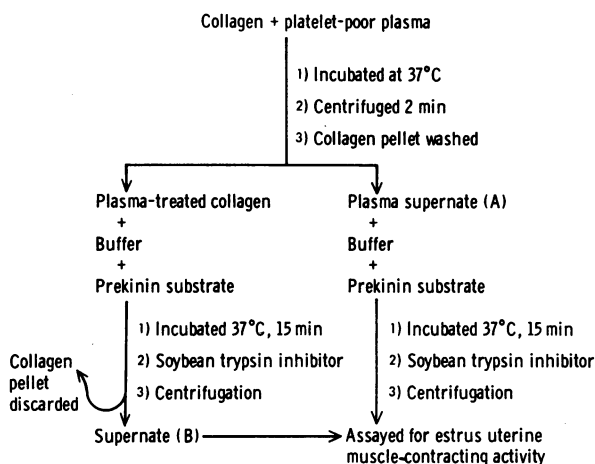


FIGURE 1 Schematic outline of the experimental procedure for the study of the surface-oriented interaction between collagen and a plasma kallikrein-like enzyme system.

vested by centrifugation, and washed twice in buffer. The collagen was then incubated with 4.0 ml 1.7 M NaCl, 0.05 M Tris, pH 8.0, 1 hr at 37°C with mixing. The eluate was dialyzed 2 hr against distilled water followed by 48 hr of dialysis against large volumes of tris-saline buffer. Portions were frozen at -20°C. The protein concentration was 340 µg/ml with a total protein of 1.7 mg. This eluate was tested for kinin-generating activity by incubating equal volumes of the eluate with heated plasma substrate in polypropylene tubes for 30 min at 37°C. After incubation, SBTI was added. This incubation mixture was active in stimulating rat uterine-muscle contractions, whereas the eluate incubated with buffer was totally inactive, indicating that the eluate contained kinin-generating activity.

A fibrinolytic assay for plasmin in the plasma-treated collagen eluate was performed by a modification of a previously described method (28). Plasminogen-free bovine fibrinogen was obtained from Pentex Biochemical, Kankakee, Ill. The fibrinogen was dissolved in 0.05 M tris-HCl buffer, pH 8.0, containing 0.3 M NaCl at a concentration of 5 mg/ml and stored at -20°C. 0.4 ml of the fibrinogen solution was added to 12 × 75-mm glass test tubes in an ice bath. 0.05 M tris-HCl buffer, pH 8.0, containing 0.02 M NaCl and serial dilutions of human plasmin (The American National Red Cross) or of collagen eluate were added to a final volume of 0.9 ml. 0.1 ml plasminogen-free bovine thrombin (Leo Pharmaceutical Products, Denmark) containing 40 NIH U/ml was rapidly added, and the contents of the tubes mixed and incubated at 37°C. Lysis of the fibrin clot was observed within 24 hr in tubes containing 0.1 caseinolytic U or more of plasmin, whereas lower concentrations had no effect. 0.5 ml of the collagen eluate failed to produce clot lysis. A fibrin clot control containing 50 Ploug U of human urokinase (Leo Pharmaceutical Products) failed to lyse, thereby ruling out significant concentrations of plasminogen in the system.

RESULTS

The binding of kinin-generating activity by collagen after incubation with human plasma. Collagen, after preincubation with platelet-poor human plasma and

TABLE I

Kinin-Like Activity Generated by Collagen after Preincubation with Plasma

Incubation mixtures*	Volume added to muscle bath	Uterine contraction†
	ml	mm
(A) Plasma-treated collagen incubated with heated plasma.	0.2	116
	0.1	40
(B) Supernate from plasma, collagen preincubation mixture incubated with heated plasma.	0.6	0
(C) Plasma-treated collagen incubated with buffer.	0.6	0
(D) Collagen incubated with heated plasma.	0.6	0
(E) Plasma-treated collagen incubated with heated plasma, then 50 µg carboxypeptidase B added.	1.0	0
(F) Hageman-deficient plasma-treated collagen incubated with heated plasma.	0.6	0
(G) Bradykinin, 5 ng		100

