Guinea Pig Hageman Factor as a Vascular Permeability Enhancement Factor

TETSURO YAMAMOTO, MD, and
CHARLES G. COCHRANE, MD

From the Department of Immunopathology, Research Institute of Scripps Clinic, La Jolla, California

Hageman factor was purified from guinea pig plasma by successive column chromatography. The guinea pig Hageman factor appeared homogeneous as a single-chain protein on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol. The apparent molecular weight was 76,000 daltons by SDS-polyacrylamide gel electrophoresis and 105,000 daltons by gel filtration with a Sephadex G-150 column. Amino acid composition of the guinea pig Hageman factor was similar to that reported for human, bovine, and rabbit Hageman factors. The purified guinea pig Hageman factor, as well as guinea pig plasma, showed strong clotting time correction activity in Hageman-factor-deficient human plasma. The activity could be blocked by the IgG fraction of antiserums against guinea pig Hageman factor raised in rabbits or a goat. The concentration of Hageman factor in guinea pig plasma was determined to be 120 μg/ml by quantitative radial immunodiffusion assay. The 28,000-dalton active form of Hageman factor (β-HFa) was prepared from guinea pig Hageman factor by treatment with plasma kallikrein. β-HFa caused an increase in vascular permeability when injected into guinea pig skin at concentrations as low as 3×10^{-10} M (0.8 ng). Native, or zymogen Hageman factor did not cause an increase in permeability at concentrations of up to 2×10^{-7} M. The increased permeability induced by β-HFa was short lasting, with about a 50% decrease in activity apparent within 6 minutes after intradermal injection. The permeability enhancement activity of β-HFa was inhibited by pretreatment of β-HFa with diisoprophylfluorophosphate. It may be concluded that active Hageman factor in the interstitial space of guinea pigs acts as a vascular permeability factor of far greater potency than bradykinin. (Am J Pathol 1981, 105: 164–175)

A PROTEASE-LIKE PERMEABILITY factor derived from guinea pig skin has been reported previously.[1,2] The permeability factor was shown to have properties similar to Hageman factor in human, bovine, or rabbit plasma; among these properties were similarity in molecular weight and the ability to be activated by contact with kaolin to a form that could cause prekallikrein activatin.[3,4] It therefore became essential to determine whether Hageman factor purified from guinea pig plasma possessed permeability enhancement activity.

Other investigators have conducted studies into the nature of permeability enhancement factor(s) in serum or plasma. More than 25 years ago, Miles et al reported a powerful permeability factor in guinea pig serum, which existed as an inactive precursor in the α_2-globulin fraction of plasma and was transformed into an active form by 200-fold dilution of the serum with saline solution or by treatment with ethyl-ether.[5–8] This permeability factor—permeability factor of dilution (PF/dil)—has been extensively studied through the use of many other mammalian serums or plasmas.[9–11] Margolis showed that contact of plasma or serum with glass was required for generation of PF/dil,[12] and further studies showed that not only Hageman factor,[13] but also prekallikrein[14,15] and kininogen[16] of high molecular weight were required in plasma for the generation of PF/dil. The precursor of PF/dil was not prekallikrein.[17] Although Ratnoff et al had proposed that the precursors of PF/dil and Hageman factor were different,[13,18] Johnston et

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Address reprint requests to Charles G. Cochrane, MD, Department of Immunopathology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037.

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al[15] and Oh-ishi and Webster[19] presented evidence that PF/dil and active Hageman factor were identical.

Some evidence has been reported suggesting that Hageman factor may possess permeability enhancement activity. Ratnoff and Miles observed a permeability enhancement phenomenon when purified active Hageman factor derived from human plasma deficient in clotting Factor XI was injected into guinea pig skin; however, this material contained measurable contamination with plasminogen and clotting Factor IX.[13] Johnston and Barrow[20] failed to observe a direct association of activated human Hageman factor and the permeability activity in rabbits. Kellermeyer and Ratnoff,[18] however, reported that human Hageman factor that was purified from normal plasma caused an increase in permeability when it was injected into guinea pig skin. Although the preparation of Hageman factor was contaminated with other proteins, the permeability activity was lost after absorption with rabbit anti-human Hageman factor. Temme et al[21] reported that active bovine Hageman factor mixed with prekallikrein enhanced vascular permeability in rabbit skin, while active Hageman factor alone did not. Movat et al[22,23] observed that the permeability enhancement activity and prekallikrein-activating activity were cofractionated during column chromatography with active Hageman factor of small molecular weight (less than 40,000 daltons) generated in guinea pig serum. Furthermore, with partial purification of Hageman factor, they observed generation of permeability activity and prekallikrein-activating activity after trypsinization of the fractions containing Hageman factor.

Thus, the capacity of highly purified Hageman factor to cause a permeability reaction has not been shown unequivocally, and a quantitative assessment of the presumptive permeability activity of purified Hageman factor is not available. Such information is essential in order to ascertain the biologic role of the contact (Hageman factor) system.

In order to examine this question further and to examine the relation of Hageman factor to other known permeability factors, especially the skin permeability factor, we have purified Hageman factor from guinea pig plasma and prepared an active Hageman factor, β-HFa. These purified components have been used to examine quantitatively the capacity of activated Hageman factor to enhance vascular permeability.

Materials and Methods

Animals and Reagents

Albino-Hartley strain guinea pigs of both sexes, 300–700 g in body weight, were used. Diisopropylfluorophosphate (Dip-F) was obtained from Calbiochem (La Jolla, Calif). 2-methyl-2-D-glycopyranoside, rabbit brain cephalin, and L-lysine were obtained from Sigma (St. Louis, Mo). Leupeptin and ε-aminocaproyl-p-chlorobenzylamide were products of Protein Research Foundation (Osaka, Japan). Kaolin was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Benzamidine hydrochloride hydrate and polybrene were purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wis). D-Pro-Phe-Arg paranitroanilide and Bz-Pro-Phe-Arg paranitroanilide were from Kabi Diagnostica (Stockholm, Sweden). Carboxymethyl (CM)-Sephadex C-50, diethylaminoethyl (DEAE)-Sephadex A-50, Sulphopropyl (SP)-Sephadex C-50, concanavalin A-Sepharose 4B, Sephadex G-150, Sepharose 4B, and CNBr-activated Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals (Madison, Kent, England). Plasma deficient in Hageman factor was generously donated by Mrs. Gunda Hiatt.

p-Chlorobenzylamine-ε-aminocaprolyl-Sepharose 4B (PCB-Sepharose) was prepared by covalent coupling of ε-aminocaproyl-p-chlorobenzylamide (500 mg) to CNBr-activated Sepharose 4B (15 g) at pH 8.5. The remaining nonreacted sites on the beads were blocked by monoethanolamine. Lysine-Sepharose 4B was prepared by covalent coupling of L-lysine (15 g) to Sepharose 4B (500 g), pH 9.5, which was previously activated by CNBr (40 g). The remaining nonreacted sites on the beads were blocked by monoethanolamine.

