A PREALBUMIN ACTIVATOR OF PREKALLIKREIN

II. Derivation of Activators of Prekallikrein from Active Hageman Factor by Digestion with Plasmin*

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Bradykinin is generated in human plasma by the action of plasma kallikrein upon kininogen (1, 2). Although Hageman factor is required for the formation of kallikrein (3, 4), the role of Hageman factor in the formation of the prekallikrein activator is not clearly delineated. Kaplan and Austen (5) have described a prekallikrein activator present in human serum which has a prealbumin mobility on disc gel electrophoresis, an estimated molecular weight of 35,000 by Sephadex G-100 filtration (Pharmacia Fine Chemicals Inc., Uppsala, Sweden), and the capacity to correct selectively the coagulation defect of Hageman factor-deficient plasma. Prolonged dialysis of a partially purified preparation of active Hageman factor resulted in the development of prealbumin bands in the position previously noted for the prekallikrein activator derived from serum; the eluates of these bands had prekallikrein-activating activity and corrected Hageman factor deficiency. It was therefore postulated that active Hageman factor dissociates into fragments which retain clot-promoting activity while manifesting a striking ability to activate prekallikrein (5). Since plasminogen was recognized in preparations of highly purified Hageman factor (6), and since plasmin has been implicated in the activation of prekallikrein (7), it seemed possible that plasmin was responsible for the development of prekallikrein-activating activity from active Hageman factor. The cleavage of active Hageman factor by streptokinase-activated, highly purified plasminogen to form the prekallikrein activator is described herein.

Materials and Methods

Bradykinin triacetate (Sandoz Pharmaceuticals, Basel, Switzerland, or New England Nuclear Corp., Cambridge, Mass.) was used as the standard for native bradykinin. Antisera

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to IgG, IgA, IgM, β-lipoprotein, transferrin, albumin, and whole human serum were purchased from Behring Diagnostics, Inc., Woodbury, N.Y. Hexadimethrine (polybrene) was a gift of Dr. Floyd McIntire of Abbott Laboratories (North Chicago, Ill.). Hageman factor-deficient plasma was supplied by Dr. O. Ratnoff (Cleveland, Ohio). Plasma thromboplastin antecedent (PTA)[1]-deficient plasma was supplied by Dr. Anthony Britton (Boston, Mass). Purified plasminogen and streptokinase were gifts of Dr. E. C. DeRenzo of Lederle Laboratories (American Cyanamid Co., Pearl River, N. Y.).

Diethylaminoethyl (DEAE)-Cellulose Chromatography.—Sera and plasma were drawn for the isolation of active enzymes and proenzymes respectively and were processed as previously described (5). 35 ml of dialyzed serum or plasma was applied to a 3.5 × 30 cm column of packed DEAE-cellulose equilibrated with 0.01 M PO_4 buffer, pH 7.8. The column was washed with 500 ml of the equilibrating buffer and eluted with a linear salt gradient of 2 liters of the equilibrating buffer and 2 liters of 0.01 M PO_4 buffer, pH 7.8, containing 0.3 M NaCl. The column was run at 60 ml/hr and 12 ml fractions were collected. The eluate fractions were assayed for protein content (OD 280 mμ), conductivity, and various intermediates of the kinin-forming sequence. Peaks having bradykinin-generating capacity were concentrated by ultrafiltration using a UM-10 membrane (Amicon Corp., Lexington, Mass.) to 5–10 ml and were then further concentrated by wall vacuum using Collodion bags No. 100 (Schleicher and Schuell, Inc., Keene, N. H.).

Carboxymethyl (CM)-Cellulose Chromatography.—A 3.5 × 20 cm column of packed CM-cellulose was equilibrated with 0.01 M PO_4 buffer, pH 6.0. 20–30 ml samples, dialyzed in 0.01 M PO_4 buffer, pH 6.0, were applied to the column; the column was washed with 350 ml of equilibrating buffer, and a linear salt gradient using 1.5 liters of equilibrating buffer and 1.5 liters of 0.01 M PO_4 buffer, pH 6.0, containing 0.75 M NaCl was applied. The column was run at 60 ml/hr and 12 ml fractions were collected.

Disc Gel Electrophoresis.—Analytic disc gel electrophoresis was performed at a running pH of 9.3 using the equipment and directions supplied by Buchler Instruments, Inc. (Fort Lee, N.J.). Samples of either 100 μl or 150 μl were applied to the gels. After electrophoresis, the protein bands were stained with Coomassie blue stain (8) and inspected visually. In order to correlate the bands seen with their functional activity, a series of disc gels was run simultaneously. After staining one gel with Coomassie blue, this gel and the unstained gels were sliced into either 1 mm or 2 mm sections using the gel column cutter described by Heideman (9). The unstained gel sections were crushed and the proteins were eluted from each section by suspension in 0.5 ml of 0.15 M NaCl. After standing at room temperature for 2 hr and at 4°C overnight, the polyacrylamide was sedimented by centrifugation and the supernatant was separated.