* (A) Plasma-treated collagen was prepared by incubating 10 mg collagen with 0.5 ml normal platelet-poor plasma in polypropylene tubes at 37°C for 15 sec. After recovery by centrifugation at 2500 rpm for 2 min at 4°C, the collagen pellet was resuspended in 8.0 ml tris-saline buffer, centrifuged, and suspended in 2.0 ml buffer. This washed plasma-treated collagen suspension was incubated in a new polypropylene tube with 1.0 ml heated plasma substrate with frequent mixing at 37°C for 15 min. After incubation, 0.5 ml SBTI (4 mg/ml) was added, the collagen removed by centrifugation, and the supernate tested for rat uterine muscle-contracting activity. (B) 0.2 ml of the plasma supernate recovered after incubation of collagen with plasma (A), was incubated 15 min at 37°C with 1.4 ml buffer and 1.0 ml heated plasma substrate. 0.5 ml soybean inhibitor was added and the mixture tested in the muscle bath. (C) 2.0 ml of washed plasma-treated collagen suspension was prepared as in A, and was incubated with 1.0 ml buffer 15 min at 37°C. This mixture was assayed after the addition of soybean inhibitor and centrifugation. (D) 10 mg collagen which had not been exposed to plasma was incubated with 2.0 ml buffer and 1.0 ml heated plasma substrate as described in A. (E) After incubation of a washed plasma-treated collagen suspension with heated plasma substrate as described in A, 0.1 ml carboxypeptidase B containing 50 µg of the enzyme was added to the supernate and incubated for an additional 15 min before assay. (F) Hageman-deficient plasma-treated collagen was prepared by the procedure outlined in A, with the substitution of Hageman factor-deficient plasma for normal plasma.

† Uterine contractions were measured in millimeters.

washing in buffer, acquired the ability to induce rat uterine-muscle contractions after incubation with a source of prekinin (Table I). Varying concentrations of the supernate from the collagen, platelet-poor plasma preincubation mixture failed to generate kinin-like activity when incubated with buffer or with heated plasma. Heated plasma substrate was necessary for the evolution of kinin-like activity, as plasma-treated collagen incubated with buffer failed to stimulate smooth muscle contractions. Collagen not previously exposed to plasma failed to generate this kinin-like activity. This activity was inactivated by the addition of carboxypeptidase B to the heated plasma after kinin-like activity had been generated. Collagen preincubated

with Hageman factor-deficient plasma failed to generate uterine muscle-contracting activity when incubated with heated plasma.

The effect of collagenase and elastase on the ability of collagen to bind plasma kinin-generating activity. Treatment of collagen with collagenase completely abolished the ability of collagen to activate and bind kinin-generating activity when preincubated with plasma (Table II). Suitable controls eliminated the possibility that this inhibition was due to the direct effect of collagenase on the plasma in the preincubation mixture. Elastase had no inhibitory effect on the collagen-induced binding of plasma kinin-like inducing activity.

The effect of time of incubation of collagen and plasma on the kinin-generating activity of plasma-treated collagen. Maximum kinin-generating activity was obtained after 15 sec of incubation of collagen with plasma followed by 2 min of centrifugation at 4°C (Table III). The kinin-generating activity of the collagen exposed to plasma for 5 min was less than that found at 10 min in three separate experiments. This activity, which fell progressively with time after the 10 min sample, was still demonstrable after 30 min of incubation. The plasma supernates were entirely inactive in generating uterine-contracting activity. Experiments utilizing collagen suspended in tris-saline

TABLE II

Effect of Collagenase and Elastase on the Ability of Collagen to Bind Plasma Kinin-Generating Activity

Incubation mixtures*	Uterine contraction‡
	mm
(A) Collagen preincubated 48 hr with buffer, then incubated with plasma.	69
(B) Collagen preincubated 48 hr with collagenase, then incubated with plasma.	0
(C) Collagenase preincubated 48 hr with buffer, then collagen added, and incubated with plasma.	>125
(D) Collagen preincubated 48 hr with elastase, then incubated with plasma.	>125
(E) Elastase preincubated 48 hr with buffer, then collagen added and incubated with plasma.	>125
(F) Bradykinin, 10 ng	75

