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# BRASIL FACTOR

## A NEW PREKALLIKREIN ACTIVATOR IN HUMAN PLASMA*

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**Abstract**—A new activator of prekallikrein, provisionally called Brasil factor, has been found in human plasma. While developing a radiochemical method for the detection of plasma deficient in Hageman factor, prekallikrein and/or high molecular weight kininogen, it was found that plasma deficient in high molecular weight kininogen was capable of activating prekallikrein in the presence of a negatively charged particle (celite). Also, prekallikrein added to this deficient plasma shortens its clotting time. These results suggested that, in addition to Hageman factor, another activator of prekallikrein existed in human plasma. Further studies have shown that Brasil factor elutes from DEAE-cellulose after prekallikrein and Hageman factor and close to high molecular weight kininogen. Brasil factor, like Hageman factor, is adsorbed to celite and activates prekallikrein most rapidly in the presence of the particle. It differs from Hageman factor by its pattern of elution from DEAE-cellulose, its molecular weight which was estimated as 70,000 by gel filtration, and in that it is inhibited by hexadimethrine bromide (1–2μg/ml) added after it is adsorbed to the negative particle. Sodium chloride (0.1 to 0.5 M) inhibits the activation of prekallikrein by Brasil factor. It is proposed that this new factor may be the missing one previously described or provide an alternative method for the activation of the intrinsic clotting system.

It is now well established that Hageman factor adsorbed to a negative particle remains essentially inactive unless two components of the plasma kallikrein–kinin system, prekallikrein [1–3] and its specific substrate high molecular weight (HMW) kininogen [4–8], are also present. Thus, both prekallikrein and HMW kininogen are early components of the intrinsic coagulation system. However, mixtures of inactive Hageman factor, prekallikrein and HMW kininogen in amounts equivalent to that found in plasma [8] failed to fully activate Hageman factor, making it reasonable to suggest that other factor(s) may be involved.

In the present studies, a new prekallikrein activator, provisionally called Brasil factor, has been found in normal human plasma. Some of the properties of this new factor and evidence differentiating if from Hageman factor and its fragments are described.

### MATERIALS AND METHODS

Hexadimethrine bromide and sodium azide were products of the Aldrich Chemical Co. (Milwaukee, WI 53233, U.S.A.) and Sigma Chemical Co. (St. Louis, MO 63178, U.S.A.) respectively. Plasma deficient in HMW kininogen (Fitzgerald factor) was kindly supplied by Dr. O. A. Carretero, Henry Ford Hospital (Detroit, MI); plamsa deficient in Hageman factor, by Dr. P. G. Iatridis, Indiana University School of Medicine, (Gary, IN); and plasma deficient in prekallikrein (Fletcher), by George King Bio-Medical Inc. (Salem, NH 03079, U.S.A.).

*Preparation of plasma proteins.* Fresh ACD human plasma, to which 0.01% hexadimethrine bromide and 0.1% sodium azide had been added, was dialyzed overnight at room temperature against three changes (4, 4 and 12 h) of 0.01 M Tris, pH 7.0, containing the same concentration of hexadimethrine bromide and sodium azide (1 vol. of plasma/10 vol. of buffer). After centrifugation to remove insoluble material, the dialyzed plasma was chromatographed on a DEAE-cellulose column (Whatman DE-32, Whatman Inc., Clifton, NJ, U.S.A.) (2.0 ml of column/1.0 ml of dialyzed plasma) equilibrated with 0.01 M Tris, pH 7.0, at flow rates of 80–200 ml/hr, as recommended by Dr. Y. Hojima, National Heart, Lung and Blood Institute (Bethesda, MD). After washing with two column volumes of buffer, the proteins adsorbed to the DEAE-cellulose were eluted with a gradient of equal volumes of 0.01 M Tris, pH 7.0, and 0.35 M Tris, pH 7.0 (total volume was 1.2 to 1.5 times the column volume). The fraction volume equaled $\frac{1}{10}$ the plasma volume. Prekallikrein eluted with the proteins which failed to adsorb to DEAE-cellulose and, in general, the activity paralleled the main protein peak. The fractions were pooled and stored at −20°. These preparations had no detectable arginine esterase activity and could be frozen and thawed more than once. Hageman factor eluted early in the gradient. The fractions were pooled, stored at − 20° and remained inactive provided they were frozen and thawed only once. Brasil factor eluted next. These preparations were quite stable to repeated freezing and thawing or to storage at room temperature. HMW kininogen eluted last and those fractions which were essentially devoid of Brasil factor were pooled, stored at − 20°, and frozen and thawed only once.