Assay of Components of the Bradykinin-Generating System.—Bradykinin was routinely determined by bioassay utilizing the isolated guinea pig ileum (10) or by radioimmunoassay (11). Kallikrein, prekallikrein, and prekallikrein-activating components were determined as previously described (5). The shortening of the partial thromboplastin time of Hageman factor-deficient plasma was used as an assay for Hageman factor. 0.0050 ml of Hageman factor source was incubated for 2 min at 37°C with 0.05 ml cephalin reagent (12) and 0.05 ml Hageman factor-deficient plasma in siliconized glass tubes. 0.0050 ml of 0.05 M $CaCl_2$ was added and the clotting time determined at room temperature. The tubes were tilted each minute and the end point was defined as the time interval required for the clot to adhere to the glass tube. Utilization of the cephalin reagent with and without kaolin, 10 mg/ml, revealed whether or not Hageman factor was already active. Plasma thromboplastin antecedent activity was assayed in the same manner utilizing PTA-deficient plasma in place of Hageman factor-

[1] *Abbreviation used in this paper*: PTA, plasma thromboplastin antecedent.

deficient plasma. Plasmin was measured by digestion of azocasein as described by Hummel et al. (13). Plasminogen, the inactive precursor of plasmin, was determined by incubating 0.5 ml of plasminogen source with 250 units of streptokinase for 15 min at 30°C, followed by measurement of the plasmin generated.

RESULTS

Sequential Change in Charge and Size of Prekallikrein-Activating Activity Derived after Activation of Hageman Factor.—As demonstrated previously (5), five areas having bradykinin-generating activity were found when serum was chromatographed on DEAE-cellulose and the fractions assayed using fresh plasma as substrate. Peak 1 eluted with the starting buffer, released bradykinin from either fresh plasma, heat-inactivated plasma, or purified kininogen, and therefore contained kallikrein activity. Plasma thromboplastin antecedent assessed by correction of the coagulation defect of PTA-deficient plasma was also found in this fraction. The four peaks eluted after application of a linear gradient had bradykinin-generating activity when incubated with fresh plasma but not with heat-inactivated plasma or purified kininogen, and therefore contained prekallikrein-activating components. Activated Hageman factor was found overlapping peaks 2 and 3 with trailing of this activity through peaks 4 and 5. When either peak 2 or peak 3 was pooled, concentrated, dialyzed, and rechromatographed on DEAE-cellulose under identical conditions, the bradykinin-generating activity was found in the position of peak 5; when peak 5 was rechromatographed, all of the applied activity was recovered in the same position. Peak 5 therefore appeared to be derived from material initially eluting as peaks 2 and 3.When purified by Sephadex G-100 filtration, CM-cellulose chromatography, and elution from disc gels after electrophoresis at pH 9.3, peak 5 was shown to be a prealbumin with an approximate molecular weight of 35,000 (5).

In order to examine the possible derivation of the prekallikrein activator from active Hageman factor, a study was carried out in which a mixture of unactivated Hageman factor and prekallikrein were isolated together from plasma free of prekallikrein-activating activity. The mixture was activated, fractionated by DEAE-cellulose chromatography and disc gel electrophoresis at pH 9.3, and each fraction assayed for Hageman factor activity and bradykinin-generating activity. 100 ml of plasma was processed for the preparation of proenzymes of the human kinin-forming system (5), dialyzed against 0.05 M PO_4 buffer, pH 7.5, and applied to a 5 × 100 cm column of DEAE-cellulose equilibrated with the same buffer. The effluent contained unactivated Hageman factor and prekallikrein and was devoid of prekallikrein-activating activity and kallikrein. The effluent was concentrated 10-fold and activated by storage in a glass vessel for 36 hr at 4°C; generation of active kallikrein was indicated by the capacity of 50 μl of effluent to produce 100 ng of bradykinin upon incubation with 0.2 ml of heat-inactivated plasma. The effluent was then dialyzed

against 0.01 M PO_4 buffer, pH 7.8, and chromatographed on DEAE-cellulose with the results shown in Fig. 1. Peak 1 contained kallikrein, PTA, and IgG and gave a broad band upon disc gel electrophoresis due to the heterogeneity of IgG. The subsequent three peaks were eluted at a concentration of NaCl corresponding to peaks 2–3, 4, and 5 observed upon chromatography of whole serum (5). Fig. 2 shows the pattern obtained after disc gel electrophoresis of the fractions designated peaks 2–3, 4, and 5; a single unstained gel of each peak was sliced and each slice eluted in order to define the position of bradykinin-gener-

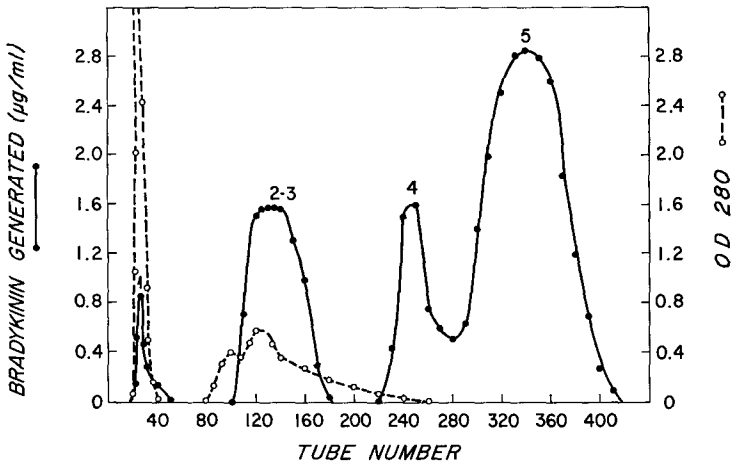

FIG. 1. Bradykinin generated when a plasma fraction obtained by batch elution from DEAE-cellulose at 0.05 M PO_4 buffer, pH 7.5, is activated and rechromatographed on DEAE-cellulose. 0.2 ml of the fractions obtained was incubated with 0.2 ml of fresh plasma for 2 min at 37°C and the bradykinin generated determined by bioassay.