* 20 mg collagen was preincubated 48 hr at 37°C with 3.0 ml tris-saline buffer containing 0.0025 M CaCl₂, or with this buffer containing 1.0 mg collagenase (240 U/mg) or 0.5 mg elastase (75 U/mg). Control tubes contained the enzymes and buffer without collagen, which was added immediately before the addition of 1.0 ml platelet-poor plasma. The contents of the preincubation mixture were incubated at 37°C for 15 min with mixing every 30 sec with plasma. The collagen was harvested by centrifugation, washed in 8.0 ml buffer and resuspended in 2.0 ml tris-saline buffer. 0.8 ml of the collagen suspensions were incubated with 0.4 ml buffer plus 0.4 ml heated plasma substrate 15 min at 37°C, after which 0.4 ml SBTI (4 mg/ml) was added. 0.5-ml portions were added to the test muscle bath.

‡ Uterine contractions greater than 125 mm represent the maximum excursion of the recorder.

buffer and resulting in a final plasma dilution of 1:4 demonstrated less inhibition with time than that found with undiluted plasma. With diluted plasma approximately 75% of the kinin-generating activity remained at 15 min incubation, as compared with 41% in the undiluted sample.

Reduction of ellagic acid-induced kinin-generating activity in plasma by the addition of varying concentrations of collagen. Collagen, added to plasma preincubated with ellagic acid, failed to bind kinin-like generating activity. Collagen, preincubated with plasma, caused a concentration-dependent reduction in the kinin-like activity which was subsequently generated by the addition of ellagic acid to the treated plasma (Table IV). The kinin-like generating activity bound to the

TABLE III
Effect of Time of Incubation of Collagen and Plasma on the Kinin-Generating Activity of Plasma-Treated Collagen

Time of incubation*	Volume added to muscle bath	Uterine contraction	Activity remaining§
	<i>ml</i> ‡	<i>mm</i>	%
15 sec	0.2	113	100
	0.15	49	
	0.1	33	
5 min	0.2	40	63
10 min	0.2	53	74
15 min	0.2	25	41
30 min	0.5	57	25
	0.2	0	
Bradykinin, 5 ng		100	

* 10 mg collagen was incubated with 0.5 ml normal platelet-poor plasma prewarmed to 37°C in polypropylene tubes for the time periods indicated. The collagen was centrifuged for 2 min at 4°C, washed, and incubated with heated plasma substrate as indicated in Table I. The plasma supernates from each incubation mixture were also incubated with the heated plasma substrate as outlined in Table I, and possessed no uterine-contracting activity.

‡ Varying volumes of the incubation mixtures of the plasma-treated collagen and heated plasma substrate were added to the muscle bath in order to quantitate the kinin-like activity generated.

§ The per cent activity remaining was calculated from the results of three separate experiments. The collagen-associated activity found at 15 sec of incubation of collagen and plasma was arbitrarily assigned a value of 100%. The activity of the incubation mixtures was compared with the activity of the 15 sec mixture. This was accomplished by added a bracketing volume of the 15 sec incubation mixture to the muscle bath which would produce contractions similar in amplitude to that of the test incubation mixture. Bradykinin standards were also added to the bath at frequent intervals to check for variations in muscle sensitivity. The per cent activity remaining was calculated by comparing the volumes of the 100% and test sample used to produce similar uterine contractions.