Guinea Pig Plasma

Blood was collected from large guinea pigs by cardiac puncture with 22-gauge needles and plastic syringes and then 9 parts blood was mixed with 1 part 3.8% sodium citrate (pH 7.4). After centrifugation, the plasma for the purification of Hageman factor was made up to 0.05% polybrene with 100 mg/ml polybrene solution and was kept frozen at −70 C until used. The standard plasma used for clotting and immunodiffusion assays was from a pool of the plasma from 15 animals.

Clotting Assay for Guinea Pig Hageman Factor

Diluted samples (50 μl) were incubated at 37 C for 8 minutes with 50 μl of Hageman-factor-deficient human plasma and 50 μl of kaolin-cephalin mixture (5 mg of kaolin in 1 ml of 2 mg/ml cephalin solution in saline). Fifty microliters of 50 mM $CaCl_2$ were

then added, and the clotting time was determined. Activity was calculated from a calibration curve where the log of Hageman factor concentration was plotted against the log of the clotting time. This plot was linear from 80 seconds to 180 seconds when citrated normal guinea pig plasma was used as a source of Hageman factor. One unit of activity is defined as the amount of activity present in 1 ml of normal guinea pig plasma. When assayed in the human Hageman-factor-deficient plasma, 1 ml of guinea pig plasma had about 10 times the clot-promoting capacity of 1 ml of normal human plasma.

For studies on the inhibition of the clotting activity of Hageman factor by antibodies, 10 μl of guinea pig plasma diluted 3 times was mixed and incubated with 10 μl of monospecific anti-guinea pig rabbit IgG fraction or normal IgG fraction for 10 minutes at 22 C. The mixture was then diluted 100 times with 980 μl of Tris-buffered saline containing 1 mg/ml bovine serum albumin before it was used as a clotting assay sample. For calculation of the percentage of inhibition, a calibration curve where the log of the plasma dilution was plotted against the log of the clotting time was used.

Purification of Hageman Factor

Hageman factor was purified from guinea pig plasma by successive column chromatography. All procedures were performed by 0–4 C with plastic or siliconized glass containers. The pH of the buffer was measured at 22 C, and conductivity was measured at 4 C. For assays of Hageman factor, the standard clotting assay was used.

Step 1—First DEAE-Sephadex Column Chromatography

Five hundred milliliters of guinea pig plasma were dialyzed against 1.5 l of 40 mM Tris-HCl buffer containing 3 mM ethylenedinitrilotetraacetic acid disodium (EDTA), 4 mM benzamidine, and 50 mg/l polybrene (pH 8.0) for 18 hours. The conductivity and pH of the dialyzed sample was close to that of the equilibration buffer for DEAE-Sephadex A-50 column chromatography. The dialyzed sample was applied to a DEAE-Sephadex column (bed volume 500 ml, cm × 25.5 cm) equilibrated with 20 mM Tris-HCl buffer containing 3 mM EDTA, 4 mM benzamidine, 50 mg/l polybrene, and 40 mM NaCl (pH 8.0, conductivity, 4.3 mmho). After the column was washed with equilibration buffer, Hageman factor was eluted by salt gradient elution with 4.5 l of the equilibration buffer and 4.5 l of the same buffer containing 300 mM NaCl instead of 40 mM NaCl. Hageman factor was eluted in the ascending portion (conductivity around 8.5 mmho) of the main protein peak (Figure 1). The Hageman-factor-rich fractions were pooled, Dip-F was added to the pool to a final concentration of 1 mM, and the pool was stirred for 18 hours. After the treatment, it was stored at −70 C until use.

Step 2—Second DEAE-Sephadex Column Chromatography

The Hageman-factor-rich pools from three different preparations from the first DEAE-Sephadex column chromatography were pooled and dialyzed against 8 l of 20 mM Tris-HCl buffer containing 3 mM EDTA, 4 mM benzamidine, and 50 mg/l polybrene (pH 8.0) for 18 hours. The conductivity and pH of the dialyzed sample were similar to the equilibration buffer for the second DEAE-Sephadex A-50 column. The dialyzed sample was applied to the second DEAE-Sephadex column (bed volume 450 ml, 5 cm × 23 cm) equilibrated with the same buffer as used for the first DEAE-Sephadex column chromatography. After washing, the absorbed Hageman factor was eluted by salt gradient elution. For gradient elution, 1600 ml of the equilibration buffer and 1600 ml of the same buffer containing 300 mM NaCl instead of 40 mM NaCl were used. The concentrated Hageman factor was eluted in the front half of the bulk protein peak, the Hageman-factor-rich fractions were pooled (750 ml), Dip-F was added at a final concentration of 1 mM, and the mixture was stirred for 18 hours.

Step 3—Lysine-Sepharose 4B Column Chromatography

The pooled fraction was passed through a lysine-Sepharose 4B column (bed volume 30 ml, 2.6 cm × 5.7 cm) equilibrated with 20 mM Tris-HCl buffer containing 3 mM EDTA, 4 mM benzamidine, 50 mg/l polybrene, and 100 mM NaCl (pH 8.0). In the breakthrough fraction, which contained the bulk of Hageman factor activity, Dip-F was added at final concentration of 1 mM, and the fraction was stirred for 18 hours.

Step 4—PCB-Sepharose 4B Column Chromatography

The pooled fraction from the lysine-Sepharose 4B column was dialyzed against 1260 ml (1.6 times sample volume) of 20 mM Tris-HCl buffer containing 3 mM EDTA, 4 mM benzamidine, and 50 mg/l polybrene (pH 8.0) for 18 hours. The dialyzed sample was applied to PCB-Sepharose 4B column (bed volume 10 ml, 1.4 cm × 6.5 cm) equilibrated with 20 mM

Tris-HCl buffer containing 3 mM EDTA, 4 mM benzamidine, 50 mg/l polybrene, and 40 mM NaCl (pH 8.0). In the breakthrough fraction containing the bulk of Hageman factor activity, Dip-F was added at a final concentration 1 mM, and the fraction was stirred for 18 hours.

