Detection of Factor XIIIa (Active Fibrin-stabilizing Factor) in Normal Plasma

By John C. Nelson and Robert G. Lerner

Factor XIIIa (active fibrin-stabilizing factor) generated in heat-defibrinated plasma by the addition of thrombin can be measured by 14C-putrescine incorporation into casein. Modification of this assay by substituting 3H-putrescine of high specific activity as the donor amine permits measurement of amine incorporation by plasma even in the absence of added thrombin. Incorporation is calcium dependent, inhibited by iodoacetamide, and absent from congenital factor XIII-deficient plasma and from normal platelets. The transamidating activity detected by radioenzymatic assay catalyzed the formation of γ-γ dimers and α polymers of fibrin and was thus biologically functional. This fibrin cross-linking activity was absent from factor XIII-deficient plasma. These experiments show (1) some factor XIII is present in plasma as factor XIIIa; (2) this factor XIIIa can cross-link fibrin and thus has biological activity as well, and (3) this activity is not present in factor XIII-deficient plasma. Factor XIIIa in normal plasma is possibly activated in vivo, perhaps by circulating thrombin, factor Xa, or other proteolytic enzymes.

PLASMA FACTOR XIII (fibrin-stabilizing factor, FSF), when activated to factor XIIIa, in the final steps of normal blood coagulation catalyzes the covalent cross-linking of fibrin by formation of selective γ-gutamyl-ε-lysine bridges between adjacent fibrin molecules yielding covalently cross-linked γ and α chains. This stabilized, cross-linked fibrin has increased mechanical strength, elasticity, and resistance to fibrinolysis and is insoluble in dilute acids or 5 M urea. It has been generally accepted that factor XIII exists in plasma as an inactive precursor and must be activated to factor XIIIa to develop its transamidase and fibrin cross-linking activity. Thrombin and, as recently shown, factor Xa both activate factor XIII, in the case of thrombin by cleavage of small polypeptides, thus providing an interrelationship with activation of the intrinsic and extrinsic clotting pathways. Previous investigations quantitating factor XIII zymogen have shown that it is consumed during clotting in vitro and is decreased in clinical conditions such as disseminated intravascular coagulation that are accompanied by consumption of other clotting factors.

Despite these observations, studies have not been specifically directed at detection or quantitation of factor XIIIa in normal plasma or in pathologic conditions. The radiolabeled putrescine assay for factor XIIIa permits investigation of this aspect of factor XIII behavior. In this type of assay, factor XIIIa, generated in heat-defibrinated plasma by the addition of thrombin, can be measured by incor-
poration of $\text{^{14}C}$-putrescine as a pseudodonor into casein in the presence of calcium and a reducing substance.

We describe a modification of this assay that substitutes $\text{^3H}$-putrescine of high specific activity as the donor amine, which permits measurement of amine incorporation by plasma even in the absence of added thrombin, indicating circulating factor XIIIa. The description of this assay and investigations of some properties of the plasma transamidase activity detected in normal plasma are the subject of this report.