The pooled Brasil factor (3.0 ml, 53.4 $A_{280}$) obtained from the DEAE-cellulose column was filtered through a 1.5 × 145 cm column of Sephadex G-75 (Pharmacia, Uppsala, Sweden) previously equilibrated with 0.15 M

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NaCl and developed with the same solution. Flow rate was 14 ml/hr and 2.0-ml fractions were collected. The standards used and their molecular weights by gel filtration were: aldolase (158,000), ovalbumin (45,000), chymotrypsinogen (25,000) and ribonuclease (13,000).

*Determination of plasma components.* Prekallikrein, Hageman factor and Brasil factor were determined by measuring the amount of kallikrein formed employing *p*-tosyl-L-arginine[$^3$H]-methyl ester ([$^3$H]TAME) as substrate [9]. In these experiments, 10 μl [$^3$H]TAME (0.047 μCi, 210 Ci/mole, Biochemical and Nuclear Corp., Burbank, CA, U.S.A.) was added to a polypropylene micro tube (Eppendorf, Arthur H. Thomas Co., Philadelphia, PA 19105, U.S.A.) containing 50 μl of solution for assay; the tube was placed in a scintillation vial containing 10 ml of toluene–liquiflor:stop solution and incubated for 30 min at room temperature. The reaction was terminated by shaking and the solution counted. Prekallikrein was determined by mixing 10 μl of 0.5 M Tris, pH 8.0, with 1–20 μl prekallikrein of distilled water in a final volume of 30 μl and incubating for exactly 10 min at room temperature with 20 μl of active Hageman factor adsorbed to supercel and then for 30 min with 10μl [$^3$H]TAME. The active Hageman factor employed in these experiments was prepared by suspending 50 mg hyflo supercel (Fisher Scientific Co., Silver Spring, MD 20910, U.S.A.) in 5.0 ml of 0.04 M Tris, pH 8.0, containing 0.15 M NaCl, washing once by centrifugation, resuspending the supercel in 5.0 ml buffer and mixing with 100 μl of human plasma. The adsorbed supercel was washed three times with 5.0 ml of buffer, resuspended in the same buffer, and stored frozen except when in use. Hageman factor was determined in the various fractions by the addition of excess HMW kininogen [8]. In these experiments, the supercel (5 mg) was washed once with 0.5 ml of buffer as described above, resuspended in 0.5 ml of buffer, and mixed at room temperature with 2–20 μl of fraction and 5–10 μl of HMW kininogen. The adsorbed supercel was washed three times with buffer, resuspended in 0.5 ml of buffer, and 20 μl of this suspension was added to 10 μl of 0.05 M Tris, pH 8.0, incubated for exactly 10 min with 20 μl prekallikrein (containing 0.015 TAME units) and then for 30 min with 10 μl [$^3$H]TAME. The presence of intact active Hageman factor in these fractions could be measured simultaneously by adsorption of the fraction to supercel in the absence of HMW kininogen. Brasil factor, unless otherwise stated in the text, was determined by mixing 10 μl of 0.5 M Tris, (pH 8.0) with 20 μl of 0.375 M NaCl, and adding (at exactly 1-min intervals) 1–5 μl of fraction, 10 μl of dialyzed prekallikrein (1 vol. prekallikrein/300 vol. 0.02 M Tris, pH 8.0, dialyzed for 4 hr, volume changed at second and third hr and stored in an ice bath until used), 10 μl celite 572Å (3 mg/ml of 0.15 M NaCl) and 10 μl [$^3$H]TAME. The reaction was terminated after 30 min by shaking.

*Total kininogen.* Total kininogen in the fraction was measured by diluting 0.1 to 0.2 ml of fraction with 1.6 ml Tris, pH 7.8, and heating the solution in a boiling water bath for 10 min to destroy kininases. The solution was then incubated with 0.2 ml of 2.5 mg/ml bovine trypsin (two times recrystallized, Worthington Biochem. Corp., Freehold, NJ, U.S.A.) for 30 min at 37° and the trypsin was destroyed by heating in a boiling water bath for 15 min. The amount of kinin present in these solutions was determined by bioassay employing the guinea pig ileum. HMW kininogen was determined by direct bioassay employing the guinea pig ileum and using plasma kallikrein to generate the kinin.