ating activity and Hageman factor activity (Table I). The eluates of peak 2–3 contained two peaks of bradykinin-generating activity located at 5–8 mm and 11–12 mm, and a possible small third peak at 15–16 mm. A major peak of Hageman factor activity was found at 5–8 mm and a small peak of Hageman factor activity was seen at 11–12 mm. The eluates of peak 4 revealed bradykinin-generating activity at 15–16 mm. A major peak of Hageman factor activity was found extending from 14–18 mm; however, Hageman factor activity was found throughout the first 13 slices. The disc gel eluates of peak 5 revealed bradykinin-generating activity from 20 mm through 26 mm and a peak of Hageman factor activity from 23 mm through 26 mm, corresponding in position to the prealbumin bands seen in the stained disc gel of the same peak 5 in Fig. 2. Some Hageman factor activity was also found from 10 to 12 mm and 17 to 22 mm. The peaks of bradykinin-generating activity and Hageman factor

TABLE I

*Bradykinin-Generating Activity and Hageman Factor Activity of Disc Gel Eluates of Peaks 2–3, 4, and 5**

Peaks 2–3

(*mm*)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Bradykinin generated (*ng*)	10		15		50		50		5		40		15		20	←							0	→						
Partial thromboplastin time (*min*)	16		14		9		9		26		22		27		30	←							40–45	→						

Peak 4

(*mm*)	1	2	3	4	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Bradykinin generated (*ng*)	←							0	→						25	50	←						0	→						
Partial thromboplastin time (*min*)	21	31	33	33	29	19	24	30	35	31	21	35	37	18	17	15	28	28	←				40–45	→						

Peak 5

(*mm*)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Bradykinin generated (*ng*)	←									0	→									10	20	50	75	125	150	75	←	0	→	
Partial thromboplastin time (*min*)				←	40–45	→				28	37	30	←	40–45	→	35	31	33		27	35	30	20	18	11	22	←	40–45	→	

* 75 μl of the disc gel eluates were incubated with 0.2 ml of fresh plasma and the bradykinin generated determined by bioassay.

activity were both found in slice 25 corresponding to the densest of the prealbumin bands seen, and an abrupt decline in both activities was observed at 27 mm.

When 50 μl of the concentrates of peaks 2–3, 4, and 5 obtained from DEAE-cellulose chromatography were each incubated with 0.2 ml of heat-inactivated plasma for 2 min at 37°C, bradykinin was not generated, indicating the absence of active kallikrein in these fractions. When 50 μl of each concentrate was incubated with 25 μl of prekallikrein for 5 min at 37°C and then incubated with

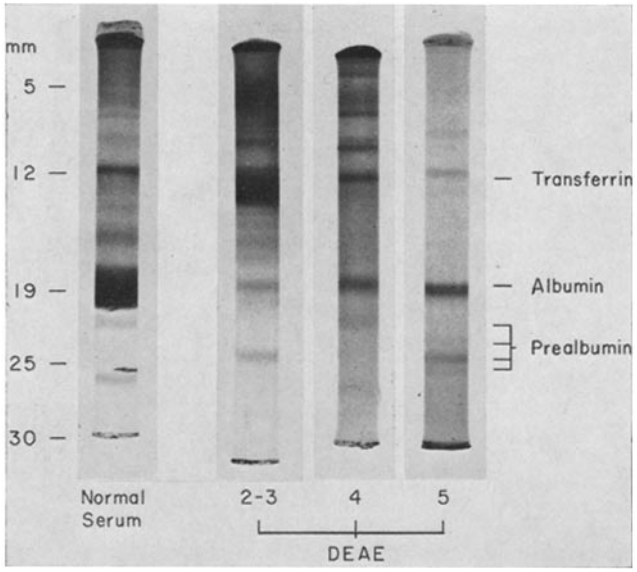

FIG. 2. Disc gel electrophoresis of peaks 2–3, 4, and 5 obtained from DEAE-cellulose.