**MATERIALS AND METHODS**

Normal blood was collected from healthy laboratory volunteers by venepuncture into plastic syringes using the two-syringe technique and anticoagulated with 9 parts blood to 1 part citrate anticoagulant (0.06 M sodium citrate, 0.04 M citric acid). Platelet-poor plasma (PPP) was obtained by centrifuging at 13,000 g for 15 min at 4°C. The supernatant PPP was pipetted with a plastic pipette and kept in a plastic vial in an ice bucket until use. Normal gel-filtered platelets, free of plasma factor XIII, were obtained by a modification of the method of Tangen et al. Platelet-rich fractions were pooled and the platelets were centrifuged and resuspended in a volume of eluate calculated to yield a final concentration of 10^6 platelets/ml. Factor XIII deficient plasma, obtained from a male patient with known congenital factor XIII deficiency, was purchased from George B. King Biomedical, Salem, N.H. $\text{^3H}$-putrescine was obtained from Amersham Searle, Arlington Heights, Ill., had a specific activity of 12.3 Ci/m mole, and was stored as an 81.3 $\mu$M aqueous solution at 4°C. Casein (Hammarsten, Gallard Schlesinger, New York) 5.5% in Tris buffer (hereafter referred to as Tris buffer), was stored at -20°C. Factor XIII free fibrinogen was obtained by dissolving 1 g lyophilized human fibrinogen (Armour Pharmaceutical, Phoenix, Ariz.) in 100 cc 3.3 M urea in 0.1 M sodium phosphate buffer pH 7.6 for 12 hr, inactivating factor XIII as described previously. After dialysis against 0.3 M NaCl, the fibrinogen was stored at -20°C in small aliquots. This fibrinogen was free of factor XIII activity when tested by clot solubility and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by our radioenzymatic assay.

Thrombin was prepared by reconstituting a vial of Parke Davis bovine thrombin, 5000 units, with 5 cc of a fluid made by adding 1 cc of 2% sodium oxalate to 5 cc 0.85% NaCl. The thrombin solution was adsorbed with barium sulfate 100 mg/ml for 10 min at 37°C and stored in small aliquots at -20°C. Glycerol thrombin was made by adding 20 ml 0.85% NaCl and 25 ml glycerol to the above solution, giving a final concentration of 100 U/ml. Purified thrombinlike enzyme from Agkistrodon rhodostoma (Ancrod), free of factor XIII activating activity, was kindly supplied by Grant Barlow, Abbott Laboratories, North Chicago, III.

Factor XIII free fibrin monomers were prepared by first adding 3.3 ml 5 M urea adjusted to pH 5.3 with monochloroacetic acid to 5 ml factor XIII free fibrinogen, 9.2 $\mu$g/ml. Fibrin was formed by addition of glycerol thrombin (20 units) to the solution followed by incubation for 30 minutes at 37°C. The fibrin was polymerized by diluting in 20 vol 0.025 Tris 0.1 35 acid urea adjusted to pH 5.3. These steps were repeated four more times to remove remaining thrombin, and the fibrin was finally dissolved in 5 M urea pH 5.3.

SDS-PAGE was performed according to the method of Weber and Osborn as modified by Schwartz et al. The acrylamide:bis ratio was maintained at 42:1.

Diisopropylfluorophosphate (DFP) (ICN, K&K Laboratories, Plainview, N.Y.) was freshly dissolved in dry isopropanol just prior to use. Iodoacetamide (Mann Research Laboratories, New York, N.Y.) was dissolved in Tris buffer 0.3 M pH 7.4 just prior to use.

Radioenzymatic assay for factor XIII/XIIIa using $\text{^3H}$-putrescine. This assay was modified from that of Dvilkansky et al. First 0.1 ml citrated PPP in a 10 x 75 mm polystyrene test tube was heated at 56°C for 4 min to denature fibrinogen and immediately chilled on ice. To this was added 0.10 ml stock casein diluted 1:3 in Tris buffer, 0.010 ml 1.2 M mercaptoethanol in Tris buffer, 0.010 ml 0.5 M calcium chloride in Tris buffer, 0.010 ml $\text{^3H}$-putrescine, and either 0.010 ml thrombin (1000U/ml) or Tris buffer. The reaction mixture was vortexed briefly (Vortex-Genie, Scientific Industries, Springfield, Mass.) and incubated at 37°C in a water bath. The reaction was stopped at times specified in the text by removing 0.1 ml of the reaction mixture onto a 3-cm-sq piece of Whatman 3mm filter paper and immersing the filter paper in cold trichloroacetic acid (TCA) (5 g/dl) for at least 20 min or until completion of the experiment. For continuous rate assays, parallel samples were used. The filter papers
FACTOR XIIIa IN NORMAL PLASMA

