Using the substrate H-o-Pro-Phe-Arg-p-nitroanilide-HCl, an amidolytic assay was designed to measure prekallikrein in plasma. At a substrate concentration of 1 mM, the amidolysis of purified kallikrein at 1 coagulant unit/ml was observed to be 2.47 μmole/min/ml. Conditions for plasma prekallikrein activation were optimized to approach complete activation when compared to the amidolytic activity of the purified plasma kallikrein. Plasma treated with chloroform to destroy inhibitors of kallikrein was activated with dilute kaolin (final concentration 1 mg/ml) for 1 min at 25°C. Activated plasma prekallikrein had 78% (1.92 μmole/min/ml) of activity of purified kallikrein at plasma concentration. Comparison of this amidolytic assay with immunochemical, esterolytic, and coagulant assays of plasma prekallikrein have been available for a decade and have led to considerable information regarding the participation of this plasma protein in human disease states. Immunochemical assays including radial immunodiffusion and radioimmunoassay allow accurate quantification of the concentration of prekallikrein antigen protein. However, the antisera currently available fail to distinguish between prekallikrein and kallikrein, and furthermore, measure kallikrein Cl-inhibitor complexes in plasma that are apparently physiologically inactive. Thus, to detect kallikrein in disease states, assays measuring its enzymatic activity are required.

Functional assays of prekallikrein have been fraught with technical and theoretical pitfalls. To measure prekallikrein in plasma it must be quantitatively converted to kallikrein. This transformation is dependent on contact phase of coagulation activation that requires the activation of factor XII (Hageman factor) and a sufficient concentration (>10% of normal) of the protein cofactor, high molecular weight (HMW) kininogen. The concentration of kallikrein formed after activation depends on the kinetics of activation of factor XII by the solid-phase or soluble activator selected. Not only must HMW kininogen be present in sufficient amounts to allow optimal factor XII activation but supplementation of normal plasma with additional HMW kininogen (0.2 U/ml) can increase the apparent prekallikrein activity measured on immunochemical and esterase assays up to 30%. Furthermore, once kallikrein is formed in plasma the enzyme is rapidly inhibited by the action of Cl-inhibitor and alpha-2-macroglobulin. Thus, the concentration of inhibitors influences the assay of prekallikrein, and methods must be developed to mitigate the effect of inhibitors on the assay.

Previous functional assays of prekallikrein have measured the ability of the activated enzyme, kallikrein, to liberate bradykinin from crude preparations of kininogen, to hydrolyse synthetic ester substrates, to correct the abnormal activated partial thromboplastin time (APTT) of prekallikrein-deficient (Fletcher factor) plasma, or to hydrolyse chromogenic tripeptide substrates. The liberation of bradykinin depends on measurement of this peptide with a sensitive but semiquantitative bioassay or the time-consuming radioimmunoassay. The use of synthetic esters has been helpful, but these substrates have a Km that is about tenfold higher than the tripeptide substrates, and the assay requires many steps, each one multiplying the pipetting error. The correction of the mildly abnormal partial thromboplastin time of prekallikrein deficiency yields a relatively flat standard.

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curve limiting the accuracy of the coagulation assay. The time of incubation is critical, since correction of the defect occurs with prolonged incubation. Recent investigations have utilized amidolytic assays for measuring the kallikrein derived from prekallikrein but no comparative studies of the functional and immunochemical assays have been performed.

This study compares three functional assays for prekallikrein using coagulation, esterolytic, and amidolytic substrates with a radial immunodiffusion reference assay in three subject populations—normals, women receiving oral contraceptives, and patients with severe liver disease. In addition, this study directly compares our plasma prekallikrein amidolytic assay with several different plasma prekallikrein amidolytic assays from other laboratories against a purified kallikrein standard. Our findings allow us to recommend a rapid and simple plasma prekallikrein amidolytic assay that measures 78% of the absolute amount of prekallikrein in plasma.