0.2 ml of heat-inactivated plasma for 2 min at 37°C, peaks 2–3, 4, and 5 generated 75, 60, and 100 ng of bradykinin respectively, demonstrating the presence of prekallikrein activator in each concentrate. The disc gel eluates of peaks 2–3, 4, and 5 were assessed for the presence of prekallikrein activator by incubating 100 μl of disc gel eluate with 50 μl of prekallikrein and then incubating the mixture with 0.2 ml of heat-inactivated plasma. The eluates of peak 2–3 generated 20 ng of bradykinin at 5–6 mm and at 7–8 mm, and 10 ng of bradykinin at 11–12 mm; the eluates of peak 4 generated 10 ng of bradykinin at 16 mm, and the eluates of peak 5 generated 25, 30, 40, 50, and 25 ng of bradykinin from 22 mm through 26 mm. The positions of the peaks of bradykinin-generating activity of the disc gel eluates of peaks 2–3, 4, and 5, when assessed for the presence of prekallikrein activator, corresponded to the positions of the peaks of bradykinin-generating activity shown in Table I when fresh plasma

was used as substrate. When the disc gel eluates of peaks 2–3, 4, and 5 were also examined for their ability to shorten the partial thromboplastin time of PTA-deficient plasma, clot-promoting activity was not found.

When peak 3 or peak 5 obtained from DEAE-cellulose chromatography of sera was subjected to Sephadex G-100 filtration, major peaks of bradykinin-generating activity were found at 55 and 59% bed volume and a minor peak at 50% bed volume (5). Peaks 2–3, 4, and 5 obtained from DEAE-cellulose chromatography of the activated plasma fraction were therefore subjected to Sephadex G-100 filtration in order to ascertain their relative sizes. Peak 2–3 gave peaks of bradykinin-generating activity at 40, 50, and 60% bed volume; peak 4 gave a single peak of bradykinin-generating activity at 52% bed volume, and peak 5 gave a single peak of bradykinin-generating activity at 61% bed volume. After Sephadex G-100 filtration of peak 4, the material was concentrated and again analyzed by disc gel electrophoresis. When a single gel was sliced, eluted, and assayed for bradykinin-generating activity, the single activity previously observed at 15–16 mm (Table I) was no longer apparent, and two peaks of activity at 19–21 mm and 24–25 mm were observed. The activity at 19–21 mm was intermediate in position relative to the original activity of peaks 4 and 5, while the activity at 24–25 mm corresponded to the position of peak 5. Analysis of peak 5 by disc gel electrophoresis after filtration on Sephadex G-100 did not reveal a shift in elution position; bradykinin-generating activity was found between 23 and 26 mm.

Formation of the Prealbumin–Prekallikrein Activator by the Interaction of Active Hageman Factor and Streptokinase-Activated Plasminogen.—

Enhancement of bradykinin-generating activity by the interaction of activated Hageman factor with streptokinase-activated plasminogen: The observation that a plasma fraction could be separated, activated, and then fractionated to yield materials of progressively greater negative charge and smaller size, each of which possessed the ability to correct Hageman factor deficiency and to convert prekallikrein to kallikrein, suggested that progressive digestion of active Hageman factor had occurred. Any enzyme responsible for this digestion must have been present in the effluent of the initial batch eluate of plasma from DEAE-cellulose in which the column and plasma were equilibrated with 0.05 M PO_4 buffer, pH 7.5; further, the enzyme must also elute from DEAE-cellulose close to Hageman factor since prekallikrein-activating activity has been shown to be generated from partially purified preparations of Hageman factor (5). Plasmin has been implicated in the activation of prekallikrein (7); therefore, plasminogen and plasmin were sought in the fractions obtained by DEAE-cellulose chromatography of whole serum. Fractions were pooled, concentrated 10-fold, and assessed for plasminogen and plasmin content by cleavage of azocasein with and without the prior addition of streptokinase. Plasminogen

and plasmin were found between 0.06 and 0.12 M NaCl overlapping peak 3 and the descending half of peak 2. The peak of plasmin activity just preceded plasminogen.

An experiment was designed in which partially purified active Hageman factor was incubated with streptokinase-activated plasminogen to determine

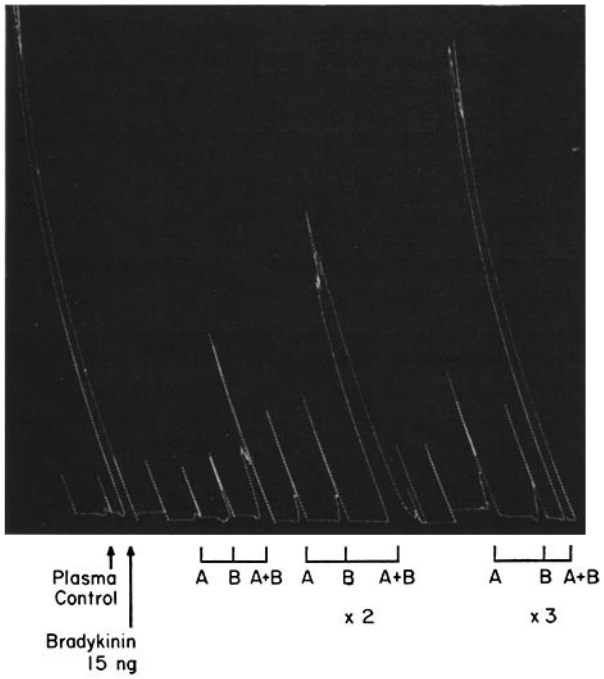

FIG. 3. A chymograph recording of contractions of the guinea pig ileum obtained when active Hageman factor (A), streptokinase-activated plasminogen (B), and a mixture of active Hageman factor preincubated with streptokinase-activated plasminogen (A + B) are incubated with fresh plasma and assayed for bradykinin. The first determination contains 10 μl of active Hageman factor (A) and 25 μl of streptokinase-activated plasminogen (B); the second and third determinations contain double and triple the quantity of the reactants, respectively.

