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Research Article

Plasma from an individual with a hereditary deficiency of kininogens is deficient in kininogen antigens; heterozygous relatives are partially deficient in plasma kininogen antigens. In addition, plasma from the proband is partially deficient in functional and antigenic properties of a plasma prekallikrein, and the relatives heterozygous for kininogen deficiency are also partially deficient in the plasma prekallikrein. It is possible that the defects are both inherited and that the inheritance of a deficiency of prekallikrein is genetically linked to the inheritance of a deficiency of kininogen. Alternatively, it is possible that the deficiency of prekallikrein may be due to its hypercatabolism which could be a consequence of a deficiency of high molecular weight kininogen that may stabilize the prekallikrein in plasma. Evidence to support this possibility is presented by the fact that prekallikrein and high molecular weight kininogen apparently exist as a complex in normal plasma, because monospecific antiserum to kininogen removed both high molecular weight kininogen and prekallikrein from plasma, and vice versa. Moreover, prekallikrein was not adsorbed from kininogen-deficient plasma by antiserum to kininogen unless high molecular weight kininogen was first added to the plasma. Low molecular weight kininogen did not participate in these reactions.

Prekallikrein Deficiency in a Kindred with Kininogen Deficiency and Fitzgerald Trait Clotting Defect

EVIDENCE THAT HIGH MOLECULAR WEIGHT KININOGEN AND PREKALLIKREIN EXIST AS A COMPLEX IN NORMAL HUMAN PLASMA

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ABSTRACT Plasma from an individual with a hereditary deficiency of kininogens is deficient in kininogen antigens; heterozygous relatives are partially deficient in plasma kininogen antigens. In addition, plasma from the proband is partially deficient in functional and antigenic properties of a plasma prekallikrein, and the relatives heterozygous for kininogen deficiency are also partially deficient in the plasma prekallikrein. It is possible that the defects are both inherited and that the inheritance of a deficiency of prekallikrein is genetically linked to the inheritance of a deficiency of kininogen. Alternatively, it is possible that the deficiency of prekallikrein may be due to its hypercatabolism which could be a consequence of a deficiency of high molecular weight kininogen that may stabilize the prekallikrein in plasma. Evidence to support this possibility is presented by the fact that prekallikrein and high molecular weight kininogen apparently exist as a complex in normal plasma, because monospecific antiserum to kininogen removed both high molecular weight kininogen and prekallikrein from plasma, and vice versa. Moreover, prekallikrein was not adsorbed from kininogen-deficient plasma by antiserum to kininogen unless high molecular weight kininogen was first added to the plasma. Low molecular weight kininogen did not participate in these reactions.

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INTRODUCTION

In plasma from persons deficient in high molecular weight kininogen (HMW-HGN),[1] surface-initiated reactions leading to the formation of clot-promoting activity, of kinins, fibrinolytic, permeability enhancing, and arginine esterase activity are all impaired (1–6). The markedly delayed clotting of plasma from persons deficient in plasma kininogens is not associated with a bleeding tendency (1–6). This coagulation defect can be corrected in vitro with preparations of HMW-KGN whether the plasma is deficient only in certain species of kininogens including HMW-KGN, as in Fitzgerald trait (1, 2), or in all species of kininogen molecules (3), as in Flaujeac (4) and Williams trait (5). The deficiency called Reid trait (6) appears to reflect a partial deficiency of plasma kininogen in which HMW-KGN is deficient, and thus resembles Fitzgerald trait.[2]

Deficiency of Fletcher factor (clot-promoting activity of a plasma prekallikrein) activity in Fitzgerald trait was observed (2, 3, 5, 6), and the studies to be de-

[1] *Abbreviations used in this paper*: Fitzgerald factor refers to clot-promoting activity of a high molecular weight kininogen; Fletcher factor refers to the clot-promoting activity of a plasma prekallikrein; HMW-KGN, high molecular weight kininogen; LMW-KGN, low molecular weight kininogen; PTA, plasma thromboplastin antecedent (factor XI); TAME, *p*-toluene sulfonyl-L-arginine methyl ester.

[2] Lutcher, C. L., and V. H. Donaldson. Unpublished observations.

scribed define a partial deficiency of Fletcher factor clotting activity in plasma from persons heterozygous for kininogen deficiency. When normal plasma was exposed to antibody to kininogen, both Fitzgerald factor (clot-promoting activity of a high molecular weight kininogen) and Fletcher factor clotting activities were removed or neutralized. Antibody to kallikrein also removed both clotting activities from plasma, as if Fletcher factor prekallikrein and Fitzgerald factor (HMW-KGN) exist as a complex in normal plasma.

METHODS

Plasma was prepared from blood drawn into 0.02 vol of pH 5.2 buffered citrate, 0.5 M, using silicone-coated (G. E. DriFilm, SC-87, General Electric Co., Waterford, N. Y.) syringes and test tubes to prevent blood from coming in contact with a glass surface. After separation of plasma by centrifugation, it was rendered platelet-deficient during recentrifugation in a Sorvall RC-2B at 20,000 *g*. Fletcher trait plasma was obtained from Dr. Charles Abildgaard, Department of Pediatrics, University of California at Davis, Calif.; plasma deficient in Hageman factor (factor XII) or plasma thromboplastin antecedent (factor XI) was obtained from Dr. Helen Glueck, Department of Medicine, University of Cincinnati Medical Center, Cincinnati, Ohio. Plasma deficient in kininogens was obtained from members of a kindred reported earlier (3), and was stored at −70°C in small aliquots in polyethylene vessels until used.

Materials used in experimental procedures were obtained as follows: hexadimethrine bromide (Polybrene), tri-sodium ethylene diamine tetraacetate (EDTA), and *p*-toluene sulfonyl-L-arginine methyl ester (TAME) from the Aldrich Chemical Co., Milwaukee, Wis; DE-23 Cellulose and DE-52 Cellulose (preswollen) from H. Reeve Angel, Clifton, N. J.; Centrolex-O phospholipid for partial thromboplastin times from Central Soya, Chicago, Ill.; Sephadex G-200, G-150, CM-Sephadex C-50, QAE-Sephadex A-50, and CNBr-activated Sepharose 4-B from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; kaolin (acid washed) and potassium thiocyanate from Fisher Scientific Co., Pittsburgh, Pa.; crystalline salt-free trypsin from Worthington Biochemical Corp., Freehold, N. J.; Celite 512, from the Johns-Manville Corp., Celite Division, New York; lima bean and soybean trypsin inhibitors, and Tris-HCl from Sigma Chemical Co., St. Louis, Mo.; guanidine hydrochloride from Eastman Kodak Co., Rochester, N. Y. A unit for electrophoresis of 6 × 150-mm polyacrylamide disc gels used for final purification of kininogens was obtained from Savant Instruments, Inc., Hicksville, N. Y., as was the Autogeldivider, used for fractionating these gels; analytical disc electrophoresis was performed with equipment from Shandon Southern Instruments Inc., Sewickley, Pa. Electrophoresis cells used for standard immunoelectrophoresis were obtained from LKB Instruments, Bromma, Sweden. Crystalline bovine serum albumin was obtained from Calbiochem, San Diego, Calif.; polyacrylamide from Bio-Rad Laboratories, Richmond, Calif.; agarose (Indubiose, l'Industrie Biologique Francaise, S. A.) from Fisher Scientific Co., Pittsburgh, Pa.