Step 5—SP-Sephadex Column Chromatography

The breakthrough fraction from the PCB-Sephadex 4B column was exhaustively dialyzed against 50 mM sodium acetate buffer containing 4 mM benzamidine and 130 mM NaCl (pH 5.3) for 18 hours. The dialyzed sample was applied to a SP-Sephadex C-50 column (bed volume 12 ml, 2.6 cm × 2.3 cm) equilibrated with dialysis buffer. After exhaustive washing, Hageman factor was eluted by salt gradient elution. For gradient elution, 80 ml of equilibration buffer and 80 ml of the same buffer containing 530 mM NaCl instead of 130 mM NaCl were used. Hageman factor activity appeared in the early part of the gradient elution (conductivity around 22 mmho) and corresponded with the main protein peak (Figure 2). Hageman-factor-rich fractions were pooled, and Dip-F was added to a final concentration of 1 mM. The pooled fraction was divided into six aliquots (10–20 ml) and kept frozen at −70 C until used.

Step 6—Sephadex G-150 Column Chromatography

KCl was added to one aliquot of the SP-Sephadex eluate to a final concentration of 1.5 M. The sample was concentrated with a Diaflow membrane (PM-30) to 1.5 ml and applied to a Sephadex G-150 column (bed volume 280 ml, 1.5 cm × 160 cm) equilibrated with 10 mM acetate buffer containing 2 M KCl (pH 5.3). Hageman factor clotting activity corresponded to the main protein peak (Figure 2). The fractions of this peak were pooled and used as purified Hageman factor.

Preparation of Plasma Kallikrein

For the purification, kallikrein activity was measured by synthetic peptides-paranitroanilide amidolytic assay. The 60% ammonium sulfate precipitated fraction from 250 ml of guinea pig plasma was dissolved and dialyzed against 20 mM phosphate buffer containing 50 mM NaCl (pH 6.0). The dialyzed sample was centrifuged to remove insoluble material and was applied to a CM-Sephadex C-50 (bed volume 50 ml) equilibrated with the same buffer used for dialysis. The prekallikrein was adsorbed to the column, and then spontaneously activated kallikrein was eluted from the column by salt gradient elution (400 ml) of 20 mM phosphate buffer containing 50 mM NaCl (pH 6.0) and 400 ml of 67 mM phosphate buffer containing 500 mM NaCl (pH 7.8). The kallikrein-rich fractions were pooled and dialyzed against 20 mM Tris-HCl buffer containing 40 mM NaCl and 3 mM EDTA (pH 7.8) and then applied to a DEAE-Sephadex column (bed volume 20 ml) equilibrated with the same buffer used for the dialysis. The breakthrough fraction, which was rich in kallikrein, was dialyzed against 100 mM acetate buffer containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 4 mM benzamidine (pH 6.0). The dialyzed sample was then applied to a concanavalin A-Sepharose 4B column (bed volume 5 ml) equilibrated with the same buffer used for the dialysis. The kallikrein was adsorbed to the gel and eluted by 500 mM α-methyl-2-D-glucopyranoside contained in the equilibration buffer (pH 6.0). The kallikrein-rich fractions from the column were pooled and stored at −70 C. Since no increasing of Bz-Pro-Phe-Arg-pNA amidolytic activity was observed after preincubation of the fraction with β-HFa, the amount of prekallikrein contained in the kallikrein-rich fraction was undetectable.

Generation of Guinea Pig β-HFa by Plasma Kallikrein

β-HFa was generated from guinea pig Hageman factor by immobilized kallikrein. The guinea pig kallikrein, highly purified as described above, was dialyzed against 50 mM acetate buffer (pH 6.0) and then immobilized on a CM-Sephadex C-50 column (bed volume 1.8 ml) equilibrated with the same buffer used for dialysis. The kallikrein–CM-Sephadex column was washed and equilibrated with 20 mM phosphate buffer (pH 7.2). In the upper part of the column, which had changed color to cloudy white, 0.3 ml of the gel was removed and placed into a plastic conical centrifuge tube. Highly purified guinea pig Hageman factor (pooled and concentrated sample of the SP-Sephadex fractions, 800 μg/ml) was dialyzed against 20 mM phosphate buffer (pH 7.2), and 0.7 ml of the sample (600 μg protein) was mixed with 0.3 ml of the kallikrein–CM-Sephadex gel in the conical tube. The mixture was incubated at 22 C and shaken for 48 hours, at which time the generation of β-HFa reached a plateau. Negligible kallikrein activity escaped into the fluid phase during the activation. At 48 hours, the incubation was stopped by the addition of benzamidine solution (final concentration of approximately 10 mM). The suspension was centrifuged and the supernatant was removed. β-HFa contained in the supernatant was further purified with a DEAE-Sephadex, which removed any trace amount of kallikrein.

Human β-HFa

Human β-HFa was prepared from acetone-treated plasma by means of DEAE-cellulose, isoelectric focusing, and Sephadex G-100 column chromatography, in that order.[24] The β-HFa showed a single band in SDS-gel electrophoresis in the presence or absence of reducing agents. The purified material, which was kindly given by Dr. Hojima in our laboratory, was used for analysis of the amino acid composition and for permeability assay for rabbit skin.

Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was carried out by the method of Weber and Osborn[25] with 7.5% acrylamide gel for Hageman factor and 10% gel for β-HFa analysis. Proteins were added to 10 mM sodium phosphate buffer containing 10% SDS and 8 M urea and were boiled for 3 minutes with or without 5 μl of 2-mercaptoethanol.

Alkaline-PAGE for β-HFa was carried out by the method of Davis[26] with 7% polyacrylamide gel and Tris-glycine buffer. Fifty μl of β-HFa solution (about 15 μg protein) was mixed with 20 mg of sucrose and was subjected to electrophoresis at 4 mA/gel for 2 hours. Staining and destaining was performed by the same method used for SDS-PAGE.