*Partial thromboplastin time.* Partial thromboplastin time was measured by incubating (for 1 min at 37°) HMW kininogen-deficient plasma (0.05 ml) or normal

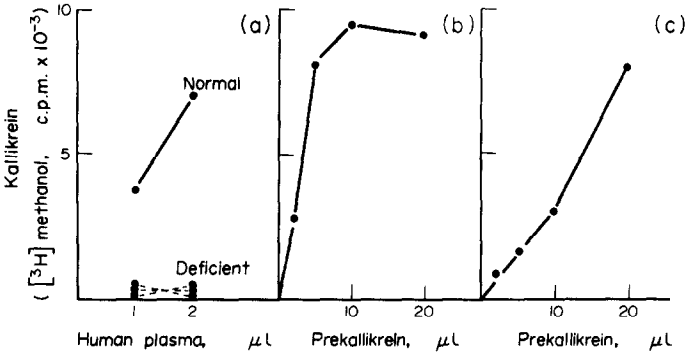


Fig. 1. Panel A: radiochemical method for the detection of plasma deficient in prekallikrein, Hageman factor or HMW kininogen. In these experiments, 10 μl of 0.5 M Tris, pH 8.0, was mixed with 30 μl of 0.15 M NaCl. The reaction was initiated by the addition of 1 or 2 μl of human plasma and, at 1-min intervals, 10 μl celite (1.0 mg/ml of 0.15 M NaCl) and 10 μl [$^3$H]TAME were added. The mixture was incubated for an additional 30 min at room temperature in closed counting vials and terminated by mixing. Panel B: replacement of prekallikrein in plasma deficient in prekallikrein. The reaction was initiated by the addition of the deficient plasma (2 μl) and at exactly 1-min intervals prekallikrein (2–20 μl), celite (10 μl) and [$^3$H]TAME (10 μl) were added. Panel C: addition of prekallikrein to plasma deficient in HMW kininogen (2 μl). Values are corrected for cpm formed in the absence of plasma or prekallikrein [usually less than 700 cpm except for the largest amount of prekallikrein (20 μl) which formed 2000cpm].

human plasma (0.05 ml) with a mixture of 0.2 ml of 0.02 M Tris, pH 8.0, containing 0.15 M NaCl or the solution to be tested and 0.05 ml celite 572Å (5 mg/ml): human cephalin suspension. After the addition of 0.1 ml of 0.05 M $CaCl_2$, the time for clot formation was followed by the tilting method. Human cephalin was kindly supplied by Dr. L. Nahas, Dept. of Haematology, Instituto Butantan (São Paulo, Brasil).

## RESULTS

*Radiochemical method for detection of plasma deficient in prekallikrein, Hageman factor or HMW kininogen.* Because both prekallikrein and Hageman factor are activated when plasma comes in contact with a negative particle, it appeared that the clotting test used for their detection could be replaced by measuring the amount of kallikrein formed rather than the time required for clot formation. This procedure was based, like the clotting test, on the addition of a negative particle (celite, 10 $\mu$g) to human plasma (1–2 $\mu$l) and the reaction was quantitated by measuring the formation of active kallikrein using a radioactive substrate, [$^3$H]TAME. As shown in Fig. 1A, seven normal human plasmas (2 and 1 $\mu$l, respectively) formed 7036 ± 479 and 4643 ± 381 cpm [$^3$H]methanol, while less than 500 cpm were formed by plasma deficient in either prekallikrein, Hageman factor or HMW kininogen.

*Attempt to identify factor missing in deficient plasmas.* It was thought that the radiochemical method could also be utilized to determine the factor which was deficient in these plasmas. As shown in Fig. 1B, when prekallikrein was added to 2 $\mu$l of plasma deficient in prekallikrein, increasing amounts of prekallikrein resulted in increasing formation of active kallikrein. This solution of prekallikrein contained approximately 14 percent of the prekallikrein found in normal plasma so that reconstitution of 35 percent of the prekallikrein of normal plasma formed as much kallikrein as that formed by normal plasma. Maximum amounts of kallikrein were formed with 10 $\mu$l of this prekallikrein solution or by reconstituting the prekallikrein to 70 percent of its normal concentration.