Kininogens were isolated from normal human plasma at room temperature by modifications of published methods (5, 7–10). Blood was drawn into silicone-coated vessels containing 0.02 vol of 0.5 M citrate buffer, pH 5.2, hexadimethrine bromide to give a final concentration of 50 μg/ml and EDTA to give a final concentration of 0.003 M. The plasma was separated during centrifugation at room temperature and made platelet-deficient by recentrifugation. Initially, 240 ml of plasma was diluted with 3 vol of distilled water and the pH adjusted to 6 with HCl. Then 30 g of DEAE-cellulose (DE-23), which had been washed with water, 0.5 N sodium hydroxide, 0.5 N HCl, distilled water, and then with distilled water containing 50 μg/ml hexadimethrine bromide and 0.003 M EDTA was added while stirring at room temperature. After stirring for approximately 2 h, the cellulose was washed with 4–6 liters of distilled water containing hexadimethrine bromide and EDTA on a suction funnel, and crude kininogens were then eluted in 0.4 M sodium chloride. This kininogen-rich eluate was concentrated by lyophilization, dissolved in distilled water, dialyzed against 0.1 M Tris buffer, pH 8.0, and applied to a 2.5 × 60-cm column of QAE-Sephadex A-50 or of DE-52. After the column was washed with about 750 ml of the Tris buffer, kininogens were eluted with a linear gradient to 1.5 M sodium chloride in the Tris buffer and were identified by their capacity to release kinin after incubation with trypsin. Each pool of kininogen was concentrated by lyophilization, dissolved in distilled water, dialyzed against 0.03 M sodium acetate pH 5.8, and applied separately to a 2.5 × 30-cm column of CM-Sephadex C-50, already equilibrated with the sodium acetate. Kininogens were eluted with a linear gradient to 1.4 M sodium chloride in the acetate buffer, and fractions containing kininogen pooled and concentrated by precipitation in 60% ammonium sulfate. After thorough dialysis against 0.05 M Tris buffer, pH 8, the redissolved precipitates were applied to a 2.5 × 100-cm column of Sephadex G-150 equilibrated with pH 8.0, 0.05 M Tris. Fractions containing kininogen were pooled, and the materials soluble in solutions of 20%, but insoluble at 60% saturation with ammonium sulfate, were recovered, redissolved, and dialyzed thoroughly against Tris-glycine buffer, pH 9.0, used for polyacrylamide disk gel electrophoresis. Preparations of HMW-KGN used in experiments contained two bands of protein in analytical disc gel electrophoresis (Fig. 1), both of which contained Fitzgerald factor clotting activity and released kinin upon exposure to trypsin. Samples of the kininogens from G-150 which still contained other proteins were applied to 6 × 150-mm polyacrylamide disc gels and after electrophoresis at 5 mA per tube for 3 h, the gels were divided with an Autogeldivider into approximately 3-mm sections and each was eluted in pH 7.4 barbital saline buffer, and kininogen-containing fractions were pooled. After polyacrylamide particles were removed, these kininogens were exhaustively dialyzed against the barbital saline buffer. Two fractions containing HMW-KGN were identified by the release of kinin activity upon exposure to trypsin and because each contained Fitzgerald factor clotting activity. The anodal HMW-KGN was used in these experiments because there was a greater amount than in the cathodal fraction. The specific activity of the HMW-KGN prepared in this way was 4 μg bradykinin released/mg of protein by trypsin, but only 0.3 U of Fitzgerald factor activity/mg protein (1 U = the amount in 1 ml of normal plasma). The HMW-KGN shown in Fig. 1 contained 2.5 U of Fitzgerald factor and released 6 μg of bradykinin/mg protein.

The specific activity of two preparations of LMW-KGN used was 16 and 20 μg bradykinin released/mg of protein by trypsin. Preparations of HMW-KGN appeared to have lost some kinin during purification because despite large and comparable amounts of Fitzgerald factor clotting activity, the trypsin-releasable kinin was variable. The preparations of HMW-KGN had a specific activity such that 4–6 μg of bradykinin was released/mg of protein by trypsin. Free

kinin in these preparations was removed by dialysis. Free kinin was not found in kininogen-rich fractions after chromatography on CM-Sephadex C-50. The HMW-KGN did not contain Hageman factor, plasma thromboplastin antecedent (PTA), Fletcher factor clotting activities, alpha-2 macroglobulin, or C1 inhibitor antigens, measured in agarose double immunodiffusion.

All buffers used during the purification of kininogens contained hexadimethrine bromide, 50 μg/ml, and EDTA, 0.003 M, until the preparation was subjected to gel filtration on G-150 Sephadex. All procedures were carried out at room temperature except the precipitation with ammonium sulfate and the elution and dialysis of kininogens purified on polyacrylamide gels, which were carried out at 4°C.

Kallikrein was isolated from normal plasma which had been separated from blood drawn into 0.10 vol of 0.1 M sodium oxalate, according to a published method (11). This plasma was adsorbed with tricalcium phosphate, 10 mg/ml, and the supernatant fluid was adsorbed with 20 mg of Celite/ml of plasma at room temperature. This Celite was washed with cold, distilled water until no visible precipitate occurred upon addition of an equal volume of 10% TCA to the wash. Crude kallikrein eluted from the Celite with 10% sodium chloride in pH 8 Tris buffer, 0.05 M, was passed over a lysine Sepharose column prepared according to the method of Deutsch and Mertz (12) to remove plasminogen, and then applied to a 2.5 × 30-cm column of CM-Sephadex C-50 which had been equilibrated with 0.15 M sodium acetate, pH 5.2. After washing the column with about 200 ml of 0.067 M pH 6.0 sodium phosphate buffer, a linear gradient to 0.5 M sodium chloride was added to the phosphate buffer. Fractions containing kallikrein, identified by their arginine esterase, kinin-releasing, and Fletcher factor clotting activities, were pooled, dialyzed against pH 8, 0.05 M Tris buffer containing 0.0001 M EDTA, and applied to a 2.5 × 35-cm column of QAE-Sephadex, already equilibrated with the Tris-EDTA buffer. Two kallikrein-rich fractions were separately eluted by the addition of a linear gradient to 0.5 M sodium chloride in the 0.05 M Tris-EDTA buffer, both of which contained traces of PTA activity. The first of these peaks, containing the higher concentration of Fletcher factor clotting activity, was concentrated by pressure dialysis over an Amicon UM-10 filter (Amicon Corp., Lexington, Mass.). This kallikrein, eluted from the QAE-Sephadex in solutions of a conductivity of 5.5–7.4 mmho, had a specific activity of 109 μM methanol released/h per mg protein when tested for TAME esterase activity according to the method of Seigelman and associates (13). This pool of concentrated kallikrein was purified during electrophoresis in 7% polyacrylamide disc gels at pH 9 for use as immunogen. One gel of each set was stained and the others sectioned and eluatesl in barbital saline buffer, pH 7.4, according to the proteins stained. Kallikrein activity was identified in those eluated by measuring their capacity to release kinin from normal plasma which had been heated at 61°C for 2 h, and measuring the kinin-releasing activity which was not inhibited by lima bean trypsin inhibitor.

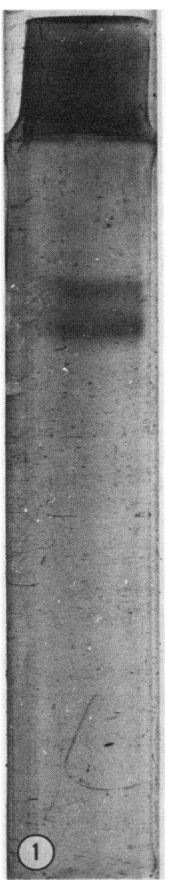

FIGURE 1 A preparation of HMW-KGN, 100 μg, was subject to electrophoresis in 7% polyacrylamide disc gel at pH 9 and stained with Coomassie Brilliant Blue.