MATERIALS AND METHODS

Materials

H-d-Pro-Phe-Arg-p-nitroanilide-HCl (PPAN) (S-2302, Ortho Diagnostics, Raritan, NJ) was used as a synthetic substrate of kallikrein. H-d-Val-Leu-Lys-p-nitroanilide-HCl (VLLN) (S-2251, Ortho Diganostics) was used as a synthetic substrate of plasmin. Tris and p-tosyl-l-arginine methyl ester (TAME) (Sigma Chemical); and Thrombofax (Ortho Diagnostics, Raritan, NJ) was used as a synthetic substrate of plasmin. Tris and p-tosyl-l-arginine methyl ester (TAME) (Sigma Chemical Corp., Pittsburgh, Pa.); HSA agarose (Litiex, Denmark); sodium barbital buffer, pH 8.6 (B-2) (Harleco, American Hospital Supply Corp., Gibbstown, N.J.); inosithin (Associated Concentrates, New York, N.Y.); dextran sulfate (Sigma Chemical); and Thrombofax (Ortho Diagnostics), were purchased from the designated supplier. All other materials used were reagent grade.

Plasma and Protein Preparations

All plasma samples to be assayed for prekallikrein determination regardless of methodology employed (enzymatic, immunologic or clotting) were prepared as follows. Prior to venipuncture informed written and verbal consent were obtained from each donor. Twenty milliliters of blood were drawn from a clean venipuncture site through a 19-gauge siliconized butterfly needle into a plastic syringe containing 3.8% trisodium citrate (1:9 v/v). After adequate mixing, blood was immediately centrifuged at 2500 G for 15 min at 4°C to produce platelet-poor plasma and then frozen in 1-ml aliquots in plastic tubes at −70°C for later analysis. In the experiments where fresh plasma was used, it was collected in a similar manner and used within 30 min after collection.

Plasma was exposed to chloroform (CHCl₃) to inactivate inhibitors of plasma kallikrein as previously described. Each sample was processed just prior to its assay. An equal volume of CHCl₃ cooled to 4°C was added to freshly thawed plasma, mixed for 1 min using a vortex mixer, and then centrifuged at >2000 g for 5 min at 4°C. The centrifuged CHCl₃-treated plasma, which separated into two phases, was not transferred to another plastic tube, and only the top half was used for the assay by carefully removing the amount necessary for each test.

Prekallikrein-deficient plasma was donated by Dr. C Abildgaard (University of California, Davis, Calif.). Pooled normal human plasma (lot 02), which was used for the comparison of the different amidolytic assays and as a pooled normal reference plasma, was purchased from George King Biomedicals Inc., Overland Park, Kans. Kallikrein was prepared by the method of Bagdasarian et al.2 It contained no detectable HMW kininogen, CI-inhibitor, alpha-2-macroglobulin, alpha-1-antitrypsin, plasminogen, or plasmin, but contained IgG, which on radial immunodiffusion was found to be 8% of the total protein present in the kallikrein preparation. This kallikrein preparation was used for the production of antiserum. Antiserum to kallikrein was prepared in rabbits by the method of Bagdasarian et al.2 It was adsorbed with prekallikrein-deficient plasma to render it monospecific. Antiserum to kallikrein and prekallikrein cross-reacts with both the zymogen and the enzyme. Prekallikrein for functional studies was prepared by the method of Scott et al.12 and was activated to kallikrein by incubating it with Hageman factor fragments that were prepared as previously reported. The specific activity of the kallikrein was 29 coagulant units/mg.