Amino Acid Analysis of Proteins

Twenty-microgram samples were hydrolyzed *in vacuo* with HCl for 24, 48, and 72 hours and analyzed with a Beckman model 121-M amino acid analyzer. The values of threonine and serine were determined by extrapolation to zero hydrolysis time. Tryptophan was estimated by the method of Hugli and Moore.[27] Half cystine was determined as cysteric acid by the method of Moore.[28]

Measurement of Permeability Activities

β-HFa, bradykinin, or Hageman factor (Sephadex G-150 fraction) were diluted to required concentration for each individual experiment with phosphate-buffered saline containing 0.1 mg/ml bovine serum albumin (pH 7.4). Aliquots (0.1 ml) of the test sample were injected intracutaneously into the clipped flank of a guinea pig or a rabbit immediately after intravenous administration of Evans blue. The permeability enhancement activity of the samples was determined by quantitative measure of the extravasculated Evans blue in the guinea pig skin lesion according to the dye extraction method of Udaka et al,[29] as previously described.[1] In the present experiment, 30 mg/kg of Evans blue (2.5% solution in 0.6% saline) was injected. The activity was expressed in μg of dye per lesion after subtraction of bovine serum albumin containing saline-injected control site.

Prepartion of Antiserum with IgG Fraction

Antiserum to guinea pig Hageman factor was raised in rabbits and in a goat by intradermal multiple-site injections of the protein solution (Sephadex G-150 fraction) emulsified with the same volume of complete Freund's adjuvant. For the sensitization of each rabbit, 100 μg of Hageman factor were injected initially, and then 25 μg of protein were inoculated every other week three or more times. For the sensitization of a goat, 200 μg of the protein were injected initially followed by weekly inoculations of 50 μg for at least 3 weeks. For preparation of the IgG fraction, a euglobulin fraction (precipitated at 40% ammonium sulfate) was dissolved, dialyzed against phosphate-buffered saline (pH 7.4) at 4 C. The volume was adjusted to 1/4 of the serum, and the fraction was incubated twice with kaolin (1 g/20 ml) for 20 minutes at 22 C to remove or to fully activate the remaining rabbit or goat prekallikrein and Factor XI. The kaolin-treated euglobulin fraction was then incubated with Dip-F (final concentration, 1 mM), for at least 1 hour at 22 C to block active proteases in the fraction. After these treatments, the euglobulin fraction was dialyzed against 20 mM phosphate buffer (pH 7.8) and passed through a DEAE-cellulose column (bed volume was one third of the starting serum volume) equilibrated with the same buffer used for the dialysis. The bulk of the pooled γ-fraction was precipitated again with ammonium sulfate (40% saturation) and stored at −20 C. Aliquots of the γ-fraction were dialyzed against phosphate-buffered saline (pH 7.4) and were used as an anti-guinea pig Hageman factor rabbit or goat IgG fraction.

Normal rabbit or goat IgG fractions were prepared in the same way from normal rabbit or goat serum.

Double Immunodiffusion

Immunodiffusion was carried out according to the method of Ouchterlony.[30]

Quantitative Radial Immunodiffusion Assay

Quantitative radial immunodiffusion assay was carried out by the method of Mancini.[31]

Results

Purification and Characterization of Guinea Pig Hageman Factor

Purification of Hageman Factor

The purification steps and the yield for a typical preparation of guinea pig Hageman factor are shown in Table 1. Detailed descriptions of each step are given in Materials and Methods. With the first DEAE-Sephadex column, Hageman factor was clearly separated from the bulk of prekallikrein and also the bulk of kininogen of high molecular weight. Lysine-Sepharose was used to remove plasminogen. The second DEAE-Sephadex column was used for further separation of Hageman factor from the small amount of contaminating kininogen of high molecular weight and for concentration of Hageman-factor-rich fraction. PCB-Sepharose was used to remove any possible trace of prekallikrein. Elution of Hageman factor from the SP-Sephadex column was usually complete within 5 hours. Pooled fractions of SP-Sephadex column showed that over 90% of the proteins in the fraction were Hageman factor by SDS-gel electrophoresis. Twenty mg of protein were recovered from 1.5 l of guinea pig plasma. Recovery of Hageman factor clotting activity was 10% and 8.1 guinea pig units per milligram protein were obtained for the specific clotting activity. D-Pro-Phe-Arg-paranitroanilide amidolytic activity at pH 7.8 was

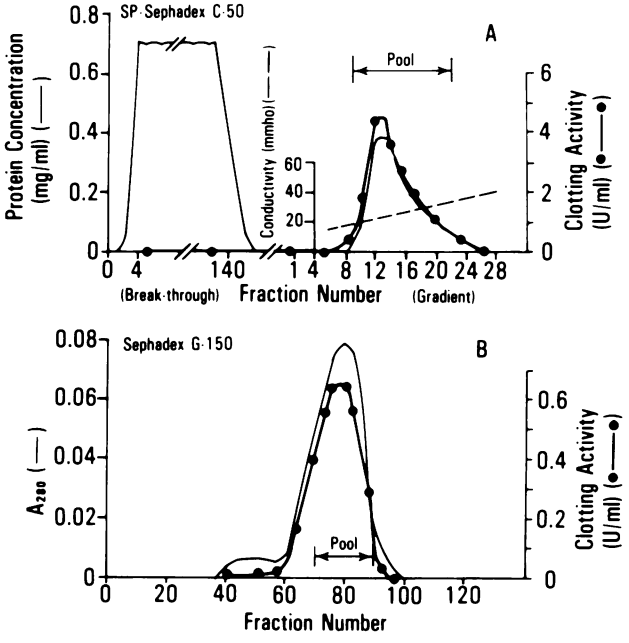

Figure 2—Column chromatographies of Hageman factor. The *thick line* denotes the clotting activity measured in Hageman-factor-deficient human plasma; the *thin line* denotes the protein concentration. **A**—SP-Sephadex column chromatography of Hageman factor pool from PCB-Sepharose column chromatography. The sample, as well as 12 ml of SP-Sephadex C-50 in the column, was equilibrated with 50 mM sodium acetate buffer containing 4 mM benzamidine and 130 mM NaCl (pH 5.3). A linear NaCl gradient elution from 130 mM to 530 mM was used with 80 ml buffer in each reservoir. The *broken line* denotes conductivity in the fractions. **B**—Sephadex G-150 column chromatography of Hageman factor pool from SP-Sephadex column chromatography. Of the concentrated sample, 1.5 ml was gel filtered in the column (bed volume 280 ml, 1.5 cm × 160 cm).