Antibody to kallikrein. Kallikrein-rich fractions eluted from polyacrylamide disc gels were pooled, mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and injected into the foot pads of lightly anesthetized rabbits at weekly intervals, until the rabbit serum developed activity which blocked Fletcher factor clotting activity of normal human plasma. The serum obtained from rabbits at this time was absorbed with Fletcher trait plasma to render it monospecific; the adsorbed antiserum gave a single precipitin line when reacted with normal plasma in agarose gel, but no band of reaction with the Fletcher trait plasma. The monospecific antiserum was heated to 61°C for 2 h, adsorbed with tricalcium phosphate, 10 mg/ml of serum. The gamma globulin fraction was isolated after adjustment of the pH of this serum to 4.8 with 0.1 M sodium acetate (pH 4). 1 vol of octanoic acid (Eastman Kodak Co., Rochester, N. Y.) was then added to each 10 vol of this pH 4.8 solution (14). After centrifugation, the supernatant fluids were filtered through a Whatman no. 1 filter (Whatman Inc., Clifton N. J.) on a Buchner funnel, and the precipitate washed on this filter with about 50 ml of the pH 4 sodium acetate. The filtrates were combined, and the pH adjusted to 7.4 with 0.1 N sodium hydroxide, and the materials precipitated at 50% saturation with ammonium sulfate were recovered, dialyzed extensively against running water and then against barbital saline buffer. These precipitated antibody globulins to kallikrein were used in experimental procedures.

Plasma prekallikrein and kallikrein were quantified immunologically with a rabbit antihuman prekallikrein which gave a precipitin reaction with both kallikrein and prekallikrein. A sensitive radioimmunoassay was used (15) to measure the low levels of prekallikrein in some of the studies to be described. Plasma prekallikrein can be quantified at concentrations as low as 0.3% of the concentration found in normal pooled plasma with this assay.

Antibody to kininogen. This was prepared with mixtures of

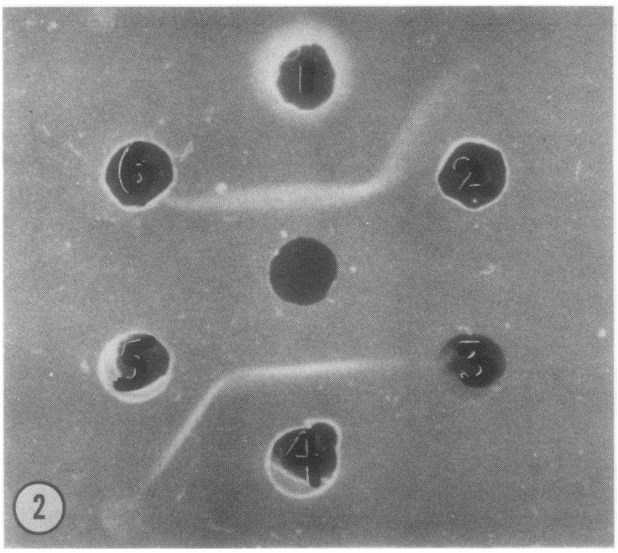

FIGURE 2 The center well contained sheep antibody to LMW-KGN from Dr. Jack Pierce. Contents of other wells from the top and clockwise: 1, normal human plasma; 2, goat antihuman kininogen prepared as described; 3, kininogen-deficient plasma; 4, Fitzgerald plasma; 5, goat antihuman kininogen as in 2; 6, kininogen-deficient plasma.

HMW-KGN that had been purified on polyacrylamide disc gels, mixed with an equal volume of complete Freund's adjuvant and residual polyacrylamide. These mixtures were injected intramuscularly into the back of a goat at weekly intervals until serum obtained could delay the clotting of normal plasma and deplete normal plasma of Fitzgerald factor clotting activity. This antibody was not monospecific, but after adsorption with kininogen-deficient plasma (3), the antiserum was monospecific, and gamma globulin fractions were prepared just as for antibody to kallikrein. The monospecific antibody gave a reaction of complete identity with a sheep antibody to human LMW-KGN prepared by Dr. Jack Pierce, National Institutes of Health, Bethesda, Md., when these preparations were reacted against normal human plasma and Fitzgerald trait plasma in agarose gel double diffusion (Fig. 2). When this antibody was reacted with the preparations of HMW-KGN and LMW-KGN in agarose gels, the precipitin reactions gave bands of complete identity between both kininogens and normal plasma (Fig. 3), but no precipitin reaction with plasma from the proband deficient in all species of kininogen molecules described earlier (3).

Protein concentrations in purified reagents were determined by comparing optical density at 280 nm in a Gilford 240 spectrophotometer, with the optical densities of a standard curve of dilutions of a solution of a weighed amount of crystalline bovine serum albumin. Antibody globulins to kininogens and Fletcher factor kallikrein were coupled to Sepharose 4-B activated according to the method of March et al. (16) to provide insoluble antibody.

Specific assays for coagulant activities were carried out by testing the capacity of test solutions to correct the clotting defect of plasma from an individual with a severe inherited deficiency of the factor in question, using an activated partial thromboplastin time (17) and procedures detailed earlier (2). Volumes of 50 μl of each reactant, instead of 100 μl of each, were used in performing these assays. The percent of clotting activity was derived by comparing the clotting times of at least two dilutions of test plasma to a double logarithmic plot of the clotting times of four dilutions of a pool of 10 normal plasma samples. The buffer used in coagulation assays contained 2.76 g barbital, 2.06 g sodium barbital, and 7.3 g sodium chloride/liter.

Electroimmunodiffusion was carried out in 1% agarose gels containing antibody globulin to kininogen in a concentration of 3 ml/100 ml solution, using the procedure of Laurell (18). Immunoelectrophoresis was carried out with a 1% agarose medium at pH 8.6 in barbital buffer containing calcium lactate (19).

Tryptic digestion of kininogen containing fractions was carried out at pH 8 using 50 or 100 μg of trypsin/ml of 0.1 Tris buffer. The mixtures were incubated at 37°C for 30 min or more and an amount of soybean trypsin inhibitor, which slightly exceeded the amount of trypsin used, was added before assay. The assay for kinin activity was carried out at 29–31°C with a uterine horn from a rat in which estrus had been induced 18–48 h earlier by intraperitoneal injection of 25 μg diethylstilbestrol (Eli Lilly and Company, Indianapolis, Ind.) in an 8-ml organ bath containing de Jalon's buffer (20).