Assays for Prekallikrein and Kallikrein

Radial Immunodiffusion

Radial immunodiffusion was performed according to the method of Mancini et al.29 using 1% agarose in B-2, 1/2 = 0.0375. Kallikrein antiserum was used at final concentrations of 3%. Diffusion proceeded for 24 hr. No further increase in ring diameter was observed at 48 or 72 hr. In our laboratory, the concentration of plasma prekallikrein antigen has been determined to be 39 µg/ml. This value correlates well with those reported by two other laboratories.21,22

Kallikrein Coagulant Activity

This was measured by preparing a standard curve from normal pooled plasma diluted in sodium barbital buffer in saline (VBIS), pH 7.4. The dilutions ranged from 1/40 to 1/320. In this assay, a modified one-stage APTT,20 50 µl of diluted pooled normal plasma or test sample was added to a mixture of 50 µl of prekallikrein-deficient plasma, 50 µl of 0.1% inosithin in VBIS, and 50 µl of 10 mg/ml kaolin in normal saline. This mixture was then incubated for 1 min at 37°C before recalcification with 50 µl of 0.025 M CaCl₂. One coagulant unit was defined as that amount that is present in 1 ml of normal pooled plasma.

Kallikrein Esterolytic Activity

Kallikrein esterolytic activity was assayed for its ability to hydrolyze the synthetic substrate TAME according to the method of Siegelman et al.31 as modified by Colman et al.31 using kaolin to convert prekallikrein to kallikrein. Kallikrein inhibitors were removed by chloroform prior to activation. Briefly, prekallikrein in plasma is activated by kaolin. The amount of activated kallikrein was then permitted to react with a known amount of the substrate at 37°C. The amount of esterase activity generated was then determined colorometrically by quantitating the release of methanol, which was oxidized to formaldehyde with KMnO₄ and finally coupled to chromotropic acid to yield a purple color. The absorption of this purple solution was determined at 580 nm spectrophotometrically. Spontaneous arginine esterase activity was determined as above, substituting phosphate buffer for kaolin. Without an activating agent, plasma prepared under these conditions hydrolyzed less than 0.05 µmole TAME/min/ml. This value was subtracted from the value with kaolin to yield the plasma prekallikrein concentration.
Kallikrein Amidolytic Activity

This was determined using the chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide-HCl (PPAN). Plasma (50 μl) in a plastic test tube at 25°C was mixed with 50 μl of 0.1 M sodium phosphate, pH 7.65, containing 0.15 M NaCl and kaolin (final concentration 1 mg/ml) and incubated at 25°C for 1 min. Ten microliters of this mixture was then added to a cuvette that contained 60 μl of PPAN stock solution (4 mM) and 170 μl of sodium phosphate-NaCl buffer, pH 7.65, at 37°C. The final concentration of PPAN in the assay was 1 mM. After mixing, the initial rate of increase in absorbance at 405 nm was recorded in a Gilford 240 Spectrophotometer with a Gilford 6015 chart recorder (Gilford Instr., Oberlin, Ohio). The initial rate without kaolin activation was less than 5% of the rate with kaolin added and was subtracted to calculate the prekallikrein concentration. The micromoles of PPAN hydrolyzed/min/ml plasma was calculated using an assumed molar extinction coefficient of 10,600 for p-nitroaniline at 405 nm.

Initial studies were performed with purified kallikrein to determine the optimal buffer and substrate concentration for the assay. All dilutions of purified kallikrein were performed with buffer containing 3 mg/ml bovine serum albumin (BSA). Initial studies with kaolin-activated plasma were performed to determine the necessity of treatment of plasma with CHCl₃ to remove inhibitors and the optimal temperature of activation. Subsequent studies were performed with CHCl₃ treated plasma to determine the optimal kaolin concentration and the sensitivity of the assay over a wide range of plasma dilutions.

Studies to Compare Plasma Spectrophotometric Assays

The present kaolin-activated plasma kallikrein assay was compared with other published plasma kallikrein assays and against purified kallikrein. The assay of Kluft using dextran sulfate as the activator was performed as described and in a modified version that increased the final concentration of the substrate (PPAN) to 1 mM and decreased the plasma dilution to 1:48. The assay of Friberger et al. using ellagic acid as the activator was performed as written. All comparison studies were performed with one lot of commercial pooled normal human plasma. As performed with the kaolin assay, the initial rate of amidolysis without the activator was determined and was subtracted to calculate the prekallikrein concentration. Moreover, spectrophotometric studies were performed on dextran-sulfate-activated plasma according to Kluft to see if plasmin was generated. Plasma plasmin was assayed using the substrate VLLN according to the procedure of Teger-Nilson et al.