However, as shown in Fig. 1C, when this same concentration of prekallikrein was added to plasma deficient in HMW kininogen, the prekallikrein was also activated. This result was unexpected since prekallikrein would not be activated by Hageman factor in the absence of HMW kininogen. Prekallikrein also shortened the partial thromboplastin time of this plasma. The addition of 0.1, 0.05 and 0.03 ml of dialyzed prekallikrein, at a concentration similar to that found in plasma, to 0.05 ml of HMW kininogen-deficient plasma gave clotting times of 323, 288 and 350 sec, respectively, as compared to 446 sec for control values. On the other hand, addition of 0.05 ml of prekallikrein to 0.05 ml of normal human plasma prolonged the clotting time somewhat (136 sec as compared to control values of 112 sec). These results suggested to us that still another activator of prekallikrein, provisionally called Brasil factor, might exist in human plasma.

*Partial separation of Brasil factor by chromatography on DEAE-cellulose.* Brasil factor could be partially separated from other plasma components by chromatography on DEAE-cellulose. As expected from earlier studies, prekallikrein eluted with the proteins which failed to adsorb to DEAE-cellulose (Fig. 2). Hageman factor eluted shortly after the gradient commenced, followed by low molecular weight (LMW) kininogen. Brasil factor eluted shortly after the main protein peak and between low and high molecular weight kininogens. As can be seen, a clear separation of the activities could not be obtained at the flow rates employed (80–180 ml/hr). However, as reported by Dr. Y. Hojima of the National Heart, Lung and Blood Institute (personal communication), fractionation of plasma on DEAE-

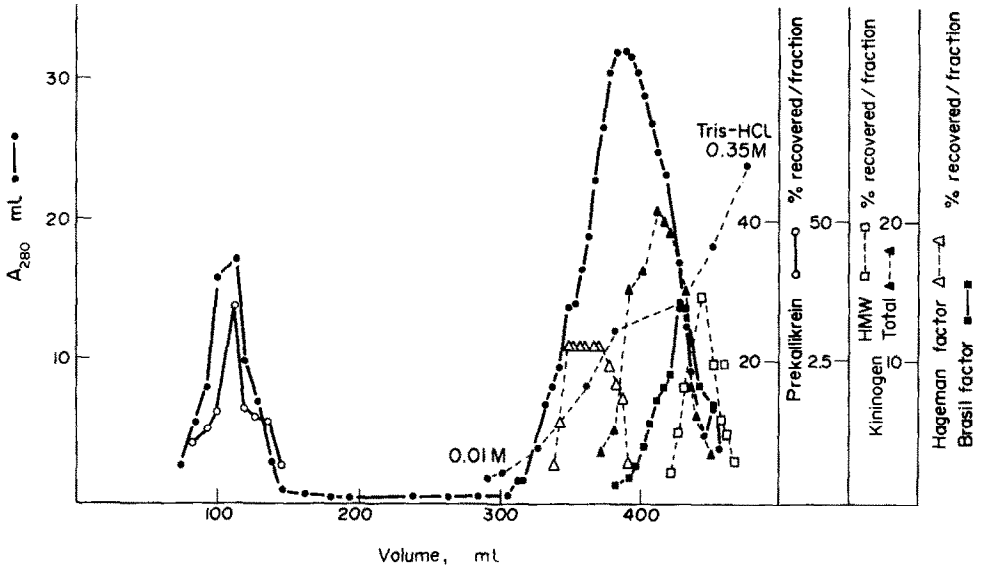


Fig. 2. Partial separation of prekallikrein, Hageman factor, kininogens and Brasil factor by chromatography of dialyzed normal human plasma (56 ml) on DEAE–cellulose (3.6 × 10 cm). Conditions: flow rate 90 ml/hr. Washed with 220 ml of 0.01 M Tris, pH 7.0; gradient 0.01 to 0.35 M Tris, pH 7.0, 75 ml each; washed with 130 ml of 0.35 M Tris, pH 7.0.