were then washed together six times for 15 min with the TCA using at least 10 ml TCA per filter paper by shaking in a Dubnoff horizontal incubator. The filter papers were then washed once for 15 min in acetone-ethanol (1:1 v/v) and once for 15 min in acetone and dried in a 60°C oven for at least 30 min. The filter papers were counted in 10 ml Bray’s solution in a liquid scintillation counter (Packard Tri Carb spectrometer model 3320). The radioactivity from a reaction mixture prepared with Tris buffer substituted for plasma was uniformly low over a 6-hr incubation period, with about 1500–2500 counts per minute (cpm). The radioactivity of this blank reaction was subtracted from the experimental assays to obtain the net incorporation of H-putrescine into casein. The SD of multiple blanks was always less than 5% of the mean. Other controls without casein showed H-putrescine incorporation into TCA-precipitable material, presumably other plasma proteins.

Fibrin cross-linking activity of normal plasma. In these experiments, Factor XIII free fibrin monomers were allowed to polymerize and form gels in various test plasmas. Plasma samples comprised normal citrated PPP, citrated factor XIII-deficient plasma, or EDTA-anticoagulated plasma. Each was heated to denature fibrinogen, the precipitate was removed by centrifugation, and 0.25 ml was then diluted 1:2 with 0.3 M Tris buffer pH 7.4 in a dialysis bag. To this was added 0.5 ml factor XIII-free fibrin monomer solution, 19 mg/ml in 5 M urea pH 5.3, 0.01 ml 2.2 M calcium chloride in Tris buffer, 0.01 ml 6 M mercaptoethanol in Tris buffer, and either 0.1 ml glycerol thrombin, 0.1 ml heparin (600 U/ml) or 0.1 ml 0.3 M Tris buffer pH 7.4. These were dialyzed overnight at 4°C against 200 vol 0.025 M Tris HCl and 0.135 M NaCl, pH 7.3, with 20 mM CaCl, and 0.05 M mercaptoethanol. The EDTA plasma-fibrin monomer mixtures were dialyzed against the same dialysate without calcium. The dialysis bath was then brought to 37°C and further incubated for 1 hr. The gels were removed from the bags and rinsed by stirring each gel in 500 ml normal saline. Then some gels were placed in 2.0 ml 5 M urea pH 8.0 for 72 hr at 37°C. Dissolved fibrin was quantitated spectrophotometrically by absorption at 280 nm and was expressed as percentage total fibrin of a parallel gel completely dissolved in alkaline urea (40 g/dl in 0.2 M NaOH). Other parallel gels were solubilized and subjected to SDS-PAGE.

RESULTS

Radioenzymatic assay using H-putrescine for measurement of factor XIII. The incorporation of H-putrescine into casein by plasma with a reaction mixture containing added thrombin is depicted in Fig. 1, curve A. The initial 5–20-min delay in incorporation corresponds to the period required for complete activation of the zymogen factor XIII by thrombin and dissociation of its subunits and has
been noted by others.\textsuperscript{12,13} After this delay, the incorporation was linear for at least 2 hr, beyond which the rate of incorporation sometimes decreased.

**Detection of transamidase activity in normal plasma.** When thrombin was omitted from the reaction mixture, \(^{3}H\)-putrescine incorporation into casein by plasma could still be detected, as shown in curve B of Fig. 1. Other experiments showed that there was no initial lag phase and that this incorporation was linear for up to 6 hr. Net incorporation in 2 hr, after subtraction of the blank reaction, generally was about 6,000–12,000 cpm. With this assay for factor XIIIa determination in 28 normal subjects, the incorporation into TCA-precipitable protein was 4.2 ± 0.96 (2 SD) pmole putrescine incorporated/120 min incubation. Using undiluted thrombin-treated plasma to measure total factor XIII in these 28 normal subjects, a mean of 71.1 (range 50–116) pmoles \(^{3}H\)-putrescine was incorporated per 2-hr incubation. A mean of 5.9% (range 4.0%–8.3%) of the total factor XIII was activated. Dilution of plasma with Tris buffer resulted in an apparently greater proportion of activated enzyme.