Statistical Methods

Mean, standard deviation, and standard error of the mean were determined for all assays performed on every patient group. Correlation coefficients were determined between assays in each patient group. Students’ t test (unpaired) was performed to determine significance between particular assays in all groups.

RESULTS

Standardization of the Amidolytic Assay With Purified Kallikrein

Initial studies were performed with purified kallikrein to define the optimal conditions of the chromogenic assay. Using purified kallikrein, the $K_m$ for kallikrein on PPAN was determined by a Lineweaver-Burke plot to be 0.2 mM. In accordance with the findings of Friberger, the $K_m$ for this protein on this substrate was not altered in buffer systems using 0.1 M sodium phosphate containing 0.15 M NaCl, pH 7.65, or 0.01 M Tris-Cl containing 0.15 M NaCl, pH 8.0. Moreover, the amidolytic activity of kallikrein on the substrate PPAN was proportional to the enzyme concentration from 0.1 to 1.0 coagulant units in the original sample.

Standardization of the Amidolytic Assay With Plasma

Studies were performed with undiluted plasma, native or chloroform treated, at 0°C and 25°C. As shown in Fig. 1, activation of native plasma with kaolin (final concentration 1 mg/ml) at 25°C showed an initial rapid formation of amidolytic activity with a 40% decrease at 5 min, similar to the previously
described activation using the synthetic ester TAME.12 The maximum activity observed was only 65% of that in CHCl3 treated plasma as previously observed.8 Activation with kaolin at 0°C, the same temperature used in the dextran sulfate assay,14 resulted in suboptimal amidolytic activity, reaching only 30% of the maximum value in CHCl3-treated plasma at 5 min. In contrast, activation with kaolin of CHCl3-treated plasma gave the highest values at 1 min and only a 20% decrease in activity at 5 min.

Proper handling of CHCl3-treated plasma required assaying the material directly from the tube that was centrifuged. Transferring the supernatant of CHCl3-treated plasma to another plastic tube resulted in a 50% decrease in the measured amidolysis.

The optimum concentration of kaolin was next determined (Fig. 2A). Activity was maximal at a final concentration of kaolin from 1 to 5 mg/ml in CHCl3-treated plasma. For all subsequent studies, the final kaolin concentration in the activation mixture was 1 mg/ml. The amidolytic activity after kaolin activation of CHCl3-treated plasma was linear for plasma volumes in the assay from 0.5 μl to 7.5 μl (Fig. 2B).

Comparison of Kallikrein Amidolytic Assays

Using commercially available pooled normal human plasma, a direct comparison was performed among purified kallikrein, the proposed kaolin-activated plasma kallikrein assay with normal plasma, and prekallikrein-deficient plasma reconstituted with purified prekallikrein (39 μg/ml), and previously published plasma kallikrein assays (Table 1). With purified kallikrein (1 unit coagulant activity/ml), the amount of amidase activity was 2.47 μmole/min/ml. The presence of BSA in the buffer used to dilute the stock kallikrein stabilized the amidase activity of the diluted kallikrein for at least 1 hr. The BSA in the buffer at this concentration (3 mg/ml) prevented surface adsorption of the kallikrein.19 With native plasma and kaolin (final concentration 1 mg/ml), only 39% of the available plasma prekallikrein was measurable on the amidase assay. The partial measurement of the available prekallikrein must have been due to the presence of plasma inhibitors, since pretreatment of plasma with chloroform resulted in activation of 78% of the available plasma prekallikrein, as calculated from the amidolytic activity of purified kallikrein when used in a concentration of 1 unit coagulant activity/ml. Moreover, reconstitution of prekallikrein-deficient plasma with purified prekallikrein (39 μg/ml), followed by CHCl3 treatment and kaolin activation, lead to amidolytic measurement of 71% of the available prekallikrein.