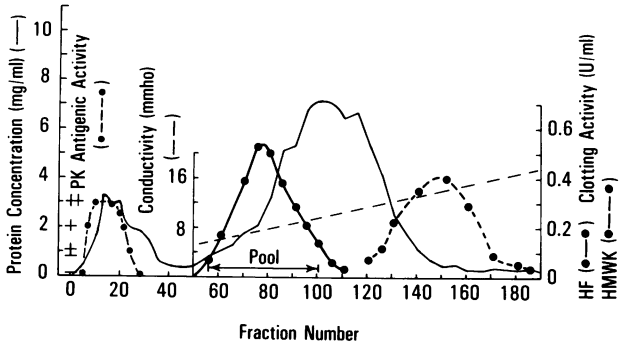

Figure 1—DEAE-Sephadex column chromatography of guinea pig plasma. Five hundred ml of plasma, as well as 500 ml of DEAE-Sephadex A-50 in the column, were equilibrated with 20 mM Tris-HCl buffer containing 3 mM EDTA, 4 mM benzamidine, 50 mg/l polybrene, and 40 mM NaCl (pH 8.0). A linear NaCl gradient elution from 40 mM to 300 mM was used with 4.5 l buffer in each reservoir. The *thick solid line* denotes the clotting activity measured in Hageman-factor-deficient human plasma; the *thick broken line* denotes the clotting activity measured in high-molecular-weight kininogen-deficient human plasma; the *dotted line* denotes the arbitrary units of prekallikrein antigenic activity measured in double immunodiffusion plate; the thin solid line denotes the protein concentration; the *thin broken line* denotes conductivity in the fractions. The double-headed arrow represents pooled fractions for Hageman factor purification.

undetectable for the fraction, which suggested that all the Hageman factor was in a precursor form.

To remove the remaining 5–10% contaminating protein, which was of a significantly higher molecular weight than the Hageman factor, aliquots were gel filtrated with a Sephadex G-150 column after 10–15 times concentration. Recovery of the clotting activity from the column was around 80%. Analysis by SDS-PAGE under nonreducing conditions showed no evidence of visible contaminating protein (Figure 3). The purified Hageman factor showed around 8.5 guinea pig clotting units/mg protein which was equivalent to 85 human clotting units, when the protein concentration was measured by the absorbance of ultraviolet light at 280 nm (A_{280}), and $E^{1\%}_{280}$ = 14.2 was used following the data for bovine Hageman factor.[32] In a few preparations with the Sephadex G-150 column, the fractions showed detectable amidolytic activity and a partially cleaved pattern in SDS-gel electrophoresis in the presence of reducing agents, which sugested that up to 10% of the Hageman fac-

Table 1—Purification of Hageman Factor

Procedure	Volume (ml)	Total protein* (mg)	Total HF activity† (units)	Specific activity (units/mg)	Recovery of activity (%)
Plasma‡	1470	59,290.0	1470.0	0.023	100.0
First DEAE-Sephadex‡	4715	15,864.5	606.8	0.038	41.2
Second DEAE-Sephadex	750	6487.5	262.5	0.040	17.9
Lysine-Sepharose 4B	790	6320.0	213.3	0.034	14.5
PCB-Sepharose 4B	840	5838.0	316.2	0.062	24.6
SP-Sephadex	102	19.4	158.1	8.158	10.8

* Protein concentrations were determined by Bradford's method using bovine serum albumin solution as the standard.

† Contents of Hageman factor measured by the clotting activity in Hageman-factor-deficient human plasma. One unit of the activity is defined as the amount of activity present in 1 ml of normal guinea pig plasma.

‡ The number is the total or average of three separate preparations, which started at 490 ml plasma each. The samples of the three preparations were pooled before the second DEAE-Sephadex column chromatography.

HF = Hageman factor.

tor in these preparations was in a two-chain active form (α-HFa) (Figure 3).

Molecular Weight of Hageman Factor

On the basis of its elution position from the Sephadex G-150 column, the molecular weight of Hageman factor was estimated to be 105,000. It was measured at 76,000 by SDS-polyacrylamide gel analysis in the presence of reducing agent.

Amino Acid Composition of Hageman Factor

The amino acid composition of the guinea pig Hageman factor is shown in Table 2 and compared with the compositions of human,[33,34] bovine,[32] and rabbit[35] factors, which were previously reported. A high degree of similarity, except for higher methionine content in the guinea pig molecule, is observed.

Double Immunodiffusion Test

Normal guinea pig plasma and purified Hageman factor made single precipitation lines against anti-guinea pig factor rabbit or goat serum in immunodiffusion plates. As shown in Figure 4, these precipitation lines, which showed complete identity, were not observed against normal serums. Rabbit serums gave at least 4 times higher titer than the goat serum in a serially diluted immunodiffusion test.

Inhibition of Hageman Factor Clotting Activity by Monospecific Antibody

Guinea pig plasma was incubated with anti-Hageman factor rabbit IgG or normal rabbit IgG fraction for 10 minutes at 22 C, and the remaining clotting activity was measured. As shown in Figure 5, monospecific anti-Hageman factor antibody blocked the clotting activity of the guinea pig plasma, but normal rabbit IgG showed no effect on the activity.

Concentration of Hageman Factor in Guinea Pig Plasma

Guinea pig plasma and purified Hageman factor made immunoprecipitation rings in radial immunodiffusion plates that contained anti-guinea pig Hageman factor goat IgG fraction. The areas of the rings derived from either plasma or Hageman factor were in proportion to the dilution used. By this quantitative immunodiffusion method, with a purified

Figure 3—Guinea pig Hageman factor on 7.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Hageman factor in 2 M KCl-containing buffer from Sephadex G-150 column chromatography was dialyzed against 5 mM phosphate buffer (pH 7.0) containing 0.7% SDS and 3M urea. Seventy μl of the sample (10 μg protein) was boiled for 3 minutes in the absence of reducing agent (*NR*) or in its presence (*R*, final concentration, 7% β-mercaptoethanol) before being run. Protein staining was carried out with Coomassie brilliant blue R-250.