**Fibrin cross-linking activity of normal plasma.** To see if the factor XIIIa activity detected by radioenzymatic assay had biologic function and could cross-link fibrin, experiments were performed in which soluble fibrin monomers were allowed to polymerize and form gels in plasma but without activation of coagulation. This was accomplished by adding to plasma fibrin monomers solubilized in 5 \(M\) urea pH 5.3 and then dialyzing the urea under appropriate pH and salt conditions. Fibrin gels formed in the dialysis bag in physical association with factors XIII and XIIIa.

Fibrin gels formed in normal plasma with thrombin added were substantially insoluble in 5 \(M\) urea, indicating that \(\gamma-\gamma\) cross-linking and possibly \(\alpha\) cross-linking had taken place.\textsuperscript{15} Fibrin gels formed in normal PPP without added thrombin, with or without heparin, were only partially soluble in 5 \(M\) urea over 72 hr. Gels formed in factor XIII-deficient plasma, with or without thrombin, or in EDTA plasma were completely soluble in 5 \(M\) urea within 2 hr (Table 1).

The pattern of cross-linking of these fibrin gels was investigated by SDS-PAGE to separate the polypeptide chains of fibrin and allow detection of cross-linked \(\gamma-\gamma\) dimers and \(\alpha\) polymers. The gel in Fig. 2 labeled fibrin shows the \(\alpha, \beta, \gamma\) chains of non-cross-linked fibrin.

The fibrin gel that had polymerized in normal plasma containing added thrombin and calcium is shown in Fig. 2 to be partially cross-linked fibrin. In this

<table>
<thead>
<tr>
<th>Test Plasma</th>
<th>Solubility in 5 (M) Urea (37 (^{\circ})C at 72 hr) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recalculated citrated plasma with thrombin</td>
<td>15.1 ± 8.3</td>
</tr>
<tr>
<td>Recalculated citrated plasma with buffer</td>
<td>62 ± 11</td>
</tr>
<tr>
<td>Recalculated citrated plasma with heparin</td>
<td>65 ± 14</td>
</tr>
<tr>
<td>Recalculated factor XIII-deficient plasma with thrombin</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Recalculated factor XIII-deficient plasma with buffer</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>EDTA plasma with thrombin</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>EDTA plasma with buffer</td>
<td>100 ± 0</td>
</tr>
</tbody>
</table>

Factor XIII-free fibrin monomers were polymerized into gels in the presence of various test plasmas and examined for cross-linking by dissolving each gel in 5 \(M\) urea. Solubility of gels expressed as percent original fibrin dissolved ± SE.
Fig. 2. Fibrin cross-linking activity of normal plasma. When fibrin monomers were allowed to polymerize in presence of various plasmas, γ-γ dimers formed in plasma activated with thrombin (partially cross-linked fibrin) and in plasma even without added thrombin (fibrin in normal plasma) but not in factor XIII-deficient plasma (fibrin in XIII-deficient plasma). Dimers shown as prominent new bands in 7.5% SDS polyacrylamide gels.

gel, in addition to bands representing α, β, and γ chains, two prominent new bands appeared. The first with molecular weight 100,000 daltons, corresponds to γ-γ dimers; the second, of higher molecular weight and which did not penetrate the polyacrylamide gel, corresponds to α polymers. Several faint bands of higher molecular weight may represent α oligomers. Additional incubation of the gel beyond 1 hr prior to solubilization for electrophoresis resulted in further disappearance of the γ-chain band and formation of a denser α polymer band and a γ-γ dimer band (not shown).