The dextran sulfate assay of Kluft14 when performed as written exhibited 76% of the available plasma prekallikrein activation. Modifying this assay by decreasing the plasma dilution to 1:48 from 1:162 and increasing the substrate concentration to 1 mM from 0.16 mM, the same plasma prekallikrein activity as determined with the kaolin-activated assay was expressed. Moreover, in agreement with Kluft,14 the measured amidase activity with dextran sulfate as the

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Fig. 2. (A) Effect of kaolin concentration on the activation of plasma prekallikrein. Undiluted plasma [native (●●●●) or CHCl3 treated (○○○○)] was incubated with an equal volume of kaolin in 0.1 M Na phosphate buffer, pH 7.65, containing 0.16 M NaCl for 1 min. The final concentration of kaolin in the activation mixture was 0.5-5 mg/ml. (B) Relation of plasma volume (μl) to μmole/min PPAN hydrolyzed. Undiluted CHCl3-treated plasma was incubated with kaolin (final concentration 1 mg/ml) as described (see Materials and Methods). Various amounts (μl) of activated plasma was removed and the amount of μmole/min PPAN hydrolyzed was determined. In both experiments (A and B), the assay was performed as in Fig. 1, and the values recorded represent the initial rate of hydrolysis of the substrate as determined by a continuous chart recording.
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and decreasing the final plasma dilution 1:48. The kallikrein used in the assay was equivalent to 1 coagulant unit/mL.

Calculated values employ an assumed molar extinction coefficient of 10,600 for p-nitroaniline of this wavelength and pH.

normal pooled plasma (Table 2). The actual amount of amidolytic activity measured in the 19 normal individuals ranged from 29 to 44 μg/ml. There was no significant difference between the 10 male subjects (94.2% ± 4.0% of normal pooled plasma) and the 9 female subjects (97.8% ± 4.3%). The 21 women tested using birth control pills had a value of 107.8% ± 4.8%. The value was not significantly different from the value observed in the 9 normal female subjects. Seven patients with severe liver disease showed markedly decreased immunochemical levels of prekallikrein 40.1% ± 2.7% (p < 0.001).

Functional Prekallikrein by Esterase Assay

The mean of 19 normal individuals was 2.35 ± 0.09 μmole TAME hydrolyzed/min/ml (83.7% ± 3.2% of normal pooled plasma) (Table 2). The actual amount of esterolytic activity measured in the 19 normal individuals ranged from 1.43 to 3.16 μmoles TAME hydrolyzed/min/ml. There was no difference between male (2.41 ± 0.14) and female (2.28 ± 0.11) subjects. The 21 women receiving oral contraceptives had elevated esterase activity 2.50 ± 0.12 μmoles TAME hydrolyzed/min/ml (89.2% ± 4.2% of normal pooled plasma), but this did not differ significantly from the normal women. Nevertheless, 8 of these 21 women exceeded 2 standard deviations of the mean of the normal group.

Functional Prekallikrein Amidolytic Assay

The mean for 19 normal individuals was 1.60 ± 0.1 (mean ± SEM) μmoles PPAN hydrolyzed/min/ml (83.3% ± 5% of normal pooled plasma) (Table 2). The actual amount of amidolytic activity measured in the 19 normals ranged from 0.98 to 2.21 μmoles PPAN hydrolyzed/min/ml. There was no difference between male (1.60 ± 0.16) and female (1.60 ± 0.16) subjects. The 21 women receiving birth control pills had a mean of 1.94 ± 0.7 μmoles PPAN hydrolyzed/min/ml (101% ± 3.9% of normal pooled plasma), which was significantly greater than the normal controls (p < 0.02) and the normal women (p < 0.05). The liver disease patients had markedly lower levels of prekallikrein amidolytic activity 0.58 ± .1 μmoles PPAN