whether prekallikrein-activating activity was generated. Unactivated Hageman factor obtained from DEAE-cellulose chromatography of plasma (5) was pooled, concentrated, dialyzed, and chromatographed on CM-cellulose. Active Hageman factor was eluted at 0.6 M NaCl, concentrated, and further purified by elution from disc gels after electrophoresis at pH 9.3. The peak activity at 5–6 mm, free of plasminogen and plasmin, was pooled from six disc gels and concentrated to 1 ml. 50 μl of this preparation shortened the partial thromboplastin time of Hageman factor-deficient plasma from 40 to 6 min and

generated 10 ng of bradykinin when incubated with fresh plasma; the only identifiable contaminant of active Hageman factor was IgG. A preparation of purified plasminogen which gave a single band after either disc gel electrophoresis at pH 4.3 or isoelectric focusing in gels (isoelectric point 5.9) was activated with streptokinase and generated 20 azocaseinolytic units of plasmin per milliliter. The streptokinase preparation used gave four bands located between transferrin and albumin after disc gel electrophoresis at pH 9.3, each band disappearing after interaction with plasminogen.

Three mixtures of streptokinase-activated plasminogen and active Hageman factor at a volume ratio of 2.5:1 were assessed for total bradykinin-gen-

TABLE II

*Activation of Prekallikrein by a Mixture of Hageman Factor and Streptokinase-Activated Plasminogen (Plasmin)**

A	+	B	+	C	+	D	Bradykinin generated
(0.03 ml)		*(0.075 ml)*		*(0.01 ml)*		*(0.2 ml)*	*(ng)*
Buffer		Plasmin		Buffer		Heat-inactivated plasma	10
Hageman factor		Plasmin		Buffer		Heat-inactivated plasma	10
Buffer		Plasmin		Prekallikrein		Heat-inactivated plasma	15
Hageman factor		Plasmin		Prekallikrein		Heat-inactivated plasma	50
Hageman factor		Buffer		Prekallikrein		Heat-inactivated plasma	0
Hageman factor		Buffer		Buffer		Heat-inactivated plasma	0
Buffer		Buffer		Prekallikrein		Heat-inactivated plasma	0

* Column A was incubated with column B for 5 min at 37°C; C was then added and the mixture again incubated for 5 min at 37°C, and then D was added. After incubation for 2 min at 37°C, the bradykinin generated was measured.

erating activity. The response when 10, 20, and 30 μl of active Hageman factor was reacted with 25, 50, and 75 μl of streptokinase-activated plasminogen, respectively, is shown in Fig. 3. Neither the active Hageman factor nor the streptokinase-activated plasminogen alone generated a significant quantity of bradykinin when incubated with fresh plasma. The mixtures of active Hageman factor and streptokinase-activated plasminogen generated bradykinin in proportion to the quantities of the reactants present. The same results were obtained when an active Hageman factor and streptokinase-activated plasminogen mixture was incubated with prekallikrein and then assayed with heat-inactivated plasma (Table II). The plasmin preparation under these conditions was capable of generating 10 ng of bradykinin when incubated with heat-inactivated plasma, indicating some kininogenase activity. Incubation of plasmin with Hageman factor did not increase the bradykinin-generating activity of the mixture relative to that of plasmin alone. Incubation of plasmin

with prekallikrein increased the bradykinin generated by 5 ng above that accounted for by plasmin alone. Incubation of plasmin with Hageman factor and then prekallikrein produced three times more bradykinin than could be accounted for by the ability of plasmin to directly activate prekallikrein or to act as a kininogenase. A prekallikrein activator must therefore have resulted from the interaction of plasmin with Hageman factor.