TABLE IV
The Adsorption of Ellagic Acid-Induced Kinin-Generating Activity from Plasma by Varying Concentrations of Collagen

Incubation mixtures*	Volume added to muscle bath	Uterine contraction
	<i>ml</i>	<i>mm</i>
(A) Plasma activated with ellagic acid.	0.65	65
	0.50	23
(B) Plasma, preincubated with 5 mg collagen, then activated with ellagic acid.	0.65	48
(C) Plasma, preincubated with 10 mg collagen, then activated with ellagic acid	0.65	18
(D) Plasma, preincubated with 20 mg collagen, then activated with ellagic acid.	0.65	10
(E) 5 mg collagen obtained from preincubation mixture B, incubated with heated plasma.	0.4	0
	0.9	51
(F) 10 mg collagen obtained from preincubation mixture C, incubated with heated plasma.	0.4	11
	0.6	46
(G) 20 mg collagen obtained from preincubation mixture D, incubated with heated plasma.	0.3	49
	0.4	55
(H) Bradykinin, 5 ng		61

* (A) 0.9 ml tris-saline buffer was preincubated in duplicate with 0.1 ml platelet-poor plasma for 15 sec in polypropylene tubes. 0.5 ml of the mixture was added to a tube containing 0.2 ml 0.01 M *o*-phenanthroline and 0.3 ml 2×10^{-4} M ellagic acid and incubated 5 min at 37°C. 0.4 ml SBTI (4 mg/ml) was then added to stop the reaction. Portions were tested for uterine-contracting activity. (B) 5 mg collagen was preincubated with mixing, with 0.9 ml tris-saline buffer and 0.1 ml plasma for 15 sec, 37°C in plastic tubes. After centrifugation for 1 min, 0.5 ml supernate was added to a tube containing 0.2 ml *o*-phenanthroline and 0.3 ml ellagic acid and the procedure as described in A was followed. (C) 10 mg collagen was preincubated with plasma and the supernate processed as described in B. (D) 20 mg collagen was preincubated with plasma as described in C. E - G. The collagen pellets from A - D were washed individually in 8.0 ml tris-saline buffer, centrifuged, and resuspended in 2.0 ml buffer. 1.0 ml heated plasma was added to each suspension and the mixtures incubated with mixing at 37°C for 30 min. 0.4 ml SBTI was then added, the collagen removed by centrifugation, and the supernate assayed in a 1/10 dilution for uterine-contracting activity.

collagen was found to be approximately proportional to the amount of collagen initially added to the plasma.

The effect of various naturally occurring inhibitors on the kinin-generating activity of collagen previously exposed to plasma. The nature of the kinin-like inducing activity which was bound to collagen after preincubation with platelet-poor plasma was examined by utilizing several naturally occurring proteolytic enzyme inhibitors (Table V). Plasma C₁ inactivator, α_2 -macroglobulin, SBTI, and Trasylol inhibited the ability of plasma-treated collagen to induce kinin-like activity when incubated with heated plasma. LBTI or the addition of the inhibitors after incubation failed to inhibit uterine muscle contractions.

Further properties of the kinin-generating enzyme bound to collagen were examined by the preparation of an eluate of plasma-treated collagen. This eluate contained kinin-generating activity when incubated with

TABLE V
Effect of Soybean Trypsin Inhibitor, Lima Bean Trypsin Inhibitor, Trasylol, C \bar{I} Inactivator, and α_2 -Macroglobulin on the Kallikrein-Like Activity of Plasma-Treated Collagen

Incubation mixtures*	Volume added to muscle bath	Uterine contraction
	ml	mm
(A) Plasma-treated collagen + buffer	0.05 0.02	>125 86
(B) Plasma-treated collagen + 2 mg SBTI	0.1	0
(C) Plasma-treated collagen + 2 mg LBTI	0.02	121
(D) Plasma-treated collagen + 5000 U Trasylol	0.1	0
(E) Plasma-treated collagen + 2.5 mg α_2 -macroglobulin	0.1	0
(F) Plasma-treated collagen + 25 U C \bar{I} inactivator	0.1	0
(G) Bradykinin, 5 ng		118

* 10-mg portions of collagen were preincubated 10 sec with 0.5 ml platelet-poor normal plasma at 37°C in polypropylene tubes with mixing. After centrifugation, the collagen was resuspended in 8.0 ml tris-saline buffer, recentrifuged, and then dispersed in 1.5 ml buffer. This suspension of plasma-treated collagen was incubated 5 min at 37°C with 0.5 ml of the inhibitor preparations as indicated. 1.0 ml heated plasma substrate was added and the incubation was continued with mixing for an additional 15 min. The collagen was then removed by centrifugation and the supernate tested for rat uterine-contracting activity.