RESULTS

Monospecific goat antiserum to human kininogens gave precipitin reactions with both HMW-KGN and

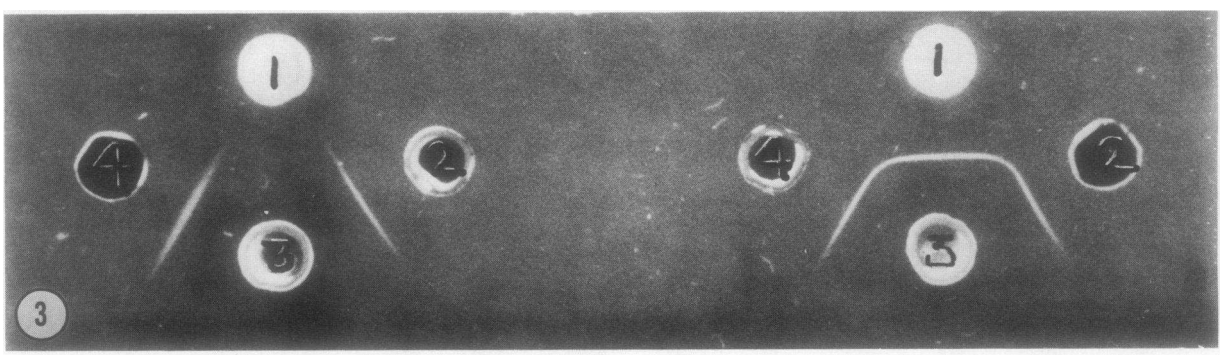

FIGURE 3 *Left*: well 1 (top), kininogen-deficient plasma; well 2 (right), LMW-KGN; well 3 (lower), goat antihuman kininogen prepared as described; well 4 (left), HMW-KGN. *Right*: well 1, normal human plasma; wells 2, 3, and 4 as on the left.

LMW-KGN in agarose (Fig. 3), indicating that these proteins share common antigenic determinants, as reported earlier (21). The plasma from the proband deficient in kininogen gave no precipitin reaction with this antiserum, confirming our earlier report (3) that this plasma is deficient in all species of kininogen molecules. The antiserum gave reactions of complete identity between the kininogen preparations and normal human plasma (Fig. 3), and in immunoelectrophoretic studies, two precipitin arcs of identical electrophoretic mobility were observed (Fig. 4). It is likely that these arcs are due to kininogens of differing diffusability, perhaps because of differing molecular size or because of the existence of complexes between some plasma kininogens and other proteins such as prekallikrein (*vide infra*). Consistent with the latter possibility was the observation that a single precipitin arc was observed in the immunoelectrophoretic study of plasma from patient Fitzgerald (Fig. 4), deficient in HMW-KGN but not in LMW-KGN. Thus, this antibody appeared to react with kininogens, but not with other plasma components. When this antiserum was used to quantify the kininogen antigens in plasmas from members of the kindred with kininogen deficiency (3), those who were partially deficient in Fitzgerald factor clotting activity were also partially deficient in plasma kininogen, as shown in Table II and Fig. 5, for example. Plasma obtained from the proband at different times was essentially void of kininogen antigens (Fig. 5) as well as Fitzgerald factor clotting activity.

Prekallikrein deficiency associated with hereditary kininogen deficiency. Samples of the proband's plasma obtained at different times contained from 12.5 to 35% of the normal amount of Fletcher factor clotting activity. Neither the deficiency of Fletcher factor nor Fitzgerald factor clotting activity could be attributed to an inhibitory property in plasma of the proband, for his plasma did not delay the clotting of normal plasma when the two were incubated together.

When plasma from other members of this kindred were tested for Fletcher factor clotting activity, each plasma which was partially deficient in kininogen contained less Fletcher factor activity than the mean value found in normal plasmas (Fig. 6 and Table I). Although different plasma samples from the proband contained from 12.5 to 35% of the normal amount of Fletcher factor clotting activity, relatives who appeared to be heterozygous for kininogen deficiency had between 29 and 71% of the normal plasma concentrations of Fletcher factor activity (Table II).

When prekallikrein antigen concentration was measured by a radial immunodiffusion technique (22), its concentration in plasma from the proband was 71% of that in normal plasma which far exceeded the quantity of Fletcher factor activity. This could

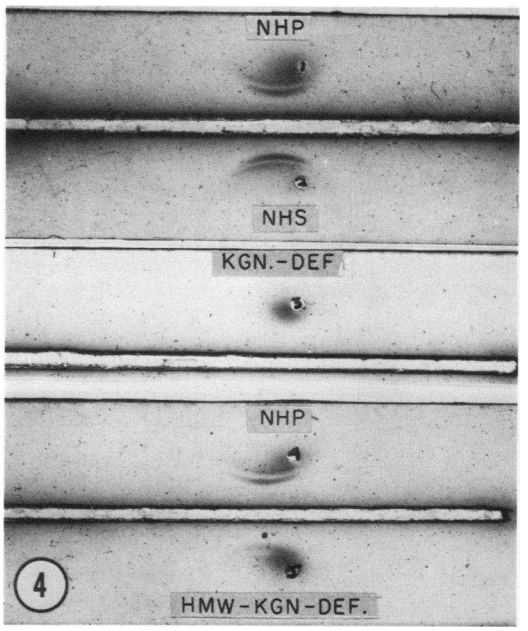

FIGURE 4 Immunoelectrophoretic comparison of kininogen in normal plasma (well 1), normal serum (well 2), kininogen-deficient plasma (well 3), normal plasma (well 4), and plasma from Mr. Fitzgerald containing kininogen lacking clotting activity (well 5).

have been due to the presence of normal amounts of poorly functioning abnormal prekallikrein protein, or to a form of prekallikrein which contained more antigenic determinants than that in normal plasma. To confirm this discrepancy, the concentration of prekallikrein antigens in plasma from members of this kindred were determined with a sensitive radioimmunoassay (Table II, column 4).

In a sample from the proband, the concentration of Fletcher factor clotting activity was 35% of that in normal plasma, and the prekallikrein antigen concentration was 32% of normal. Thus, the concentrations of prekallikrein clotting activity and protein paralleled one another. In plasma from heterozygous members of the kindred, the Fletcher factor clotting activity ranged from 29 to 71% of normal (mean = 50.2±18%; $P = <0.005$; see Table II, column 2), and prekallikrein antigen concentration ranged from 62 to 92% of normal (mean = 75±12.6%; $P = <0.1$). In most instances, there was a disproportionately high concentration of antigen. The significance of the difference between the clotting and antigen concentrations in the group of heterozygotes, however, is doubtful ($P = <0.3, >0.2$).

It was possible that this prekallikrein required HMW-KGN in order to function and that the deficiency of Fletcher factor activity was a manifestation of the kininogen deficiency. Therefore, the effect of kininogen upon the assay for Fletcher factor activity was