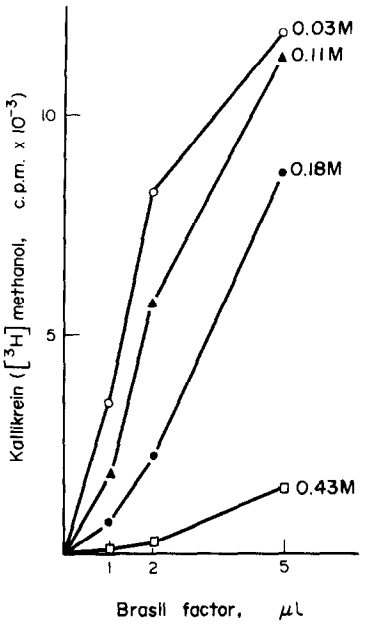


Fig. 3. Influence of NaCl on activation of prekallikrein by Brasil factor. In these experiments 10 μl of 0.5 M Tris, pH 8.0, was mixed with 20 μl of water or NaCl. The reaction was initiated by the addition of 1, 2 and 5 μl of Brasil factor and, at 1-min intervals, 10 μl of dialyzed prekallikrein, cellite (3.0 mg/ml of 0.15 M NaCl) and [³H]TAME were added. Final concentrations: NaCl, 0.03 M, 0.11 M, 0.19 M and 0.43 M.

cellulose at slower flow rates results in activation of some of the components. The Brasil factor found in these fractions was pooled and used in the subsequent studies.

*Some properties of Brasil factor.* The activation of prekallikrein by Brasil factor required the presence of a negatively charged particle, such as celite. In the absence of the particle, no activation could be detected and maximum activation occurred when 20–50 μg celite was added to each sample. In most experiments, 30 μg was employed. Also, the concentration of sodium chloride was of importance. As shown in Fig. 3, in the absence of sodium chloride, except that furnished by the celite solution (0.03 M) maximum formation of kallikrein occurred. Increasing the concentration of NaCl to 0.11 M, 0.19 M and 0.43 M inhibited the activation of prekallikrein by Brasil factor by 28, 60 and 88 percent respectively.

The molecular weight of this crude Brasil factor was estimated by gel filtration. As shown in Fig. 4, two main protein peaks were obtained and Brasil factor eluted together with LMW kininogen in the early proteins of the second peak. Recovery of activity was 47 percent for Brasil factor and 100 percent for total kininogen. A molecular weight of around 70,000 was calculated.

*Differentiation of Hageman factor and Brasil factor.* Because of the partial separation of Hageman factor and Brasil factor by chromatography on DEAE-cellulose (Fig. 2), it was possible to select fractions which contained various proportions of the two prekallikrein activators. These fractions were frozen once at − 20°, and the Hageman factor and Brasil factor again determined (Table 1A). The results are reported in units where the amount of Hageman factor found in fraction 1 and that of Brasil factor found in fraction 3 were assigned values of 100 units respectively. As shown, fraction 1 contained mainly Hageman factor, fraction 3 mainly Brasil factor, and fraction 2 was a mixture of both factors. As the Hageman factor present in fraction 1 was completely inactive, it did not activate prekallikrein under the test conditions used for detection of Brasil factor.

Brasil factor, like Hageman factor, adsorbed readily to celite (Table 1B) but, unlike active Hageman factor, its ability to activate prekallikrein was inhibited by hexadimethrine bromide (2 μg/ml) added after its adsorption to the particle (Table 1B; Refs. 2 and 4). Our earlier results [10] had shown that active Hageman factor adsorbed to a glass surface was not inhibited by