The fibrin gel that had polymerized in normal plasma without added thrombin is shown in Fig. 2 as the gel labeled fibrin in normal plasma. This fibrin gel, which was partially soluble in 5 M urea, also showed evidence of cross-linking with the formation of a prominent new 100,000 dalton band corresponding to γ-γ dimers. Although only traces of α polymers could be obtained if the fibrin gel was incubated at 37°C for 1 hr, greater amounts were obtained if the gels were incubated for 4 hr prior to solubilization for electrophoresis. Complete fibrin cross-linking with total disappearance of monomeric α and γ chains was never achieved.

In contrast to the results of others showing that complete γ-γ dimerization took place prior to α polymerization, our gels showed some formation of α polymers without complete γ-γ dimerization. The explanation for this discrepancy and the kinetics of this reaction were not investigated further, but our results may have been due to the low concentration of thrombin or partial denaturation of fibrin that could have occurred during its initial preparation from urea-treated fibrinogen or with its repeated polymerization and depolymerization. Another factor may have been the relatively impure fibrinogen used as starting material.

In contrast to partially cross-linked fibrin gels that formed in normal plasma, the fibrin gel that had polymerized in factor XIII-deficient plasma showed no evidence of cross-linking on electrophoresis by either γ-γ dimerization or α polymerization, as shown in the gel in Fig. 2 labeled fibrin in XIII-deficient plasma. The
Fig. 3. Calcium dependence of plasma transamidase. Plasma transamidase activity in citrated PPP measured by \(^{1}H\)-putrescine assay with reaction mixture containing calcium (A). In reaction mixture without added calcium, activity was restored when calcium was added at 2 hr (B) and was absent from plasma anticoagulated with EDTA (C). Mean of two determinations.

three bands present in this gel were the same as those of non-cross-linked fibrin, i.e., \(\alpha, \beta, \) and \(\gamma\) chains. This same pattern was observed with the fibrin gel formed in EDTA plasma dialyzed against a calcium-free dialysate.

Absence of transamidase activity in platelets. Normal platelets, separated from plasma proteins by gel filtration, were disrupted by freeze-thawing three times, resulting in release of platelet factor XIII. However, none of the released factor XIII was already activated when tested by radioenzymatic assay. In addition, factor XIIIa was present in the PPP of a patient with chemotherapy-induced thrombocytopenia (platelets 2000/mm\(^3\)) as well as in the PPP of two other patients with autoimmune thrombocytopenia of a similar magnitude.

Properties of plasma transamidase activity. The activity of both factor XIII and various tissue transamidases is calcium dependent. The calcium dependence of the enzymatic activity detected by radioenzymatic assay was investigated by omitting calcium chloride from the reaction mixture or by testing plasma anticoagulated with 5.4 mM EDTA instead of citrate. In the absence of added calcium, no transamidase activity could be detected (Fig. 3). If calcium chloride was added to the reaction mixture (final concentration 21 mM) at 2 hr, full enzymatic activity could be restored. It should be noted that while this calcium concentration was optimal for this assay system, most of the calcium was bound to casein.\(^{18}\)

Calcium allows unmasking of an active-site cysteine residue on the \(\alpha'\) chain that is essential for transamidase activity, and this reaction has been titrated by thiol alkylation with \(^{14}C\)-iodoacetamide.\(^{19}\) To see if the enzymatic activity detected in normal plasma could be abolished by alkylation of thiol groups, heat-defibrinated normal PPP was diluted 1:5 with Tris buffer and enough iodoacetamide and calcium chloride to give final concentrations of \(8.0 \times 10^{-9} M\) and \(0.1 M\), respectively. This mixture was incubated for 30 min at 25°C, and 0.1 ml was assayed for transamidase activity, but without mercaptoethanol in the reaction mixture. This alkylation abolishes all transamidase activity even if the test plasma is first activated by added thrombin. No radioactivity was incorporated into casein over a 4-hr incubation.