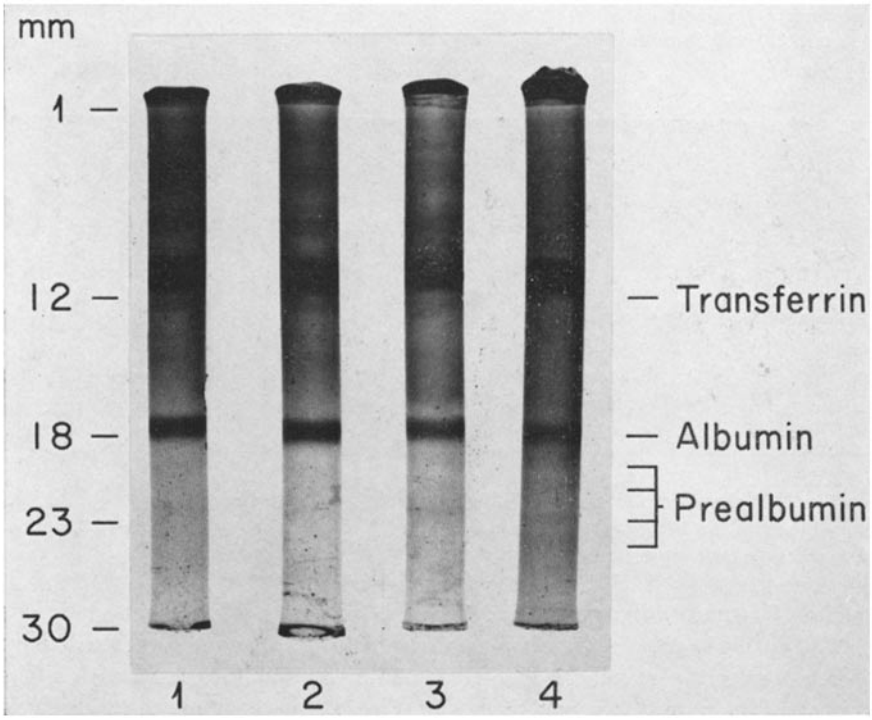

FIG. 4. Disc gel electrophoresis of mixtures of active Hageman factor with buffer (1), plasminogen (2), streptokinase (3), and streptokinase-activated plasminogen (4)

Characterization of the prekallikrein activator formed by the interaction of active Hageman factor and streptokinase-activated plasminogen: The prekallikrein activator generated by the interaction of streptokinase-activated plasminogen and active Hageman factor was sought by examination of the incubation mixture by disc gel electrophoresis. The experiment was initially carried out with highly purified Hageman factor obtained by DEAE-cellulose chromatography, CM-cellulose chromatography, and elution from disc gels after electrophoresis at pH 9.3. This experiment demonstrated that the prekallikrein-activating

activity appeared in the prealbumin region but the protein concentration was not sufficient to reveal visible protein bands. Accordingly, the study was repeated with Hageman factor eluted from DEAE-cellulose employing the ascending portion of peak 2 which was free of plasminogen and plasmin. 200 μl of active Hageman factor was allowed to react with 300 μl of either buffer, plasminogen, streptokinase, or streptokinase-activated plasminogen for 5 min at 37°C. 150 μl of each mixture was then applied to two disc gels and electrophoresed at pH 9.3. One gel of each mixture was stained and the other sliced, eluted, and assayed for bradykinin-generating activity and Hageman factor activity. As shown in Fig. 4, the disc gel containing the mixture of

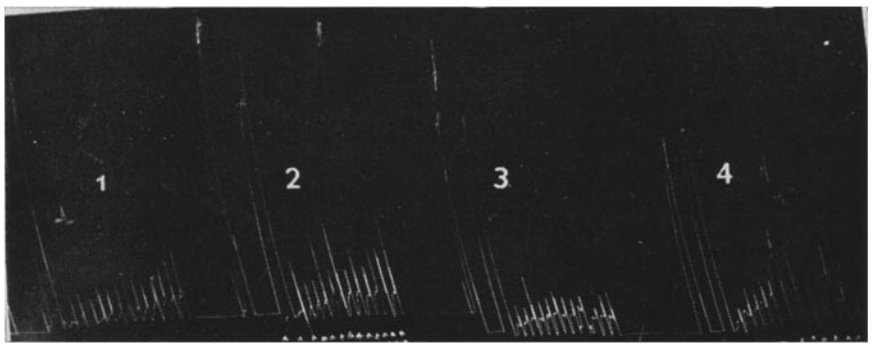

FIG. 5. A chymograph recording of the bradykinin generated when the eluates of the bottom 12 mm of the disc gels shown in Fig. 4 (30 mm–18 mm) are incubated with fresh plasma. Preceding the eluates of each disc gel is a 10 ng bradykinin standard contraction.

active Hagemen factor and streptokinase-activated plasminogen had four bands visible in the prealbumin region from 20 to 25 mm, whereas, prealbumin bands were not seen when active Hageman factor was interacted with either buffer, plasminogen, or streptokinase. The disc gel eluate of the active Hageman factor—streptokinase-activated plasminogen mixture contained bradykinin-generating activity in the prealbumin region from 21 to 25 mm (Fig. 5). The disc gel eluates of the mixtures of Hageman factor with buffer, plasminogen, and streptokinase did not contain bradykinin-generating activity. Incubation of active Hageman factor with either buffer, plasminogen, or streptokinase failed to generate clot-promoting activity in the prealbumin region; in each case active Hageman factor could be located between 1 and 9 mm. On the other hand, depletion of Hageman factor activity between 1 and 9 mm was found in the mixture of active Hageman factor and streptokinase-activated plasminogen accompanied by the appearance of such activity at 10 mm and again at 21–25 mm in the prealbumin region (Table III) corresponding to the location of the bradykinin-generating activity (Fig. 5) and the prealbumin bands

(Fig. 4). The presence of plasmin in the mixture did not interfere with the clotting test; the plasmin preparation spread between 7 and 10 mm in disc gels at pH 9.3, precipitated in the gel, and was inactivated by the procedure.