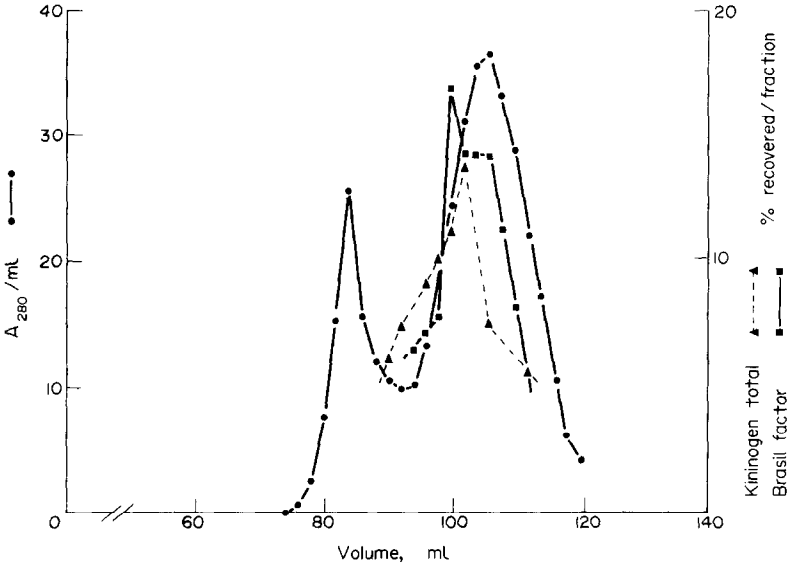


Fig. 4. Filtration of Brasil factor through a Sephadex G-75 (1.5 × 145 cm) column.

Table 1. Differentiation of Hageman factor and Brasil factor: (A) Separation on DEAE–cellulose, (B) Adsorption of Brasil factor on celite and its inhibition with hexadimethrine bromide, and (C) Determination of active Hageman factor in the presence and absence of hexadimethrine bromide.

| A<br>Fraction | Hageman factor<br>(units/ml) | Brasil factor<br>(units/ml) |
|---|---|---|
| 1 | 100 | 8 |
| 2 | 89 | 100 |
| 3 | < 20 | 100 |

| B<br>Treatment | Brasil factor<br>(cpm) | % Inhibition<br>Hexadimethrine bromide<br>(2 μg/ml) |
|---|---|---|
| (1) Centrifuged | 4,080 | 100 |
| (2) Centrifuged in presence of celite and resuspended | 4,653 | 100 |
| (3) Supernatant fraction from centrifugation in presence of celite | < 100 | |
| (4) Celite from above centrifugation resuspended | 4,049 | 100 |

| C | Prekallikrein | |
|---|---|---|
| Active Hageman factor (μl) | Hexadimethrine bromide (40 μg/ml)* | Dialyzed |
| 1 | 4,002 | 4,640 |
| 2 | 5,589 | 7,333 |
| 5 | 10,288 | 11,674 |

* Concentration of hexadimethrine bromide present in prekallikrein preparation after filtration through DEAE-cellulose (see Materials and Methods).

hexadimethrine bromide at concentrations of 100 μg/ml. These results were repeated (Table 1C) using active Hageman factor adsorbed to supercel and, as can be seen, the activity of 1, 2 and 5 μl of this fraction was essentially similar whether it was measured against prekallikrein containing 40 μg/ml of hexadimethrine bromide or on dialyzed prekallikrein.

## DISCUSSION

The initial purpose of these studies was to develop a chemical procedure for the detection of the early components of the intrinsic coagulation system. Such a procedure would be of value in screening the plasma of large numbers of individuals for deficiencies in one of the early components, such as Hageman factor, prekallikrein and HMW kininogen. Because active Hageman factor was known to activate prekallikrein [11–13], we initially developed a method for its measurement [8, 14] which involved adsorption of human plasma on a negatively charged surface (supercel), removal of inhibitors by washing, and measurement of the active Hageman factor bound on the surface of the particle by determining the amount of kallikrein formed from added prekallikrein using a radiolabeled substrate, [$^3$H]TAME. Using this procedure, plasma deficient in either prekallikrein, HMW kininogen or Hageman factor failed to form active Hageman factor. This method, however, required the preparation of prekallikrein by chromatography on DEAE–cellulose, and it was decided to investigate the possibility that the plasma's own prekallikrein could be used as substrate. In the present studies such a procedure was developed, and the amount of kallikrein formed from 1 or 2 μl of normal human plasma could easily be quantitated. As with the previous method, plasma deficient in either Hageman factor, prekallikrein of HMW kininogen failed to generate significant levels of active kallikrein.