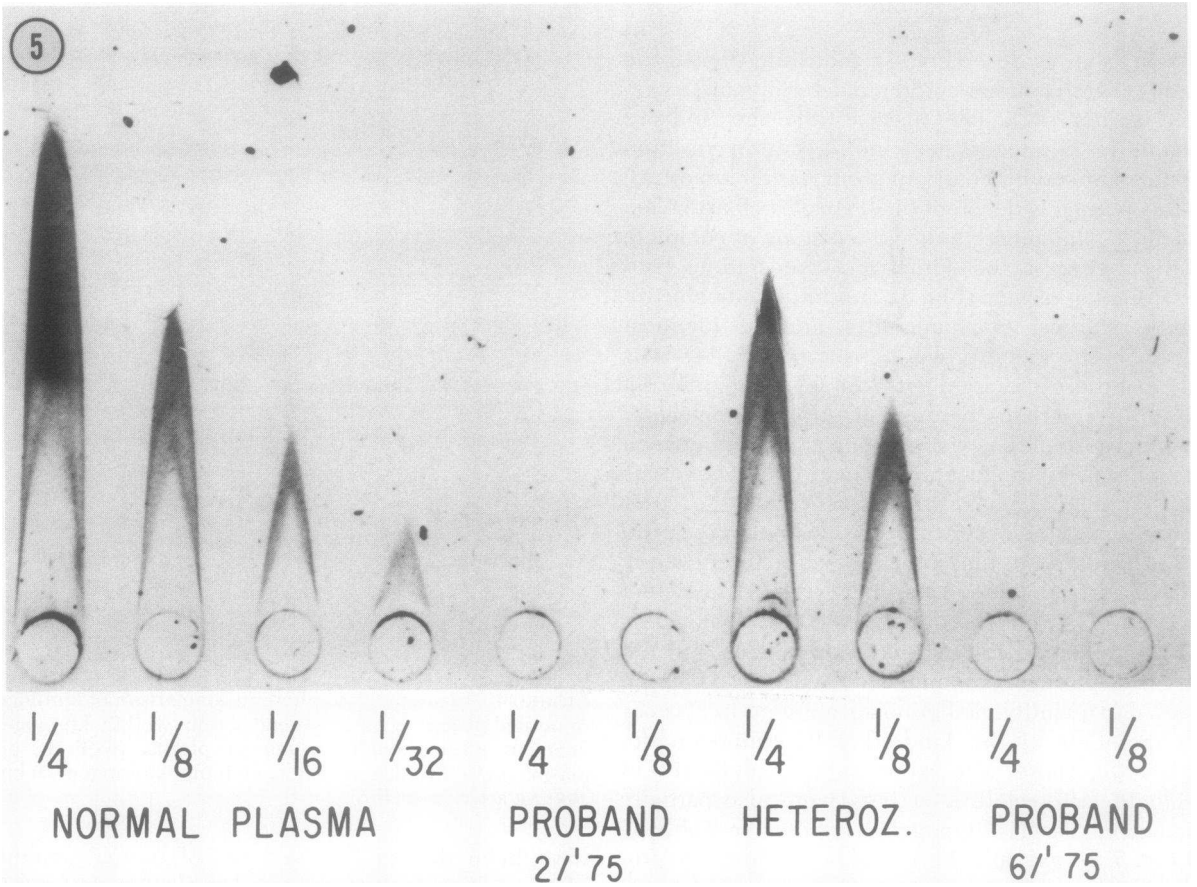

FIGURE 5 Kininogen antigens were quantified in plasma samples diluted in barbital saline buffer as noted. After electrophoresis of 7 μl of each dilution into 1% agarose containing 3% monospecific sheep antihuman kininogen gamma globulin, the plates were washed, dried, and stained with Coomassie Brilliant Blue. The concentration of kininogen proteins is proportional to the length of the "rocket" precipitin reactions. Individuals are numbered according to the pedigree shown in Fig. 6.

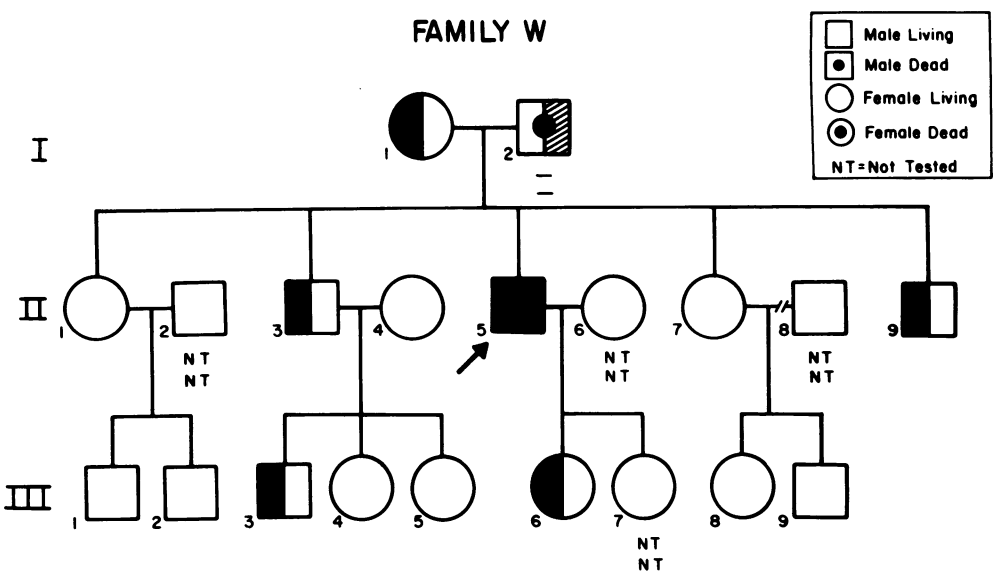

FIGURE 6 Pedigree of family W.

TABLE I
Fletcher Factor Clotting Activity in Kininogen-Deficient Plasmas

Sample of plasma	Clotting time
	s
Normal plasma pool	
1/40	87
1/80	124
1/160	160
Kininogen-deficient proband (II-5) 1/20	141
Kininogen-deficient heterozygote (II-3) 1/20	103
Kininogen-deficient heterozygote (I-1) 1/20	88
Buffer	260

The concentrations of Fletcher factor clotting activity were determined by incubating 0.05 ml of a mixture of kaolin (10 mg/ml) suspended in 0.1% Centrolex-0 in 0.15 M sodium chloride with 0.05 ml of Fletcher trait plasma and 0.05 ml of the test dilutions shown at 37°C for 1 min. Then, 0.05 ml of 0.025 M calcium chloride was added, mixed, and the clotting time measured.

tested. When a highly purified preparation of HMW-KGN was incubated with the proband's plasma before measuring Fletcher factor activity, the amount of Fletcher factor activity did not increase.

To determine whether deficient prekallikrein function in this plasma was associated with a structural alteration of the molecule causing a change in its net charge, plasma from the proband was examined by immunoelectrophoresis with a monospecific rabbit antibody to human prekallikrein which gave a faint precipitin band when reacted against normal plasma in agarose. The prekallikrein precipitin arc in the kininogen-deficient plasma extended into a more cathodal region than that in normal plasma, but was a single arc. The addition of a preparation of HMW-KGN to this plasma before electrophoresis did not change the position of the precipitin arc; LMW-KGN also failed to change the position of this arc. When a mixture of equal parts of Fletcher trait plasma (containing normal amounts of kininogens) and plasma from the proband was examined in this way, the faint precipitin arc designating prekallikrein appeared to be in a more anodal position resembling that seen in normal plasma. Thus, the prekallikrein antigens in kininogen-deficient plasma appeared to have interacted with the HMW-KGN, or some other component of the Fletcher trait plasma so that it assumed a normal electrophoretic mobility. The preparation of purified HMW-KGN may have been partially denatured during its purification so that it did not interact with plasma prekallikrein under conditions of this procedure. Alternatively, another plasma substance may be involved.

Another approach that was used to identify the prekallikrein deficiency in kininogen-deficient plasma was to measure the *p*-toluene sulfonyl-L-arginine methyl esterase (TAME) activity which developed after exposure of plasma to kaolin. As reported earlier (1, 4, 5), plasma markedly deficient in kininogen developed virtually no TAME esterase activity upon exposure to kaolin. Plasma from heterozygous relatives, partially deficient in kininogen and in functional prekallikrein, developed less esterase activity than did normal plasma so treated (Fig. 7 A).