a heated plasma substrate. The ability of the various inhibitors to inhibit this activity was compared with concentrations of partially purified human plasma kallikrein and plasmin which produced similar rat uterine-contracting activity (Table VI). Both the collagen eluate and kallikrein were inhibited by soybean inhibitor, C \bar{I} inactivator and α_2 -macroglobulin but not by lima bean inhibitor. This inhibitor profile was identical with that found for the collagen-bound kinin-generating activity. In contrast, the kinin-inducing activity of plasmin was inhibited by lima bean inhibitor, but not by C \bar{I} inactivator. That plasmin was not responsible for the kinin-generating activity of the collagen eluate was further substantiated by a fibrinolytic assay. The collagen eluate failed to lyse a plasminogen-free bovine fibrin clot sensitive to plasmin concentrations severalfold lower than those required to generate the kinin activity identified in the eluate. These results suggest that the plasma enzyme bound by collagen after exposure to plasma is similar to partially purified plasma kallikrein.

The effect of chemical treatment of collagen on the clot-promoting activity and the binding of kallikrein-like activity from plasma. Confirming the findings of Wilner, et al. (13), collagen accelerated the recalcification time of plasma (Table VII). Treatment of collagen with acid-methanol, a procedure which causes

esterification of the free carboxyl groups, and heat denaturation abolished this clot-promoting effect. As described by Wilner, removal of the telopeptides from the collagen molecule by treatment with pepsin, reduced the clot-promoting properties of collagen. Blockage of the amino groups of the collagen molecule by deamination (nitrous acid-treated collagen) or blockage of the ϵ -amino groups with DNFB failed to inhibit the coagulant activity of collagen, findings which have been previously described (13).

The ability of these collagen preparations to bind plasma kallikrein-like activity was compared (Table VIII). Esterification of the free carboxyl groups of collagen completely abolished this collagen-binding activity. Heat denaturation of collagen almost completely inhibited this activity of collagen. Pepsin-treatment and

TABLE VI
Effect of Various Inhibitors on the Kinin-Generating Activity of a Collagen Eluate, Plasma Kallikrein, and Plasmin

Substance added to incubation mixture*	Uterine contraction		
	Collagen eluate†	Plasma kallikrein‡	Plasmin
		mm	
Buffer	>125	>125	>125
Soybean inhibitor	0	0	0
Lima bean inhibitor	>125	>125	0
α_2 -macroglobulin	0	0	0
C \bar{I} inactivator	0	0	>125

* Incubation mixtures contained 0.3 ml of enzyme solution, 0.3 ml of tris-saline buffer, or 0.3 ml of inhibitor in the following concentrations: SBTI and LBTI, 4 mg/ml; α_2 -macroglobulin, 1.6 mg/ml; C \bar{I} inactivator, 16 U/ml; and 0.3 ml heated plasma substrate. The collagen eluate mixture was incubated for 30 min, whereas the plasma kallikrein and plasmin mixtures were incubated for 10 min at 37°C. 0.3 ml of the various incubation mixtures were added to the muscle bath. Addition of the various inhibitors to incubation mixtures of the enzymes and substrate after incubation failed to inhibit rat uterine contractions. 5 ng bradykinin added to the muscle bath stimulated a contraction of 121 mm.

† 100 mg collagen in 15 ml tris-saline buffer were incubated 15 min at 37°C with 5.0 ml normal plasma in a polypropylene container. The collagen was harvested by centrifugation, washed with buffer, and eluted with 1.7 M NaCl, 0.05 M tris buffer, pH 8.0. The eluate was dialyzed against tris-saline buffer.

‡ Partially purified human plasma kallikrein was prepared as previously described (27) and was diluted 1/4000 in tris-saline buffer.

|| Glycerol activated human plasmin was obtained from the American National Red Cross and contained 32.3 M.D.H. caseinolytic U/ml and 6.2 mg protein/ml. It was utilized in a concentration of 1.0 U/ml.

blockage of the amino groups of the collagen molecule did not inhibit the ability of collagen to bind the kinin-like inducing activity from plasma. These preparations were somewhat more effective than the untreated collagen in inducing uterine muscle-contracting activity after incubation with heated plasma.