activator was only due to kallikrein. No detectable plasma plasmin activity was ascertained with dextran-sulfate-activated plasma on the chromogenic substrate VLLN. In addition, performing both the kaolin and dextran-sulfate-activated assays with prekallikrein-deficient plasmas led to no amidolytic activity. The ellagic-acid-activated assay of Fribinger et al. only allowed for 17% of plasma prekallikrein activation, supporting previous findings that ellagic acid is a weak activator in the contact phase of coagulation.

Immunologic Concentration of Prekallikrein

The mean prekallikrein antigen concentration in normal individuals was 95.9% ± 2.9% (mean ± SEM) of normal pooled plasma (Table 2). The actual amount of prekallikrein antigen measured in these 19 normal

Table 1. Comparison of Amidolytic Assays

<table>
<thead>
<tr>
<th>Assay† ‡</th>
<th>μmole/min/ml † ‡</th>
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<tr>
<td>Purified kallikrein</td>
<td>2.47 ± 0.08</td>
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<tr>
<td>Kaolin assay—normal plasma</td>
<td>0.97 ± 0.06</td>
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<tr>
<td>Kaolin assay—Chloroform-treated normal plasma</td>
<td>1.92 ± 0.08</td>
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<tr>
<td>Kaolin assay—Chloroform-treated Fletcher plasma reconstituted with purified prekallikrein</td>
<td>1.76 ± 0.08</td>
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<tr>
<td>Dextran sulfate assay</td>
<td>1.87 ± 0.04</td>
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<tr>
<td>Modified dextran sulfate assay **</td>
<td>1.74 ± 0.15</td>
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<tr>
<td>Ellagic acid assay † ‡</td>
<td>0.43 ± 0.06</td>
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</table>

* Named according to activator present in reaction.
† Values represent the mean ± SEM results of 4–8 experiments.
‡ Purified kallikrein (29 coagulant units/mg) was diluted with 0.1 M NaPO₄, 0.15 M NaCl, pH 7.65, containing 3 mg/ml of BSA with syringes and test tubes precoated with this buffer. The concentration of purified kallikrein used in the assay was equivalent to 1 coagulant unit/ml.
¶ Purified prekallikrein was diluted into prekallikrein-deficient plasma so that its concentration was 39 μg/ml. After chloroform treatment of the reconstituted plasma, the assay was performed as outlined in Fig. 1.
** Modified by increasing the final substrate concentration to 1 mM and decreasing the final plasma dilution 1:48.
† ‡ Performed as described in reference 15.

Table 2. Comparison of Plasma Prekallikrein Concentration by Different Assays

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<tr>
<td>Normals (n = 19)</td>
<td>83.7 ± 3.2</td>
<td>83.3 ± 5.0</td>
<td>95.9 ± 2.86</td>
<td>94.5 ± 14.1</td>
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<tr>
<td>Oral contraceptives (n = 21)</td>
<td>89.2 ± 4.2</td>
<td>101 ± 3.9</td>
<td>108 ± 4.81</td>
<td>133.0 ± 16.2</td>
</tr>
<tr>
<td>Liver disease (n = 14)</td>
<td>ND</td>
<td>30.6 ± 5.1†</td>
<td>40.1 ± 2.73</td>
<td>34.4 ± 15.3</td>
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</table>

* All values are mean ± standard error of the mean.
† Determined on 7 patients.
ND, not determined.
I, Correlation coefficient; TAMe, kallikrein esterolytic assay; PPAN, kallikrein amidolytic assay; NS, not significant.

Fig. 3. Correlation of amidolytic and immunologic prekallikrein determinations (data not shown). However, when the two enzymatic assays and the immunologic assays in any patient group were compared to the coagulation assay, there was no significant correlation of the coagulant assay with any of the other assays (Table 2). The absence of correlation, especially between the coagulant and immunologic assays, may be due to the large standard error of the coagulant assay (Table 2).