When purified preparations of HMW-KGN were added to the kininogen-deficient plasma before agitation with kaolin, the amount of TAME esterase activity which evolved after 10 min was increased but the increase apparent after about 1 min was insignificant (Fig. 7 B); the early burst of esterase activity

TABLE II
Clotting Activities and Antigen

	Clotting activities		Antigen	
	Fitzgerald factor	Fletcher factor	KGN	Prekallikrein
	% of normal			
Homozygote				
II-5	0	12.5–35	0	32*
Heterozygotes				
I-1	50	71	45	72
II-3	45	33	39	62
II-9	46	71	50	92
III-3	55	29	24	72
III-6	32	47	41	79
Unaffected				
II-1	156	112	100	92
II-7	60	111	54	NT‡
III-1	99	116	NT	NT
III-2	84	93	NT	100
III-4	100	55	NT	NT
III-5	84	90	NT	NT
III-8	80	52	NT	NT
III-9	100	NT	NT	NT
Normal				
Pool I	100	100	NT	NT
Pool II	100	90	100	88
Individuals, normal		104±22.8		104±18§

The Fletcher and Fitzgerald factor concentrations were determined in a coagulation assay and are expressed as a percent of the concentration in a pool of 10 normal plasmas. The concentrations of kininogen shown were measured immunologically by the method of Laurell (18), as shown in Fig. 5. The concentration of prekallikrein antigen was measured in a radioimmunoassay (15).

* Fletcher factor clotting activity of this sample was 35% of that of normal pool.

‡ Not tested.

§ Determined in plasma from 40 normal individual adults.

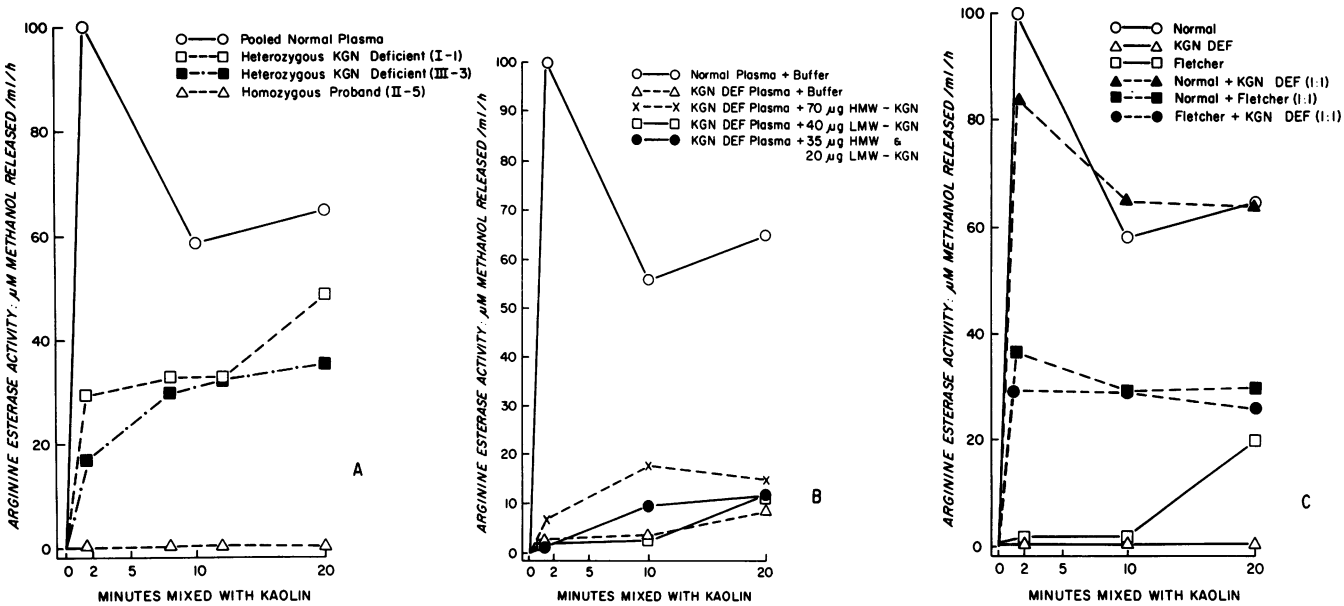

FIGURE 7 TAME esterase activity which evolved in plasmas during continuous agitation with kaolin was measured as methanol generated (13), shown on the ordinates. Mixtures of 0.15 ml of kaolin suspension (10 mg/ml of buffer) and 0.15 ml of plasma or mixtures of plasma and kininogens or buffer were agitated at room temperature for the time intervals shown on the abscissae and then 0.25 ml of TAME (42 mg/ml) was added and the mixtures transferred to a 37°C bath for 30-min incubation. Then, 1.0 ml of 10% TCA was added, and after 15 min or more at 4°C, the precipitates were removed by centrifugation, and methanol formation measured in the supernatant fluids as noted (13). Incubation and assay mixtures were buffered with 0.1 M phosphate in 0.15 M sodium chloride at pH 7.4. A. Kaolin-induced TAME hydrolysis in normal plasma, plasma from two individuals partially deficient in kininogen (heterozygotes) and the kininogen-deficient proband. B. The effect of adding 70 μg of HMW-KGN, 40 μg of LMW-KGN (measured as protein), or of equal parts of each KGN upon the TAME esterase activity of plasma from the kininogen-deficient proband. C. A comparison of the TAME esterase activity initiated by kaolin in normal, kininogen-deficient, and Fletcher trait plasmas with that which evolved in the mixtures shown. The mixture of normal and kininogen-deficient plasma contained 68% of the normal amount of Fletcher factor; the mixture of normal and Fletcher trait plasma contained about 50% of the normal amount of Fletcher factor, and equal parts kininogen-deficient and Fletcher trait plasma contained 16% of the Fletcher factor concentration in normal plasma.

found when normal plasma was treated with kaolin (11), apparently due largely to kallikrein, was not restored with this kininogen despite the fact that the amount of the kininogen preparation used shortened the clotting time of kininogen-deficient plasma from 400 to 50 s. Thus, the plasma behaved as if it were deficient in prekallikrein. A purified preparation of LMW-KGN, of even higher specific activity but similar protein concentration, did not correct defective kaolin-initiated TAME esterase activity in kininogen-deficient plasma. When mixtures of HMW and LMW-KGN were added to this plasma, the increase in TAME esterase activity after 10 min agitation with kaolin was as would be predicted if this dilution of HMW-KGN alone were added to the plasma (Fig. 7 B). Surface-initiated TAME esterase activity was deficient in plasma mixtures which were partially deficient in Fletcher factor (Fig. 7 C) and in plasma from heterozygous members of this kindred (Fig. 7 A). The mixture of normal plasma and Fletcher trait plasma with significantly decreased esterase activity contained 50% of the normal concentration of prekallikrein; the mixture of normal and kininogen-deficient plasma contained 68% of the normal amount of prekallikrein (Fig. 7 C). When a mixture of equal parts of Fletcher trait and kininogen-deficient plasma was tested (Fig. 7 C), the amount of esterolytic activity found after 2 min was 30% of that in normal plasma, as if the HMW-KGN in the Fletcher trait plasma had facilitated the activation of the prekallikrein in the kininogen-deficient plasma.