Table 2—Amino Acid Composition of Hageman Factor

	Guinea pig HF* (%)	Human HF† (%)	Human HF‡ (%)	Bovine HF§ (%)	Rabbit HF/ (%)
Lysine	3.65	6.45	4.2 ± 0.2	4.44	3.55
Histidine	5.29	3.57	4.5 ± 0.8	4.61	5.33
Arginine	5.65	5.52	5.9 ± 0.5	5.76	6.54
Aspartic acid	7.06	8.22	6.3 ± 0.8	7.67	7.09
Threonine	5.16	5.58	5.8 ± 0.3	5.55	5.45
Serine	6.53	9.73	8.5 ± 1.5	6.04	7.16
Glutamic acid	11.49	12.02	11.3 ± 0.6	11.41	11.31
Proline	9.55	5.81	8.9 ± 0.9	8.44	10.0
Glycine	8.28	9.71	10.5 ± 2.0	8.27	8.91
Alanine	7.50	7.10	9.2 ± 1.9	8.30	9.30
Cystein	6.18	3.56	4.2 ± 0.8	4.47	2.54
Valine	4.15	5.11	5.4 ± 0.4	5.25	5.60
Methionine	0.91	0.28	0.1 ± 0.1	0.24	0.22
Isoleucine	1.87	2.77	1.4 ± 0.1	2.31	1.80
Leucine	9.05	8.34	7.6 ± 0.9	8.77	9.85
Tyrosine	3.09	2.71	2.8 ± 0.2	2.31	2.31
Pheylalanine	3.31	3.54	2.9 ± 0.1	3.71	3.02
Tryptophan	1.36	NR	NR	2.44	NR
Total	99.99	100.02	99.5	99.99	99.98

* Cystein was determined as cysteic acid. See Materials and Methods for a detailed description.
† Reported by Revak et al.[33]
‡ Reported by McMillin et al.[34]
§ Reported by Fujikawa et al.[32]
‖ Reported by Griffin and Cochrane.[35]
HF = Hageman factor; NR = not reported.

Hageman factor solution as a standard and with $E^{1\%}_{280}$ = 14.2 for protein determination, 1 ml of guinea pig plasma was found to contain 120 μg Hageman factor.

By comparison of the clotting activity of plasma with that of purified Hageman factor, it was calculated that guinea pig plasma contains 115 μg Hageman factor/ml.

Preparation and Characterization of an Active Form Hageman Factor, β-HFa

Purification and Electrophoretic Analysis of β-HFa

β-HFa was generated from Hageman factor by guinea pig plasma kallikrein immobilized on CM-Sephadex beads and was further purified by DEAE-Sephadex column chromatography (see Materials and Methods). By this method, 200 μg of β-HFa was obtained from 600 μg of Hageman factor. SDS-PAGE analysis of β-HFa is shown in Figure 6. In the absence of reducing agents, a single band corresponding to an apparent molecular weight of 31,000 was observed. After reduction, a single protein band with an apparent molecular weight of 28,000 was observed. In alkaline disc gel, β-HFa migrated in the prealbumin region and, as shown in Figure 6, an electrophoretical microheterogeneity was observed.

Double Immunodiffusion Assay of β-HFa

As shown in Figure 7, the purified Hageman factor and β-HFa both gave single precipitation lines against anti-guinea pig Hageman factor rabbit IgG fraction in an immunodiffusion plate. Since the precipitation line derived from β-HFa was fused with the line derived from Hageman factor, and the latter made a spur directed toward the β-HFa-containing well, it was shown that the β-HFa molecule contained some but not all of the antigenic sites of the parent Hageman factor molecule.

Amino Acid Composition of β-HFa

Amino acid compositions of guinea pig and human β-HFa are shown in Table 3 and compared with guinea pig prekallikrein activator, which was previously reported by Treloar et al.[22] A high degree of similarity is observed between guinea pig β-HFa and human β-HFa. Although human β-HFa contains fewer lysine residues than the guinea pig molecule, it seems to be compensated by a higher content of arginine residue. The degree of similarity between numbers of residues in these two β-HFa preparations is greater than that of the guinea pig prekallikrein activator of Treloar.

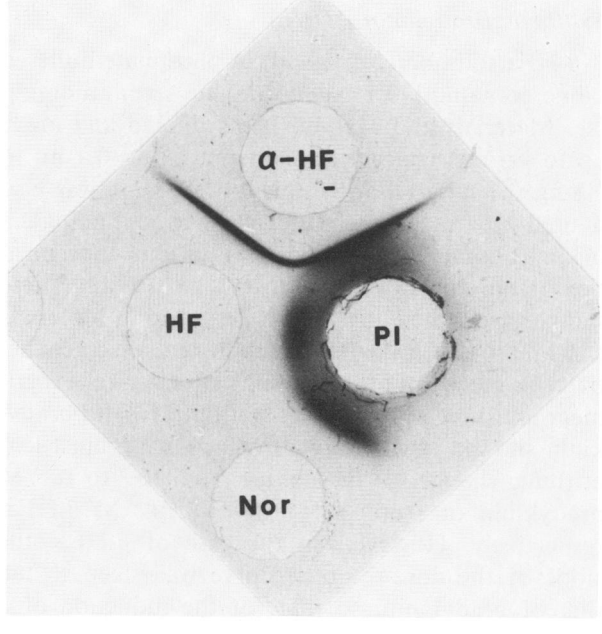

Figure 4—Double immunodiffusion reactions of Hageman factor and guinea pig plasma against rabbit anti-guinea pig Hageman factor serum. One percent agar was used. α-HF, rabbit anti-guinea pig Hageman factor serum (8 μl); HF, purified guinea pig Hageman factor (2 μg/10 μl); PI, guinea pig plasma (15 μl); Nor, normal rabbit serum (15 μl). In addition to specific precipitation lines, a nonspecific broad precipitate was observed around the well containing plasma not only in the specific antiserum part but also in the normal rabbit serum part. It was difficult to detect in either part before protein staining of the plate.

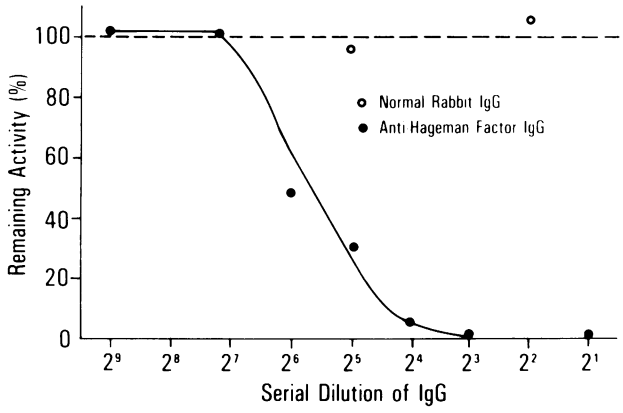

Figure 5—Inhibition of clotting activity of Hageman factor in plasma by rabbit anti-guinea pig Hageman factor IgG. Ten μl of guinea pig plasma diluted three times was mixed and incubated for 10 minutes at 22 C with 10 μl of serially diluted rabbit anti-guinea pig Hageman factor IgG fraction or normal rabbit IgG fraction. After 100-times dilution with 980 μl of Tris-buffered saline containing 1 mg/ml bovine serum albumin, the remaining clotting activity was measured in Hageman-factor-deficient human plasma. For calculation of the percentage inhibition, a calibration curve was used in which the log of the plasma dilution was plotted against the log of the clotting time. Protein concentration of neat IgG fractions used for inhibition was 50 mg/ml.