It was thought that the additi sn of the missing factor to the deficient plasma would result in activation of prekallikrein, as it had in our earlier studies when active Hageman factor was measured [8]. As expected, when prekallikrein was added to 2μl of plasma deficient in prekallikrein, increasing amounts of prekallikrein resulted in increasing formation of active kallikrein (Fig. 1B). The concentrations of kallikrein employed were similar to those found in plasma and reconstitution of the prekallikrein level to 70 percent of its normal concentration gave maximum activation of kallikrein. However, when these same concentrations of prekallikrein were added to plasma deficient in HMW kininogen, this deficient plasma was also capable of activating prekallikrein. These results were unexpected since, in the absence of HMW kininogen, neither Hageman factor nor prekallikrein should have been activated. Also, prekallikrein added to HMW kininogen-deficient plasma shortened its partial thromboplastin time as does kallikrein [7]. These data suggested to us that still another activator, provisionally called Brasil factor, might exist in human plasma.

Normal human plasma also contains this new prekallikrein activator, as chromatography of this plasma at high flow rates on DEAE–cellulose resulted in a partial separation of Brasil factor from the other plasma components being measured. Brasil factor eluted from these columns after prekallikrein and Hageman factor and

between low and high molecular weight kininogens. It was first thought that this prekallikrein activator might be Hageman factor which had been activated by either the DEAE–cellulose and/or the HMW kininogen present in these fractions. Since Hageman-deficient plasma was not available to us in sufficient quantities to chromatograph on DEAE–cellulose or to attempt to measure directly its ability to activate prekallikrein, we began to examine the properties of this new activator to see if they were similar to active Hageman factor and its fragments. Brasil factor clearly differed from inactive Hageman factor by its pattern of elution from DEAE–cellulose, and it was possible to select column fractions which contained various proportions of the two prekallikrein activators (Table 1A). It also clearly differed from the 30,000 molecular weight fragment of active Hageman factor since it adsorbed to a negatively charged particle (Table 1B) and the fragment does not [9, 13]. In addition, it differed from Hageman factor which had been activated with excess HMW kininogen in that it is inhibited by hexadimethrine bromide (1–2 μg/ml) added after adsorption of the factor to celite, while intact active Hageman factor is not inhibited by concentrations of hexadimethrine bromide up to 40 μg/ml (Table 1C) or greater [10]. The above results indicate that Brasil factor is neither Hageman factor nor its active fragments. If it is a 70,000 molecular weight fragment of Hageman factor, it has properties not described previously and can be activated by a negative particle in the absence of HMW kininogen. It is, of course, not likely to be HMW kininogen since Brasil factor can be detected readily in plasma deficient in this substrate.

The chemical nature of Brasil factor is still not known. No evidence has been found that it exists in plasma as an inactive precursor. Sodium chloride markedly inhibits its ability to activate prekallikrein as does hexadimethrine bromide. A molecular weight of 70,000 was calculated by gel filtration. When the prekallikrein in normal plasma is activated by a negatively charged particle, the proportion of kallikrein formed by Brasil factor, as compared to that activated by Hageman factor, is difficult to determine. Plasma deficient in HMW kininogen is known to have a lowered (10–45 percent) content of prekallikrein [4, 5, 7]. The Fitzgerald plasma used in these studies has only 10–15 percent of the normal content of prekallikrein. Increasing this concentration to the normal levels (Fig. 1C, 10–20 μl) gave substantial activation of prekallikrein and shortened its clotting time. Why, therefore, does Brasil factor not activate the normal levels of prekallikrein found in plasma deficient in Hageman factor (Fig. 1A)? It has been shown recently [15, 16] that prekallikrein circulates in plasma bound to HMW kininogen. It could be that this binding protects prekallikrein from activation with Brasil factor.

The role of Brasil factor in the intrinsic coagulation system remains to be determined. The possibility exists that this factor represents the missing factor previously proposed [8]. In these earlier studies, it was shown that mixtures of Hageman factor, prekallikrein and HMW kininogen in amounts equivalent to that found in plasma only partially activated Hageman factor, whereas plasma deficient in Hageman factor caused complete activation. Kallikrein has also been shown to shorten the clotting time of normal rabbit plasma [17] and, to a lesser extent, that of normal human plasma. It has also been reported to activate Factor VII through its effect as a plasminogen activator [18] and to directly activate Factor IX [19]. This latter possibility is of primary importance since Brasil factor and prekallikrein could thus provide an alternate intrinsic coagulation pathway which could explain the lack of *in vivo* hemostatic disorder of those patients deficient in Hageman factor or HMW kininogen and the relatively mild bleeding tendency of patients deficient in Factor XI.

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