Evidence that prekallikrein and HMW-KGN exist in normal plasma as complex. These observations suggested a close relationship between prekallikrein and HMW-KGN in normal plasma. To test this more specifically, normal plasma was adsorbed with

antibody globulins directed against kininogen or kallikrein and the residual coagulant activity of both of these proteins was measured. In repeated experiments, solutions of antibody to kininogen depleted normal plasma of both Fitzgerald factor and Fletcher factor clotting activities (Table III). Conversely, antibody to kallikrein removed both Fletcher factor and Fitzgerald factor clotting activities from normal plasma (Table III). Hageman factor and PTA activities were not removed by either procedure (Table III). When insoluble antikininogen globulin complexed to agarose beads was used, the normal plasma was again depleted of both Fitzgerald and Fletcher factor clotting activities (Table III), but no Hageman factor or PTA was removed. Thus, soluble or insoluble preparations of either monospecific antibody depleted normal plasma of both Fletcher and Fitzgerald factor

TABLE III

Removal of Both Fletcher and Fitzgerald Factor Clotting Activities from Normal Plasma with Antibody to Kininogen or Kallikrein

Antibody	Dilution	Activity removed: Fitzgerald factor	Fletcher factor	Hageman factor	PTA
			%		
Antikallikrein globulin solution	½	60	68	0	0
	¼	17	20	<10	0
Antikininogen globulin solution	0	35	30	0	—
Sepharose-antikininogen		40	62	0	0
Sepharose-antikallikrein		44	45	<10	—

The amount of activity attributable to each coagulation factor that remained after normal plasma was adsorbed with antibody preparations shown was determined in clotting assays described, and the quantity removed by this procedure calculated therefrom. Normal plasma was mixed with dilutions of goat antihuman kininogen gamma globulin or rabbit antihuman kallikrein gamma globulin, inclubated at 37°C for 1 h and at 4°C overnight, and the immune precipitates removed by centrifugation. The residual coagulant activities were measured by comparison to a double logarithmic plot of the residual activity in the same normal plasma that had been incubated alone and then diluted with barbital saline buffer. To test the effect of insoluble antibody, 0.5 ml of normal plasma was mixed with 0.5 ml of Sepharose-antikininogen or Sepharose-antikallikrein gamma globulin which had been washed with barbital saline buffer, centrifuged, and free buffer removed from the surface of the sedimented layer before adding the normal plasma. The mixtures were stirred gently in a small magnetic stirrer for 2 h at room temperature, chilled for 1 h and supernatant recovered for assay procedures after centrifugation at 4°C.

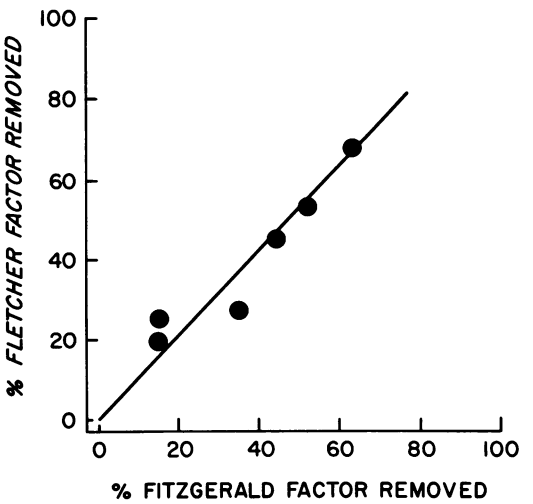

FIGURE 8 Adsorption of normal plasma with Sepharose antikallikrein. A summary of results from six different experiments in which normal human plasma was absorbed with variable quantities of antibody to kallikrein rendered insoluble by binding to cyanogen bromide activated Sepharose 4-B (see Methods). After removing the insoluble phase by centrifugation, residual Fletcher and Fitzgerald factor clotting activities were determined. Ratios of volumes of plasma to Sepharose-antikallikrein ranged from 2:1 to 1:2. In a control mixture, plasma was adsorbed with an amount of Sepharose-antikallikrein which would remove over 60% of these clotting activities from normal plasma, but which had first been incubated with normal plasma to cover antibody binding sites. Less than 10% of these clotting activities were removed from fresh normal plasma after treatment with this pretreated Sepharose-antikallikrein, and this was presumably due to dilution by buffer trapped in the insoluble phase.

clotting activities, suggesting that they may exist as a complex in the normal plasma. When kininogen-deficient plasma from the proband was adsorbed with antibody to kininogen, the small amount of the Fletcher factor clotting activity present was not removed. When Fletcher trait plasma was adsorbed with antibody to kallikrein, the Fitzgerald factor clotting activity was not removed. Therefore, the depletion of both Fletcher and Fitzgerald factor clotting activities by antibody directed against either protein was not a nonspecific effect of the antibody globulins, or of the Sepharose.

The quantitative relationship between the amounts of Fitzgerald factor and of Fletcher factor activities removed from normal plasma with insoluble antikallikrein is shown in Fig. 8. The curve illustrating the percent of the total Fletcher and Fitzgerald factor clotting activities removed from pooled normal plasma in six different experimental mixtures indicates a linear relationship. This relationship is difficult to demonstrate with antibody to kininogens, for it reacts with kininogens which do not have Fitzgerald factor

activity, as well as the HMW-KGN which does have this activity (Fig. 3). When a preparation of HMW-KGN was added to kininogen-deficient plasma, however, subsequent adsorption of the mixture with antikininogen resulted in depletion of the plasma of its prekallikrein (Table IV), which did not occur when LMW-KGN had been mixed with the plasma. Therefore, the added HMW-KGN seemed to associate with the plasma prekallikrein in this plasma.

To identify kallikrein and HMW-KGN complexed with insoluble antibody, Sepharose-antikininogen that had been exposed to normal plasma was washed 10 times with barbital saline buffer, eluted with an equal volume of 8 M guanidine hydrochloride at room temperature for 10 min, and the eluate dialyzed for 5 days against multiple changes of barbital saline buffer. This eluate contained both kininogen and kallikrein antigens detectable in agarose gels with monospecific antibody to each protein but only small amounts of Fitzgerald factor and Fletcher factor clotting activities were found. In a similar experiment, Sepharose-antikallikrein was treated with normal plasma and, after washing, was eluted with 2 M potassium thiocyanate. After dialysis, both kallikrein and kininogen antigens were also found in this eluate but only traces of Fletcher factor and Fitzgerald factor activities were identified. The concentrations of these clotting activities in the eluates did not parallel one another. The processes of elution and dialysis may have impaired the function of these proteins.

TABLE IV

Effect of Adding Kininogen to Kininogen-Deficient Plasma upon Depletion of Prekallikrein with Antikininogen

Mixtures	Prekallikrein antigen		
	Unadsorbed	Adsorbed	Loss
	% of normal plasma pool		*%*
Normal plasma + LMW-KGN	68	—	—
Normal plasma + LMW-KGN + antiKGN	34	25	26
KGN-deficient plasma + LMW-KGN	16	—	—
KGN-deficient plasma + HMW-KGN	16	—	—
KGN-deficient plasma + LMW-KGN + antiKGN	8	9	0
KGN-deficient plasma + HMW-KGN + antiKGN	8	4.6	42

Equal volumes (0.2 ml) of plasma samples and kininogen solution were mixed gently with 0.4 ml washed, packed Sepharose-antikininogen on a magnetic stirrer at room temperature for 30 min. After sedimentation of insoluble components at 4°C for 1 h, the mixtures were centrifuged and supernatant fluids were tested for prekallikrein antigens in the radioimmunoassay (15). The HMW-KGN used contained 60 μg of protein/ml (see Fig. 1). The kininogen-deficient plasma contained 32% of the normal amount of prekallikrein antigen before dilution in assay mixtures.