Properties of βHFa as a Permeability Enhancement Factor

Dose-Response Activity and Comparison with Synthetic Bradykinin Activity

β-HFa solution (2.5 μg/ml) in phosphate-buffered saline containing 0.1 mg/ml bovine serum albumin (see Materials and Methods) was diluted and made up to various concentrations from 25 ng/0.1 ml to 0.8 ng/0.1 ml β-HFa. Synthetic bradykinin was also diluted with phosphate-buffered saline containing 0.1 mg/ml bovine serum albumin at various concentrations from 100 ng/0.1 ml to 0.1 ng/0.1 ml. These samples were then assayed, as described in Materials and Methods, for their permeability enhancement activity in guinea pig skin. Results of a typical experiment are shown in Figure 8. Both β-HFa and bradykinin showed linear changes of activity against logarithmic changes of the dosage from 10^{-9} to 10^{-6} M bradykinin or from 3×10^{-10} to 10^{-8} M β-HFa, respectively. However, in the case of β-HFa, the slope of the dose-response curve was steeper than that of bradykinin, so that for the induction of a given strong permeability enhancement phenomenon, 10 to 100 times more bradykinin was required than β-HFa.

In contrast with these studies, only negligible permeability activity was observed when the zymogen form of Hageman factor was assayed at concentrations between 2×10^{-10} and 2×10^{-7} M (Figure 8).

In Vivo *Duration of the Permeability Activity*

The duration of the permeability enhancement activity of β-HFa after intradermal injection was tested by giving intravenous dye to animals bearing β-HFa lesions of varying times up to 75 minutes. The activity of β-HFa was short lasting. As shown in Figure 9, by 5 minutes the activity decreased to 60% of the maximum dye leakage, and by 15 minutes less than 2% of the maximum dye leakage was observed.

Susceptibility of the Permeability Activity to Trypsin Inhibitors

The permeability activity of β-HFa was inhibited by Dip-F and leupeptine. By treatment with Dip-F at concentrations of 1 mM, 0.1 mM, or 0.01 mM, 3%, 40%, or 82% of the dye leakage induced by untreated material was observed, respectively. 0.05 mM leupeptine resulted in a drop of dye leakage to 64% of normal.

Permeability Activity of β-HFa in Rabbit Skin

Guinea pig β-HFa, 20 ng/0.1 ml and 200 ng/0.1 ml, or human β-HFa, 50 ng/0.1 ml and 500 ng/0.1 ml, were intradermally injected into 2 rabbits at 2

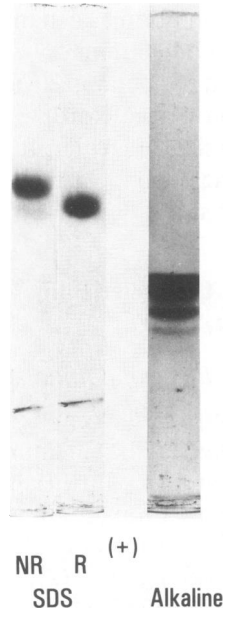

Figure 6—Guinea pig β-HFa on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) or on alkaline polyacrylamide gel electrophoresis. For the SDS-gel electrophoresis 10% polyacrylamide gel was used. Ten μg of β-HFa in 30 μl from the DEAE-Sephadex column chromatography fraction was used for the sample. Gel NR was run in the absence of reducing agent and gel R in its presence (final concentration, 10% β-mercaptoethanol). For the alkaline gel electrophoresis 7% polyacrylamide gel was used at pH 9.5. Fifteen μg of β-HFa in 50 μl was used for the sample. Protein staining was carried out with Coomassie brilliant blue R-250.

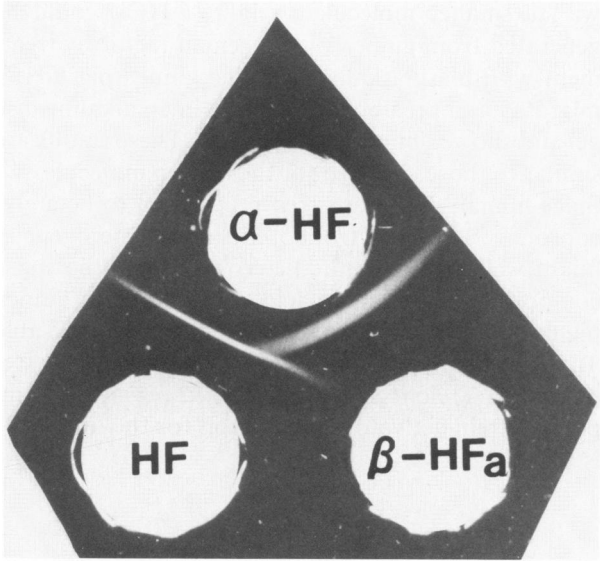

Figure 7—Double immunodiffusion reactions of Hageman factor and β-HFa against anti-Hageman factor rabbit IgG. One percent agar was used. α-HF, rabbit anti-guinea pig Hageman factor IgG fraction (15 μl); HF, guinea pig Hageman factor (2 μg/ml); β-HFa, guinea pig β-HFa (2.5 μg/20 μl).

hours, 1 hour, and 30 minutes before intravenous dye injection, or immediately after dye injection. The skin sites injected 2 hours, 1 hour, and 30 minutes before dye injection showed no permeability reaction. At the skin sites injected immediately after dye injection, faint dye leakage was observed that was similar in quantity to that at the site injected with phosphate-buffered saline. These animals showed permeability response to 100 ng/0.1 ml and 1 μg/0.1 ml of histamine. In this experiment, it was observed that rabbit skin was at least 100 times less sensitive to β-HFa than guinea pig skin for the permeability response.