The amidolytic and immunocchemical assays of all three clinical groups are compared in Fig. 3. An excellent correlation ($r = 0.86, p < 0.001$) exists.

**DISCUSSION**

A functional assay described over 10 yr ago by this laboratory measures the hydrolysis of tosyl arginine methyl ester (TAME) of plasma exposed to kaolin for 1 min. When the spontaneous activity (0 min) is subtracted, the difference was taken to represent functional prekallikrein. Recently, we have identified two variables (HMW kininogen, C1 inhibitor) that affect this assay. In both HMW-kininogen deficient and normal native plasma, the addition of 0.2 U/ml HMW kininogen increases the mean arginine esterase activity (kallikrein) 30%. Moreover, when the esterase activity is measured in HMW-kininogen deficient plasma after CHCl$_3$ treatment, the mean activity also rises 30%. However, in CHCl$_3$-treated normal plasma, the esterase activity does not rise after supplementation with HMW kininogen. The effect of HMW kininogen in normal concentrations to increase apparent kallikrein activity is due to its ability to protect kallikrein against plasma protease inhibitors. Thus, it appears that CHCl$_3$ treatment of normal plasma to inactivate inhib-

![Fig. 3. Correlation of amidolytic and immunocchemical assay for prekallikrein. The amidolytic assay employing CHCl$_3$-treated plasma and kaolin is compared with the radial immunodiffusion assay of prekallikrein ($r = 0.86, p < 0.001$). (●) Normal subjects; (X) women on oral contraceptives; (O) liver disease patients.](image)
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The effect of protease inhibitors on kallikrein activity apply equally if the substrate is an amide. The introduction of tripeptide chromogenic substrates for kallikrein, Bz-Pro-Phe-Arg-pNA and H-d-Pro-Phe-Arg-p-Na (PPAN), has several advantages over TAME, including a lower $K_m$, as well as the speed and simplicity of a spectrophotometric initial rate assay. We utilized these observations to devise an assay using an amidolytic substrate. The assay developed in this laboratory involves the activation of undiluted CHCl$_3$-treated plasma with kaolin and the measurement of the kallikrein generated using a tripeptide substrate PPAN. In our assay, incubation with kaolin in undiluted plasma at 0°C resulted in suboptimal activation; therefore, activation is performed at 25°C (Fig. 1). To obviate the effect of proteolytic inhibitors in the measurement of prekallikrein in plasma, the native plasma is pretreated with CHCl$_3$. This plasma treatment allowed for amidolytic measurement of 71%–78% of the available plasma prekallikrein antigen. The kaolin concentration employed (1 mg/ml—the final concentration when mixed with plasma) is the lowest amount that is optimal for prekallikrein activation. The final concentration of kaolin in the assay (21 µg/ml) had no apparent interference with the spectrophotometric readings. Finally, the assay is linear with respect to plasma prekallikrein concentration over a 15-fold range.