DISCUSSION

The antibody to human kininogens used in these studies reacted with both HMW-KGN and LMW-KGN and gave a single band of precipitation in an Ouchterloney analysis against normal plasma, but no precipitin band when reacted with kininogen-deficient plasma from the proband reported earlier (3 and Fig. 3). Thus, it fulfills criteria for monospecificity. This antibody revealed two precipitin arcs of similar electrophoretic mobility when normal plasma and serum were subjected to immunoelectrophoretic analysis, but a single precipitin arc with plasma from patient Fitzgerald which contains kininogen that does not have clotting activity attributed to HMW-KGN (Fig. 4). Thus, the antibody identifies antigenic determinants common to all species of kininogen molecules. With this antibody, the kininogen antigens in plasma from heterozygous members of the kindred with kininogen deficiency were quantified and found to be near half the amount found in normal plasma (Fig. 5, Table II), confirming our earlier report of partial deficiencies of functional kininogens in plasma from heterozygotes (3). In studies not illustrated, the concentrations of both HMW-KGN and LMW-KGN in plasma from a heterozygous member of this kindred were much less than in normal plasma when the two were compared with respect to their behavior during gel filtration on a column of G-200 Sephadex, and trypsin-released kinin measured. Both species of kininogen are apparently deficient in these individuals.

Prekallikrein antigen was deficient in plasma from the proband in proportion to the deficiency of Fletcher factor clotting activity (Table II). Relatives who were partially deficient in plasma kininogens were also partially deficient in Fletcher factor clotting activity, but in each instance the level of plasma prekallikrein antigens was not as low as the Fletcher factor activity (Table II). The reasons for these differences are not clear, but the assay for Fletcher factor clotting activity is susceptible to variable influences, such as plasma inhibitors, which need not influence the radioimmunoassay for prekallikrein. Even so, the mean level of plasma prekallikrein antigens was below the normal ($P = <0.1$). The possibility that nonfunctional prekallikrein protein may exist in plasma from heterozygotes was not entirely excluded.

The close relationship between prekallikrein and

HMW-KGN suggested by these observations led us to examine the relationship of Fletcher and Fitzgerald factor clotting activities in normal plasma by immunological procedures. In eight sets of experiments when kininogens were adsorbed from normal plasma with the antikininogen globulins, both Fletcher and Fitzgerald factor clotting activities were removed. Similarly, when normal plasma was adsorbed with the monospecific antibody globulin to kallikrein, the amount of Fitzgerald factor removed was directly proportional to the amount of Fletcher factor activity removed (Fig. 8). Thus, the substances behaved as though complexed to one another in normal plasma. An antibody to kininogen did not remove an amount of Fletcher factor directly proportional to the amount of Fitzgerald factor because this antibody reacts with kininogens which do not have clotting activity (LMW-KGN) as well as those which do (21) as demonstrated in Figs. 3–5. The antibody to kallikrein, on the other hand, reacts specifically with prekallikrein, which has Fletcher factor activity (9, 23), and its ability to adsorb proportional amounts of each clotting factor from normal plasma and can be explained by their existence as a complex. Nonetheless, prekallikrein antigens were removed from normal plasma by antikininogen, but not from kininogen-deficient plasma until HMW-KGN was added to the plasma (Table IV), again suggesting that a complex was formed under conditions of this experiment. Mandle and his colleagues (24) have recently provided evidence of a complex of HMW-KGN and prekallikrein in normal plasma in gel filtration and electrophoretic experiments. The complex of prekallikrein and HMW-KGN in normal plasma must not be sustained by covalent bonds, for these proteins can be dissociated during ion exchange chromatography.

Neither antibody preparation removed Hageman factor activity from normal plasma before or after its exposure to glass, as if Hageman factor were not part of the fluid phase complex of prekallikrein and HMW-KGN. In earlier studies (25), the Hageman factor in plasma that had been treated with ellagic acid, a soluble activator of Hageman factor (26), had sedimentation properties of a 5–7S protein and not of a macromolecular complex.

All of these observations point to the possibility that the deficiency of prekallikrein in kininogen-deficient plasma may be secondary to the deficiency of HMW-KGN. The catabolism of the prekallikrein might be markedly hastened if its stability in vivo depended upon the presence of the HMW-KGN. Indeed, such a situation has been described in patients with hypogammaglobulinemia who are also deficient in a portion of the first component of complement, C1q (27); the C1q interacts with gamma globulin in plasma normally but was rapidly cleared in vivo when gamma globulins were deficient. To determine whether such a mechanism is responsible for prekallikrein deficiency in kininogen-deficient plasma, the rate of clearance of intravenously administered prekallikrein from plasma of the proband before and after repletion of the plasma with HMW-KGN would have to be measured. It is difficult to conceive that the defect in plasma prekallikrein in those heterozygous for kininogen deficiency could be explained by such a mechanism, for there is considerable HMW-KGN in these plasmas. In addition, this mechanism does not explain why some (1–3, 5, 6) but not all (4) probands reported are deficient in both prekallikrein and HMW-KGN. It is hazardous to exclude the possibility without further experiment, however. It is alternatively possible that the deficiency of prekallikrein may be genetically determined and linked to the kininogen deficiency in this kindred.

Prekallikrein deficiency was also apparent in plasma from heterozygotes when kaolin-induced arginine esterase activity was measured (Fig. 7 A–C). Decreased esterase activity in HMW-KGN-deficient individuals (Fig. 7A) has been reported by others (2, 5) despite measurable amounts of prekallikrein in this plasma. The missing HMW-KGN must be required for expression of kaolin-activated Hageman factor in this assay measuring the subsequent activation of prekallikrein (28, 29). The addition of a preparation of HMW-KGN or LMW-KGN did not increase the rapidly generated esterolytic activity (1 min) to normal levels, but the amount of activity that evolved in mixtures containing HMW-KGN was greater than in those containing LMW-KGN or buffer, especially after 10 min of incubation. When mixtures of prekallikrein-deficient and kininogen-deficient plasma were tested, the amount of rapidly generated esterase activity was roughly proportional to the amount of prekallikrein therein (Fig. 7 C). Either the HMW-KGN was damaged during its isolation and could not interact with the prekallikrein in plasma during this assay or an additional factor present in the prekallikrein-deficient plasma is involved. Changes in the HMW-KGN during its purification may also explain its failure to correct the electrophoretic mobility of prekallikrein in kininogen-deficient plasma.

These studies reinforce the view that HMW-KGN is required for surface activation of plasma clotting. Relatively normal amounts of clot promoting activity evolve slowly when plasma deficient in prekallikrein is incubated with glass or kaolin (11, 30), but we were unable to shorten the clotting time of plasma from the kininogen-deficient proband by this maneuver. Prekallikrein, therefore, seems to be an acceleratory factor rather than an absolute requirement for surface-

initiated clot promoting activity, whereas HMW-KGN appears to be essential. Perhaps the HMW-KGN is effective in surface-initiated clotting in prekallikrein-deficient plasma only after a delay because it must be complexed with prekallikrein for a rapid expression of its clot promoting activity. In mixtures of purified reagents, Griffin and Cochrane (29) found that a preparation of PTA was optimally activated only when kaolin, Hageman factor, prekallikrein, and HMW-KGN were present, but deletion of the HMW-KGN alone from such an activation mixture remarkably impaired the activation of PTA. Their observations indicated a stoichiometric relationship of HMW-KGN to the activation of PTA and prekallikrein by activated Hageman factor as well as in the fragmentation of Hageman factor by kallikrein, which is consistent with the view that a complex may form on an activating surface of which HMW-KGN is an essential component.

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