The effect of chemical treatment of collagen on the binding of purified plasma kallikrein. Purified plasma kallikrein, when incubated with collagen, became bound to the particles and retained kinin-generating activity (Table IX). Paralleling the results of Table VIII, chemical treatment of the collagen molecule modified the binding of the active enzyme. Esterification of the carboxyl groups completely abolished binding, and heat denaturation reduced the binding by 67%. Treatment of collagen with pepsin or alteration of the free amino groups did not inhibit the collagen, kallikrein interaction.

DISCUSSION

The present study has shown that collagen interacts with a human plasma kallikrein-like enzyme system to gain surface-active kinin-generating activity. Collagen, after incubation with plasma and subsequent washing, acquired the ability to stimulate rat uterine contractions when incubated with heated plasma, a source of prekinin (Table I). This collagen-associated activity had the characteristics of a kinin-generating

TABLE VII
Effect of Various Collagen Preparations on the Recalcification Time of Plasma

Test substance*	Clotting time†	Residual activity‡
		%
Buffer	224.0	—
Collagen	140.0	100
Methanol collagen	226.6	0
Heated collagen	221.2	0
Pepsin collagen	153.1	76
Nitrous acid collagen	124.0	>100
DNFB collagen	126.3	>100

* 0.1 ml of the test suspension containing 100 µg of the various collagen preparations was preincubated 10 min at 37°C in siliconized glass tubes with 0.1 ml platelet-poor plasma, after which 0.1 ml platelin and 0.1 ml 0.025 M CaCl₂ were quickly added. Clot formation was followed by frequent tilting of the tube.

† Average of two determinations in seconds.

‡ 100, 50, 25, 10 µg of the control collagen preparation were tested and the clotting times plotted against collagen content on double logarithmic paper. The activity of the test collagen preparations was calculated as a percentage of that of the control collagen.

TABLE VIII
Kallikrein-Like Activity Bound to Various Collagen Preparations after Preincubation with Plasma

Incubation mixtures*	Volume added to uterine contraction‡	
	ml	mm
(A) Collagen preincubated with plasma, washed, and incubated with heated plasma.	0.2 0.1	56 18
(B) Methanol collagen, treated as in A.	0.6	0
(C) Heat denatured collagen, treated as in A.	0.6 0.2	17 0
(D) Pepsin collagen, treated as in A.	0.2 0.1	>125 46
(E) Nitrous acid collagen, treated as in A.	0.2 0.1	>125 30
(F) DNFB collagen, treated as in A.	0.2 0.1	>125 60
(G) Bradykinin, 10 µg		78

* 20 mg of collagen, or of collagen treated in various ways as detailed in the methods, were suspended in 3.0 ml tris-saline buffer and preincubated 15 min at 37°C with 1.0 ml platelet-poor plasma. After centrifugation, the collagen was washed in 8.0 ml buffer, recentrifuged, and suspended in 4.0 ml buffer. 0.8 ml of the collagen suspension was incubated 15 min at 37°C with 0.4 ml buffer and 0.4 ml heated plasma substrate with mixing, after which 0.4 ml SBTI (4 mg/ml) was added. Portions of these incubation mixtures were added to the uterine muscle bath to test for kinin-like activity.

‡ Uterine contractions greater than 125 mm represent the maximum excursion of the recorder.

enzyme as the plasma-treated collagen possessed no contracting activity when added directly to the uterine muscle bath. The smooth muscle-contracting activity generated in heated plasma was destroyed by carboxypeptidase B providing evidence for the kinin-like nature of the active agent (29). The possibility that the activity demonstrated in the plasma-treated collagen was due to entrapment of plasma was eliminated by the finding that the plasma supernate harvested after incubation with collagen failed to stimulate uterine contractions directly or after incubation with heated plasma. The Hageman factor appeared to be necessary for the surface-bound kinin-generating activity, as this activity was absent in collagen exposed to the plasma from a patient with Hageman factor deficiency. These findings are consistent with prior investigations which have shown that Hageman factor was necessary for kallikrein system activation (4-6). Collagenase destroyed the ability of collagen to react with plasma (Table II), suggesting that this effect was due to collagen itself and not to a contaminant in the preparation.