Previous investigators have concentrated on determining the optimal conditions for activating prekallikrein. Direct comparison by us of the kaolin-activated amidolytic method with the assay of Kluft$^{14}$ using diluted plasma activated by dextran sulfate at 0°C reveals agreement in the total amount of plasma prekallikrein activated. Activation in both assays proceeded to the same extent (76%–78%) when compared with purified kallikrein of 1 coagulant unit/ml. However, the absolute amount of amidolysis in normals (µmole p-nitroaniline hydrolyzed/min/ml) measured by Kluft (0.476) and by us (1.871) employing his assay differs markedly. This measured difference in the total kallikrein amidolysis by the two laboratories performing the same assay cannot be fully explained. One possible source of difference is the manner in which the test plasmas were collected, sorted, and/or handled. Another subtle, but important aspect of plasma kallikrein assays is the handling of the activated plasma specimen. Unnecessary transferring of activated plasma (both native and CHCl$_3$ treated) from one plastic test tube to another should be avoided since this manipulation alone can account for a 50% decrease in the total measured amidase activity of kallikrein on its substrate. This observation was noted in the performance of the present investigation and is probably due to the surface adsorption of activated prekallikrein to plastic test tubes and pipettes as previously reported by our laboratory.$^{19}$ In addition, the concentration of substrate employed (0.16 mM) in the Kluft assay$^{14}$ is just below the $K_m$ of the PPAN determined in this study (0.20 mM). This value of the $K_m$ is identical to that previously reported for kallikrein.$^8$ The use of a substrate at its $K_m$ means that a small change in its concentration, such as an error in weighing, may produce a large change in the rate of hydrolysis. Therefore, we employ PPAN at 1 mM, a concentration five fold in excess of the $K_m$. Modification of the dextran-sulfate-activated assay$^{14}$ to increase the substrate concentration to 1 mM and decrease the plasma dilution of 1:48 allows for an identical level of activation (76% versus 78%). However, we prefer to use the kaolin assay because the amount of expensive substrate needed in the modified dextran sulfate assay is more than twice that of our assay, and the total time needed to perform our assay is much shorter.

The values of the new kaolin amidolytic assay were compared with the same assay using TAME as substrate, and a correlation coefficient of $r = 0.89$ was obtained. Moreover, the correlation of the amidolytic and immunochemical assays for prekallikrein in both normal individuals and those taking oral contraceptives was excellent ($r = 0.72$ and 0.76, respectively). However, the correlation of the coagulant assay with either the amidolytic or immunochemical assays was not statistically significant. The coagulant assay of prekallikrein is less accurate than the enzymatic procedures as indicated by its higher coefficient of variation. The decreased correlation coefficient may reflect the reproducibility of this assay. The coagulant assay is based on the APTT$^{13}$ with congenitally deficient plasma. Recently, we have shown that the order of addition of reagents in the prekallikrein coagulant assay is critical.$^{8,19}$ If plasma prekallikrein is assayed with the addition of kaolin prior to exposure to the source of HMW kininogen (prekallikrein-deficient plasma) lower values result. Incorporation of this finding into our coagulant prekallikrein assay [i.e., adding the plasma to be assayed to prekallikrein-deficient plasma (containing HMW kininogen) prior to exposure to a surface] still results in APTT values that poorly correlated with the esterolytic and immunologic assays. Thus, since prekallikrein-deficient plasma has only a moderately prolonged APTT that self-corrects with prolonged kaolin incubation, the coagulant assay has a greater error than is desirable.

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<th><strong>ASSAY OF PREKALLIKREIN IN HUMAN PLASMA</strong></th>
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The assays have also been compared in two patient groups. As reported previously,26 women on birth control pills have a somewhat higher plasma prekallikrein concentration than women not receiving this drug. This finding was confirmed in both the amidolytic and immunochemical assays when the treated women were compared with normal controls. However, when women receiving the birth control pills were compared with normal women, the means did not differ significantly (immunochemical) or were of borderline significance (amidolytic). The increase in prekallikrein concentration when it occurs is probably due to estrogen stimulation of hepatic synthesis, since an increase in antigen protein as well as functional activity was observed. Certain women seem to hyperrespond, since a number have levels well beyond two standard deviations higher than normal (Fig. 3). Conversely, the levels in liver disease are low, similar to previous studies from this2,12 and other1 laboratories. Since the percent of normal by the antigenic, (40%), amidolytic (31%), and coagulant assays (34%) all show proportional decreases, impaired liver synthesis is probably responsible, although increased turnover cannot be excluded at this time. Patients with other signs of decreased hepatic cell synthesis, such as low albumin and long prothrombin time, seem to have lower plasma prekallikrein concentrations than those with normal prothrombin times.26

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Assay of prekallikrein in human plasma: comparison of amidolytic, esterolytic, coagulation, and immunochemical assays

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