DISCUSSION

It was previously shown that when human serum was chromatographed on DEAE-cellulose, four peaks of bradykinin-generating activity were found

TABLE III

*Hageman Factor Activity in Disc Gel Eluates**

Slices	Disc 1	Disc 2	Disc 3	Disc 4
(*mm*)				
1	14	12	43	52
2	11	12	12	52
3	8	17	17	52
4	8	15	8	33
5	10	11	8	42
6	16	15	17	48
7	34	12	14	41
8	41	20	19	41
9	36	30	28	41
10	57	43	46	33
11–20	45–60	45–60	45–60	45–60
21	52	60	55	37
22	53	54	56	33
23	53	53	58	32
24	50	55	57	36
25	57	57	44	44
26	51	54	57	57
27	60	54	57	60
28	57	58	51	58
29	55	53	51	55
30	45	57	45	60

* Disc gels of mixtures described in the legend to Fig. 4. The partial thromboplastin time in minutes is shown for the eluate of each slice of each gel.

(peaks 2–5), each of which was capable of converting prekallikrein to kallikrein (5). Hageman factor overlapped peaks 2 and 3, and this region was capable of generating a prekallikrein activator with the same characteristics as that present in peak 5. Further, the capacity of the prealbumin prekallikrein activator to shorten specifically the partial thromboplastin time of Hageman factor-deficient plasma suggested that it represented a fragment of active Hageman factor. Additional characterization of the four prekallikrein-activating peaks obtained from DEAE-cellulose was pursued by isolating a mixture

of unactivated Hageman factor and prekallikrein by batch elution from DEAE-cellulose. When this effluent was activated and then chromatographed on DEAE-cellulose, active kallikrein and three prekallikrein-activating fractions (peaks 2–3, 4, and 5) were obtained (Fig. 1) in positions similar to those observed when the kinin-forming system was fractionated from serum after its activation by clotting. After disc gel electrophoresis at pH 9.3, peak 2–3 was resolved into two peaks of bradykinin-generating activity (Table I). The first peak at 5–8 mm was associated with a major peak of Hageman factor activity and the second peak at 11–12 mm was accompanied by a small peak of Hageman factor activity. The activities observed at 5–8 mm and 11–12 mm correspond to peaks 2 and 3 eluted after DEAE-cellulose chromatography of serum, and it seems likely that peak 3 represents an early alteration of active Hageman factor resulting in a slight increase in negative charge and greater bradykinin-generating activity relative to clot-promoting activity. The demonstration that peak 4 is intermediate in size relative to peaks 2–3 and 5, has a negative charge greater than peak 3 but less than peak 5, and manifests prekallikrein-activating activity with associated Hageman factor activity suggests that peak 4 is a further breakdown product of active Hageman factor. The subsequent conversion upon disc gel electrophoresis of peak 4 to two peaks located closer to the anode than peak 4 indicates the existence of intermediate fragments as peak 4 converts to peak 5. Peak 5, as described previously (5), contained multiple prealbumin bands (Fig. 2) which possessed relatively greater prekallikrein-activating activity than clot-promoting activity when compared with peak 2 material.

Progressive fragmentation of active Hageman factor appears to result in the formation of at least three types of fragments capable of correcting Hageman factor deficiency. Fragments in the prealbumin region (Table I, peak 5: 20–26 mm) possessed strikingly greater prekallikrein-activating activity relato clot-promoting activity when compared with the parent Hageman factor molecule (Table I, peak 2–3: 5–8 mm). Fragments whose ratio of prekallikrein-activating activity to clot-promoting activity was intermediate when compared with Hageman factor and the prealbumin–prekallikrein activator were generally located between 11 and 20 mm. However, within this region are fragments having comparable clot-promoting activity but no detectable bradykinin-generating activity. It therefore appears likely that a specific site required for the conversion of prekallikrein to kallikrein is present in active Hageman factor. The formation of progressively smaller fragments which retain this site increases their efficiency in converting prekallikrein to kallikrein and decreases their ability to accelerate coagulation. Other fragments lacking this prekallikrein-activating site are apparently formed as detected by their clot-promoting activity, although one cannot rule out the possibility that these represent nonspecific aggregation of smaller fragments analogous to

the aggregation observed for the parent Hageman factor molecule (Table I, peak 2–3: 1–4 mm) (14), resulting in masking of the prekallikrein-activating site.

The possibility that plasmin might be the enzyme which cleaves Hageman factor was considered because it has been implicated in the activation of prekallikrein (7) and was detectable in preparations of highly purified Hageman factor (6). When plasmin was sought on the DEAE-cellulose chromatogram, it eluted overlapping peaks 2 and 3. Incubation of partially purified Hageman factor (peak 2) with streptokinase-activated plasminogen resulted in a striking increase in bradykinin-generating activity employing fresh plasma as substrate (Fig. 3). The mixture of active Hageman factor and streptokinase-activated plasminogen was also active in generating bradykinin upon sequential interaction with prekallikrein and heat-inactivated plasma (Table II). The latter effect is attributed to the formation of the prekallikrein activator and was not explicable by the kininogenase activity of plasmin or a direct activation of prekallikrein by plasmin.