The \(K_m\) value of the plasma enzyme for \(^{1}H\)-putrescine was determined from a double-reciprocal plot by varying the final concentration of putrescine from 3.7 to 22.2 \(\mu M\) in reaction mixtures without added thrombin. The value of \(1.4 \times 10^{-11}\) is
FACTOR XIIIa IN NORMAL PLASMA

Fig. 4. $K_m$ of plasma transamidase. Final concentration of $^3$H-putrescine was varied from 3.7 to 22.2 $\mu$M; curve derived from least-squares fit using programmed calculator. $K_m$ of plasma transamidase for $^3$H-putrescine is $1.4 \times 10^{-4}$.

Nearly identical to that previously published for factor XIII by Lorand et al.\textsuperscript{17} (Fig. 4).

It is well known that other human and animal tissues contain transamidating enzymes, some of which are not zymogens and do not require prior activation by thrombin or other proteolytic enzymes.\textsuperscript{20} The specificity of the transamidase activity detected in normal plasma was confirmed by the total absence of activity in the plasma of a male patient with congenital factor XIII deficiency. The $^3$H-putrescine incorporation by this plasma was identical to the blank (data not shown).

Finally, despite particular precautions in handling specimens, including careful venepuncture and rapid handling of specimens, it was considered possible that some factor XIII could become artifactually activated to factor XIIIa in vitro, perhaps by traces of thrombin or factor Xa present in plasma\textsuperscript{20,21} or generated during incubation. This possibility was excluded first by an experiment in which blood was drawn into a syringe containing heparin (10 or 50 U/ml final concentration) prior to immediate citrate anticoagulation. The counts were the same as controls without heparin. Also, standard samples (obtained without heparin) had the same cpm as heparin-containing samples to which exogenous thrombin (5 U/ml final concentration) had been added. This argues strongly that detectable artifactual activation by thrombin or factor X did not occur in vitro. In other experiments, heparin, (10 or 50 U/ml) was added to citrated PPP, or citrated PPP was treated with the protease inhibitor DFP $10^{-3}$ $M$ in isopropanol at 37°C for 60 min prior to factor XIIIa assay. Here, too, the counts were the same as standard samples of citrated PPP, additional evidence that there was no artifactual activation during incubation (Fig. 5). Factor XIIIa was also detectable in plasma extensively adsorbed up to five times with 2.3% aluminum hydroxide\textsuperscript{22} either before or after being heated to $56^\circ$C for 4 min.

Two experiments showed that the heating step used to denature fibrinogen did not artifactually activate factor XIII. First, one of two 1.0-ml aliquots of normal PPP was heated to $56^\circ$C for 4 min to denature fibrinogen, the precipitate was removed by centrifugation, and each sample was assayed. The heat-treated sample had the same counts as the control. Second, citrated PPP was clotted with Ancrod (4.2 U/ml final concentration), the fibrin gel was removed with a plastic stirring rod, and parallel samples were assayed for factor XIIIa with or without heating to $56^\circ$C for 4 min. The factor XIIIa level in the heated sample was the
Fig. 5. Exclusion of artifactual factor XIII activation in vitro. There was no alteration in factor XIIIa activity when blood was drawn into a syringe with heparin, where heparin was added to citrated PPP, or when citrated PPP was incubated with DFP prior to assay for factor XIIIa. Controls, standard citrated samples without heparin or DFP or standard samples to which thrombin was added. N = 3, SEM.

same as that in the unheated control. It is noteworthy that the levels of factor XIIIa and factor XIII zymogen were both decreased in the serum of the samples defibrinated by Ancrod. We do not believe that this was due to destruction of factor XIII by Ancrod, since the addition of Ancrod to plasma that has already been heat-defibrinated has no effect on either factor XIIIa or factor XIII levels. Thus the decrease in factor XIII in the serum of Ancrod-defibrinated samples may represent the loss of factor XIII by binding to polymerizing fibrin.