Discussion

From these data, we can conclude that human or guinea pig β-HFa has potent permeability enhancement activity. As shown in Figure 8, purified β-HFa caused strong permeability enhancement when it was injected into guinea pig skin at concentrations as low as 3×10^{-10} M (0.8 ng/0.1 ml). This result not only verifies previous observations,[13,18,22,23] but also shows a specific activity 100 times stronger than that in the previous reports. From the experiments with the zymogen form of Hageman factor (Figure 8) or Dip-F-treated βHFa, it was shown that only enzymatically active Hageman factor could cause the increased vascular permeability. Although as much as a few percent of the Hageman factor used for the zymogen permeability enhancement studies may have been active HFa of high molecular weight (αHFa), this material showed 1000 times less permeability activity than β-HFa.

The present data on the diminished permeability activity of rabbit skin to β-HFa may explain the lack of permeability of HFa in previous experiments.[20,21] When 20–200 ng of guinea pig β-HFa or 50–500 of human β-HFa were injected into rabbit skin, only small permeability reactions were observed, ie, over 100-fold less activity than with guinea pig skin. However, human β-HFa injected into guinea pig or rhesus monkey skin produced a similar quantity of permeability enhancement (Yamamoto, Cochrane and Revak, unpublished data). The present study does not elucidate the mechanism by which β-HFa causes the increase in vascular permeability.

The capacity of Hageman factor guinea pig plasma to correct the clotting time of Hageman-factor-deficient human plasma was found to be 10 times greater than the capacity of normal human plasma. Among the possible explanations of this observation are that the guinea pig molecule may have a higher specific activity or that Hageman factor may be present in a higher concentration in guinea pig plasma. The present study shows that the concentration of Hageman factor in guinea pig plasma is 120 μg/ml, as estimated by quantitative radial immunodiffusion. This is 4 times greater than the concentration of Hageman factor in human plasma (29

Table 3—Amino Acid Composition of β-HFa

	Guinea pig β-HFa (%)	Human β-HFa (%)	Guinea pig PKA* (%)
Lysine	2.16	0.83	5.3
Histidine	3.77	3.78	1.6
Arginine	4.29	6.58	2.4
Aspartic acid	6.02	6.08	2.5
Threonine	4.47	4.03	2.5
Serine	9.82	7.06	9.1
Glutamic acid	12.29	11.54	15.1
Proline	6.41	7.85	6.4
Glycine	10.33	11.44	11.4
Alanine	9.72	11.67	5.7
Cysteic acid	5.03	ND	5.4
Valine	5.55	7.16	4.9
Methionine	0.35	0.39	3.5
Isoleucine	2.76	2.43	3.3
Leucine	12.45	12.84	7.6
Tyrosine	2.65	3.74	10.0
Phenylalanine	1.94	2.57	3.2
Tryptophan	ND	ND	NR
Total	100.01	99.99	99.9

* Guinea pig plasma prekallikrein activator in the report by Treloar et al.[22]

ND = not determined; NR = not reported.

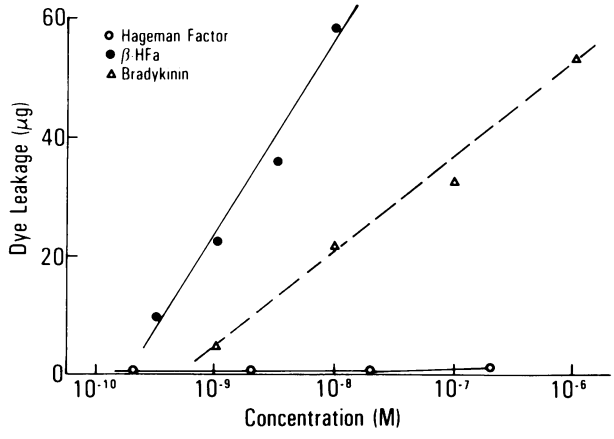

Figure 8—Dose-response curves of Hageman factor, β-HFa, or bradykinin for permeability enhancement activity. One hundred μl of each sample was injected intradermally into the guinea pig immediately after intravenous dye injection. After 20 minutes, blue lesions in the skin were harvested. Dye leakage into extravascular space was measured spectrophotometrically after extraction with formamide.

μg/ml).[33,36] However, the specific activity of the guinea pig molecule, which was 8.5 guinea pig clotting units/mg, or 85 human clotting units/mg, is similar to that reported for the human molecule.[37] Although these results suggested that the stronger clotting activity of guinea pig plasma is partially attributed to the higher plasma concentration, it does not completely explain the 10-fold difference.

The purification procedure described in the current paper yielded guinea pig Hageman factor with physical properties and amino acid compositions similar to those reported for human,[33,34] bovine,[32] and rabbit[35,38] Hageman factors. As with human[37,39] and rabbit[38] Hageman factors (but not with bovine Hageman factor[40,41]), an active form of the molecule

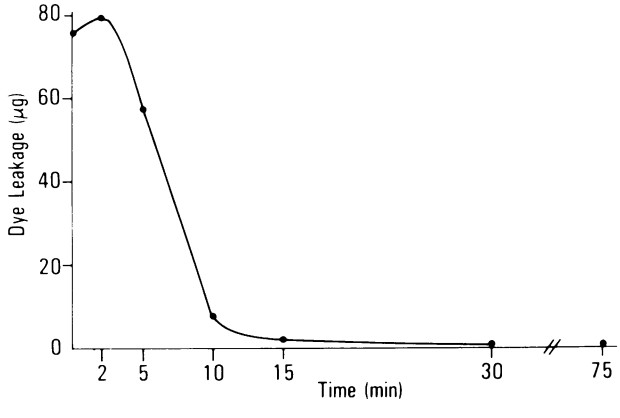

Figure 9—Time course of permeability enhancement phenomenon induced by β-HFa. β-HFa was injected into guinea pig skin at various minutes before intravenous dye injection. Time 0 means an intradermal injection immediately after intravenous dye injection. Skin was harvested 20 minutes after dye injection.

with a smaller molecular weight, β-HFa, could be generated from guinea pig Hageman factor by treatment with plasma kallikrein. The guinea pig β-HFa migrated at a prealbumin position in an alkaline disc gel and showed microheterogeneity. These results are similar to those reported for the human molecule.[24,42] Since human β-HFa was recently shown to be a glycoprotein,[43] the electrophoretic microheterogeneity might well be due to the heterogeneity of the sugar composition. The amino acid composition of guinea pig β-HFa was similar to that of human β-HFa (Table 3), but slightly different from that previously reported by Treloar et al[22] for guinea pig prealbumin prekallikrein activator. The reason for the difference is unclear.

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