The development of collagen-bound kinin-generating activity in the collagen plasma mixture occurred at a relatively rapid rate. The maximum activity was recovered at the shortest incubation time of 15 sec (Table III). The activity became progressively less potent with time at 15 and 30 min of incubation, how-

TABLE IX
The Binding of Purified Plasma Kallikrein by Chemically Modified Collagen Preparations

Test substance in the incubation mixture*	Volume added to muscle bath	Uterine contraction	Bradykinin equivalents†
	ml	mm	ng/ml
Collagen	0.2	33	33.3
Methanol collagen	1.0	0	0
Heated collagen	1.0	63	11.0
Pepsin collagen	0.2	32	32.1
Nitrous acid collagen	0.2	28	27.9
DNFB collagen	0.2	56	45.0

* 10 mg of collagen, or of collagen treated chemically as detailed in the Methods, were preincubated with 1.0 ml of partially purified plasma kallikrein containing 40 μ g protein and 6.6 TAME esterase U. After 1 min incubation at 37°C in polypropylene tubes, 8.0 ml tris-saline buffer were added and the supernate removed after centrifugation. The collagen pellet was resuspended in 8.0 ml buffer, transferred to a new tube and recentrifuged. The collagen was resuspended in 2.0 ml tris-saline buffer and incubated with 0.4 ml heated plasma substrate for 30 min at 37°C. The reaction was stopped by the addition of 0.2 SBTI (4 mg/ml) and the supernate was tested for uterine muscle-contracting activity.

† To facilitate comparison of activity of the various collagen preparations in this experiment, contraction of the rat uterus was expressed as nanograms bradykinin/milliliter by reference to a dose-response curve prepared by adding various concentrations of purified bradykinin to the muscle bath. A plot on double logarithmic paper of millimeter contractions vs. nanograms bradykinin added produced a straight line. Frequent bradykinin standards were introduced during each experiment to prevent errors due to changes in sensitivity of the uterine muscle.

ever approximately 25% of the initial activity remained at 30 min. Increasing concentrations of collagen added to plasma resulted in a concentration-dependent decrease in the kinin-like activity which could be generated by the subsequent addition of ellagic acid, a previously described activator of plasma kallikrein-like activity (24) (Table IV). Collagen added to ellagic acid-treated plasma failed to adsorb kinin-generating activity, providing additional evidence that collagen and ellagic acid activate similar enzyme systems.

Both human plasmin and plasma kallikreins have been shown to release kinin-like activity from heated human plasma (30). Evidence that the activity associated with collagen after incubation with plasma was a kallikrein-like proteolytic enzyme was provided by a study of the effect on this activity of several naturally occurring proteolytic enzyme inhibitors (Table V). C $\bar{1}$ inactivator, purified from human serum, has been shown to inhibit kallikrein, plasma permeability factor (PF/dil),

C $\bar{1}$, Hageman factor, plasma thromboplastin antecedent, and plasmin (31-34). Plasma α_2 -macroglobulin inhibits kallikrein (27, 34), plasmin (35, 36), and thrombin (37) by binding with these enzymes and forming a high molecular weight complex. Trasylol is a kallikrein, plasmin and trypsin inhibitor purified from bovine tissues (38). SBTI has been found to inhibit both plasmin and kallikrein, whereas LBTI inhibited plasmin but not plasma kallikrein (39, 40). C $\bar{1}$ inactivator, α_2 -macroglobulin, Trasylol, and SBTI, but not the lima bean inhibitor, blocked the generation of kinin-like activity when preincubated with plasma-treated collagen before the addition of heated plasma. These inhibitors had no effect when added following the heated plasma substrate. The effect of these inhibitors in preventing the generation of kinin-like activity by an eluate prepared from plasma-treated collagen, and by partially purified preparations of plasma kallikrein and plasmin was compared (Table VI). The collagen eluate and plasma kallikrein kinin-generating activities were inactivated by the same inhibitors. In contrast, plasmin was inhibited by LBTI, and was not affected by the concentration of C $\bar{1}$ inactivator utilized. The collagen eluate demonstrated no plasmin activity in a fibrin clot assay. Thus, the collagen-bound kinin-generating enzyme possessed the characteristics of a plasma kallikrein.