DISCUSSION

These experiments show that factor XIIIa can be detected in normal plasma by using a radioenzymatic assay and, furthermore, that this factor XIIIa can cross-link fibrin and thus has biologic activity as well. This activity is not present in factor XIII-deficient plasma or in normal platelets.

In decalcified plasma, factor XIII has a tetrameric structure consisting of two pairs of identical subunits designated α and β, and a molecular weight of about 330,000 daltons. Thrombin cleaves a 4000-dalton polypeptide fragment from the α chain, yielding α' and β', the catalytic capacity of factor XIIIa residing solely on the α' subunits. Activation by purified thrombin is not calcium dependent, while activation by factor Xa is calcium dependent. Subsequently, calcium also allows unmasking of a specific cysteine residue on the α' chain essential for transamidase activity. The unmasking of the reactive cysteine is coupled with the release of the α' from the β subunits, a reaction that can be inhibited or reversed by EDTA or modified by the concentration of the β subunit. Thus factor XIII activation is a complex process involving one or more calcium-dependent steps resulting in unmasking of a reactive thiol group.

When normal plasma was tested for its ability to catalyze cross-linking 1H-pu- trescine with acceptor casein, measurable incorporation occurred even when no thrombin was added to activate factor XIII, indicating that normal plasma has transamidase activity. Although factor XIII activity is detectable in plasma by radioenzymatic assay, its ability to catalyze fibrin cross-linking is needed to show biologic function. Schwartz et al. showed that plasma or platelet factor XIIIa catalyzes the rapid dimerization of fibrin γ chains followed by formation of high molecular weight polymers of α chains. Only very small quantities of factor XIII are needed to catalyze these reactions, and the degree of fibrin solubility in acetic
acetic acid is related to the relative content of \( \gamma-\gamma \) dimers and \( \alpha \) polymer cross links. In our experiments, fibrin gels formed in normal plasma were only partially soluble in 5 M urea and showed new bands of higher molecular weight corresponding to \( \gamma-\gamma \) dimers and \( \alpha \) polymers. Although liver transamidase can also cross-link fibrin and render it insoluble, the kinetics and patterns of fibrin cross-linking are different from those catalyzed by plasma factor XIIIa. Liver transamidase catalyzes \( \alpha \) polymer formation, but it causes \( \gamma \) polymers rather than pure \( \gamma-\gamma \) dimer formation.\(^{23}\) Thus the pattern of cross-linked fibrin gels that contained prominent \( \gamma-\gamma \) dimers as well as \( \alpha \) polymers is additional evidence that the transamidase activity detected in normal plasma is that of factor XIIIa rather than other tissue enzymes. The radioenzymatic assay showed that this transamidase activity was calcium dependent and was completely abolished by prior incubation of plasma with iodoacetamide, which inactivated thiol groups by alkylation to carboxymethylcysteine. Other investigators have shown that iodoacetamide binds a cysteine residue on the \( \alpha' \) protomer and does not bind to the inactive zymogen.\(^{19}\)

In plasma, factor XIIIa obeys Michaelis kinetics. The \( K_m \) of plasma factor XIIIa for \(^3\)H-putrescine, determined in these experiments to be \( 1.4 \times 10^{-4} \), is nearly identical to the values of \( 1.4-1.5 \times 10^{-4} \) previously obtained by Lorand et al.\(^{17}\) for factor XIIIa. Other tissue transamidases detected in plasma probably have different \( K_m \) for putrescine; thus the transamidase detected in plasma probably is not one of these other enzymes. Although the final concentration (\( 3.7 \times 10^{-6} \) M) of \(^3\)H-putrescine in the reaction mixture in our assay was far below this \( K_m \), it should be noted that the fractional incorporation of the isotope during incubation is so small (less than 5% of the available substrate being incorporated into casein) that the concentration of substrate is nearly constant. The velocity of the reaction thus may remain proportional to the concentration of enzyme,\(^{26}\) and other experiments have shown there is no advantage to raising the \(^3\)H-putrescine concentration in our assay.