The products of the interaction of active Hageman factor and streptokinase-activated plasminogen were therefore evaluated by elution from disc gels after electrophoresis at pH 9.3 and assayed for bradykinin-generating activity and Hageman factor activity. As shown in Fig. 4, prealbumin bands were present in a location similar to that observed upon disc gel electrophoresis of peak 5 obtained upon DEAE-cellulose chromatography of serum (5) or contact activation of a plasma fraction containing unactivated Hageman factor, plasminogen, and prekallikrein (Fig. 2). Elution of the prealbumin region (Fig. 5) confirmed the association of prekallikrein-activating activity with these bands and accounted for the enhanced bradykinin-generating activity of the starting mixture. A small peak of Hageman factor activity located between 21 and 25 mm (Table III) was associated with those slices having prekallikrein-activating activity, and a significant depletion of Hageman factor actvity was observed between 1 and 9 mm. This interaction of plasmin and active Hageman factor under the conditions described resulted in the formation of prealbumin–prekallikrein-activating activity indistinguishable from that appearing upon the contact activation of Hageman factor in the presence of other serum proteins including plasminogen.

Nagasawa et al. (15) have reported that purified activated bovine Hageman factor was capable of converting bovine prekallikrein to kallikrein; and Davies, Holman, and Lowe (16) have reported similar results in the guinea pig. Although human Hageman factor (peak 2) appears to be capable of converting human prekallikrein to kallikrein, the digestion of active human Hageman factor by plasmin appears to generate fragments having enhanced ability to activate prekallikrein. The conversion of plasminogen to plasmin therefore appears necessary for maximal generation of bradykinin when human plasma

is activated. It should be noted that the generation of plasmin activity is believed to be dependent upon the prior activation of Hageman factor (17) and the participation of a cofactor termed "Hageman factor cofactor" (18). Hageman factor cofactor elutes from DEAE-cellulose with kallikrein and PTA and after further purification could be distinguished from plasma kallikrein but not from PTA, although the activity could be detected in PTA-deficient plasma. These studies suggest a scheme by which bradykinin may be generated in human plasma (Fig. 6). It is dependent upon the initial activation of Hageman factor resulting in conversion of plasminogen to plasmin. Digestion of active Hageman factor by plasmin then liberates fragments having en-

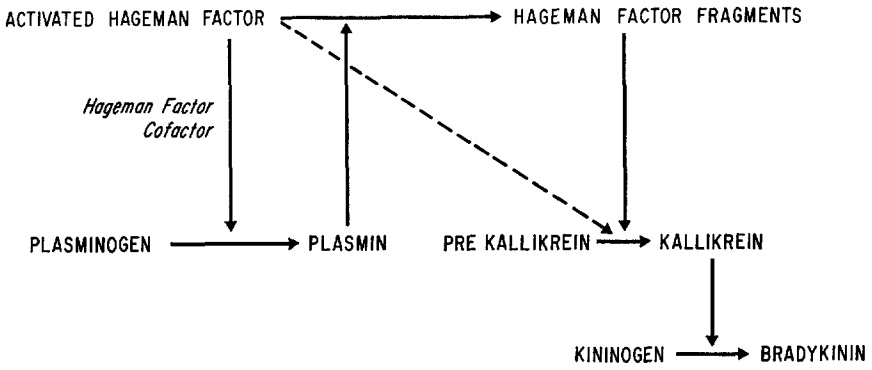

FIG. 6. Schematic diagram of the pathway by which bradykinin is generated when Hageman factor is activated in human plasma. Although activated Hageman factor may be capable of converting prekallikrein to kallikrein, shown by the dotted line, activation of plasmin and cleavage of active Hageman factor by plasmin, forming fragments of active Hageman factor which activate prekallikrein, appears to be the major pathway.

hanced ability to convert prekallikrein to kallikrein. This scheme represents one pathway in which coagulation, fibrinolysis, and inflammation are linked.

SUMMARY

Activation of a plasma fraction containing unactivated Hageman factor and prekallikrein followed by chromatography of this fraction on DEAE-cellulose revealed four peaks having bradykinin-generating activity. Peak 1 contained kallikrein; peaks 2–3, 4, and 5 each contained prekallikrein-activating activity. Elution of peaks 2–3, 4, and 5 from disc gels after electrophoresis at pH 9.3 revealed peaks of prekallikrein-activating activity located at 5–8, 11–12, 15–16, and 20–26 mm, each of which was associated with a peak of clot-promoting activity which specifically corrected Hageman factor deficiency. Conversion of peak 2 to peaks 3, 4, and 5 was associated with a progressive decrease in size, increase in net negative charge, increased prekallikrein-activating

activity, and decreased ability to correct Hageman factor deficiency. Plasminogen and plasmin were found on a DEAE-cellulose chromatogram of serum overlapping peaks 2 and 3. Incubation of active Hageman factor with streptokinase-activated plasminogen resulted in enhanced ability of the mixture to activate prekallikrein. Assessment of the products of this reaction by disc gel electrophoresis demonstrated the formation of the prealbumin prekallikrein activator corresponding to the major prekallikrein activator generated by contact activation of human plasma. The conversion of plasminogen to plasmin and the subsequent cleavage of Hageman factor by plasmin to form activators of prekallikrein represents one pathway in which coagulation, fibrinolysis, and inflammation are linked.

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