Investigations were undertaken to examine some of the physical and chemical properties of the collagen molecule necessary for its interaction with the plasma kallikrein-like enzyme system. Coagulation studies similar to those reported by Wilner, Nossel, and LeRoy (13) confirmed that esterification of collagen by treatment with acid methanol, a procedure which neutralizes 80-90% of the free carboxyl groups (41, 42), abolished clot-accelerating activity (Table VII). As previously described (13), pepsin-treatment of collagen which results in the removal of the collagen telopeptides (43), caused a slight reduction in coagulant activity, and heat-denaturation, a process which changes the structure of collagen from a triple helical to a random coil form (44-46), abolished this clot-promoting effect. Treatment of the collagen with nitrous acid, a procedure which replaces the free amino groups with hydroxyl groups and 20% of the guanidyl groups with cyanamide groups (13), or blockage of the ϵ -amino groups by DNFB treatment (42, 47), appeared to enhance the coagulant activity of collagen.

The effect of these chemically treated collagen preparations on the development of collagen-bound kallikrein-like activity paralleled their clot-accelerating properties (Table VIII). Esterification of the collagen molecule abolished, and heat-denaturation greatly diminished the kinin-generating activity bound to collagen after exposure to plasma. Pepsin, nitrous acid, and DNFB-

treatment enhanced this collagen-induced activity. The binding of partially purified human plasma kallikrein to collagen was found to have similar structural and chemical requirements (Table IX). Both the triple helical structure and the free carboxyl groups of glutamine and aspartic acid of the collagen molecule have previously been shown to be necessary for Hageman factor activation by collagen (13). The finding that these chemical requirements are also necessary for the development of the kallikrein-like activity which is bound to collagen after exposure to plasma, provides additional evidence that Hageman factor activation may represent an intermediate step in this reaction. In contrast, the binding of fully activated, purified plasma kallikrein to collagen apparently proceeds in the absence of Hageman factor, as the methods used to purify kallikrein would remove this clotting factor (34).

These studies demonstrate that a plasma kallikrein-like activity develops on the surface of the collagen molecule after incubation with plasma and that both the generation of this activity and the binding of the active enzyme to the collagen surface has specific structural requirements. Thus, the plasma kallikrein system, analogous to the human complement system (48), may be proteolytically active not only in the fluid phase, as has been described by others (1-10), but as shown in this investigation, on an appropriate and relatively specific surface. The subendothelial location of collagen (49) suggests that disruption of the vascular endothelium may result in local activation and concentration of collagen-bound kallikrein-like activity. The interaction of collagen and a plasma kallikrein may serve as a model for the examination of other supporting tissues such as basement membrane and elastin for their ability to activate and bind surface-active, kinin-inducing activity.

In addition to activating Hageman factor and thereby initiating intrinsic blood coagulation, collagen causes platelet adherence and aggregation (50-52). Collagen-induced activation of both the coagulation and kallikrein systems raises the possibility that the kallikrein-kinin vasoactive system may function in both hemostatic and thrombotic processes. The activation of these enzyme systems by collagen establishes yet another interrelationship between the coagulation and kallikrein pathways which may have pathophysiologic relevance to various inflammatory human disease states.

ACKNOWLEDGMENTS

These studies were performed with the invaluable technical assistance of Mr. Tsun-San Chang. I wish to thank Dr. Ralph Nachman for his valuable suggestions and for his review of the manuscript.

This study was supported by U. S. Public Health Service grants NB-03346, HL-13993, and in part by contract No. PH-43-67-1439 with the National Institutes of Health.

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