Other tissues as well, including lung, liver, spleen, uterus, red blood cells, placenta, and hair follicles, all contain significant transamidase activity. These enzymes are distinctly different from the transamidase of platelets and plasma.\(^{20, 27}\) We discount the possibility that one of these tissue transamidases is responsible for the transamidase activity of normal plasma, since no transamidase activity was detectable in the plasma of a male patient with congenital factor XIII deficiency who would be expected to have normal tissue transamidases.

Normal platelets also contain factor XIII, which, however, consists only of pairs of \( \alpha \) subunits apparently identical to the plasma \( \alpha \) subunits.\(^{29}\) Platelets do not appear to be the source of plasma factor XIIIa, since we found no factor XIIIa in gel-filtered platelets and normal plasma factor XIIIa in three severely thrombocytopenic patients.

It is recognized that the critical factor in detecting factor XIIIa is not the sensitivity of the methodologies utilized herein. In fact, many other radioenzymatic and fluorimetric assays and SDS-PAGE have each been successfully employed to measure equally low factor XIIIa generated by thrombin activation of plasma factor XIII or purified factor XIII. However, none of these have been applied to detect factor XIIIa in normal plasma. These data, showing for the first time that factor XIIIa is present in normal plasma, are consistent with reports of other investigators, who have remarked that purified factor XIII prepared from
normal plasma may contain some material that is already activated.\textsuperscript{9,29} The amount of activated material is apparently usually only 1\%-15\% of the total, but up to 60\%-90\% of the peak-4 eluate from DEAE-cellulose chromatography may be already activated if outdated blood is used as the starting material.\textsuperscript{30} Purified factor XIII preparations should be tested for factor XIIIa, which would be overlooked unless specifically sought, by adding calcium but omitting thrombin from the test system.

Our experiments are in accord with the concept that factor XIIIa is generated in vivo by thrombin, factor Xa, or another proteolytic enzyme, such as trypsin. The surprising finding is that substantial activated material is already present in normal plasma. Recent work by others\textsuperscript{21,31} has focused on quantitation of thrombinlike activity in normal plasma that might account for the generation in vivo of factor XIIIa. These experiments have used circulating fibrinopeptide A levels, the kinetics of fibrinopeptide A cleavage in vitro, or detection of active factor V and have resulted in conclusions that normal plasma has some thrombin present, with levels calculated to be $6.6 \times 10^{-6}$ U/ml.\textsuperscript{21}

The biologic consequences of circulating factor XIIIa remain speculative. It has been previously hypothesized\textsuperscript{32} that transamidases other than factor XIIIa may be present in normal plasma or appear under pathologic conditions such as muscle disease or liver injury. However, our experiments suggest that the only transamidase activity present in normal plasma is that of factor XIIIa. Nevertheless, tissue transamidases might still appear in plasma in various diseases. Random cross-linking of most plasma proteins by factor XIIIa is unlikely because of the low affinity of factor XIIIa for normal plasma proteins except fibrin and, to a lesser extent, fibrinogen.\textsuperscript{33} However, amine incorporation catalyzed by plasma factor XIIIa into various proteins might occur. For example, it has been hypothesized that in patients taking isonicotinic acid hydrazide (isoniazid, INH) who eventually develop autoimmune factor XIII inhibitors, the initial event is the haptenic incorporation of INH as an amine-containing pseudodonor into a protein, producing a new immunogenic antigen.\textsuperscript{34} This reaction could be catalyzed by the factor XIIIa present in normal plasma. In addition, factor XIIIa has been shown to cross-link fibronectin (cold-insoluble globulin) to the $\alpha$ chain of fibrin.\textsuperscript{35} Although a definite function for fibronectin has not yet been established, it may be important in such diverse processes as cell to cell adhesion, cell spreading, or malignant cell growth.

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