

A Rapid and Sensitive ^{125}I -Fibrin Solid-Phase Fibrinolytic Assay for Plasmin

By Leonard A. Moroz and Norbert J. Gilmore

^{125}I -fibrinogen, adsorbed to polystyrene tubes at low ionic strength and treated with thrombin, serves as a substrate for a rapid, convenient, and sensitive test tube assay for plasmin and activators and inhibitors of this enzyme. ^{125}I -labeled digestion products released from the ^{125}I -fibrin-polystyrene matrix are readily separated and quantitated and behave, on gel permeation, in the same manner as plasmin-generated degradation products from an unlabeled conventional fibrin clot. The ^{125}I -fibrin, in probable non-cross-linked form, is firmly bound to the polystyrene and is resistant to nonspecific release, with control (no enzyme) values equivalent to $15.2 \text{ ng} \pm 1.2 \text{ (SD)}$ fibrin (1% of the total bound ^{125}I -fibrin). This fact permits consistent detection of lysis of

30–50 ng ^{125}I -fibrin, which exceeds published sensitivities (1000–5000 ng) using ^{125}I - or fluorochrome-labeled fibrin clots as substrate. The sensitivity for plasmin (0.2 $\mu\text{g/ml}$) is tenfold greater than that of the fibrin-plate method (2.0–2.5 $\mu\text{g/ml}$), while sensitivities for streptokinase and urokinase activation of plasmin are 0.02 U/ml and 0.04 CTA U/ml, respectively (sensitivity of fibrin-plate method, 0.5 U/ml for both). The method provides a reasonable analogue of the solid-phase nature of fibrin under physiologic conditions, and the ease of preparation of large batches of tubes makes the method suitable for large-scale screening of factors modulating the plasminogen-plasmin system.

PLASMINOGEN IS THE proenzyme in plasma which, upon conversion to its active form, plasmin (E.C. 3.4.4.14), plays a central role in interrelationships among coagulation, fibrinolytic, and inflammatory processes.¹ Examination of physiologic factors which modulate the function of plasmin either directly or by influencing the activation of its proenzyme requires methodology which monitors its enzymatic activity on its major physiologic substrate, fibrin. Available methods, including those based on lysis of radioactively or fluorochrome-labeled fibrin clots²⁻⁴ or of fibrin gels (fibrin plate method),⁵⁻⁶ are often insufficiently sensitive for this purpose, are excessively time consuming, fail to monitor activators/inhibitors which enter the substrate poorly (in the case of gels or clots), or do not provide adequate separation of reaction products from residual substrate.

Although the mechanism is unexplained, the firm adsorption of protein (in the form of antibody or antigen) to polystyrene at low ionic strength is an established feature of solid-phase radioimmunoassay technology.^{7,8} We have used this binding as the basis for a solid-phase radioassay method. ^{125}I -fibrinogen is adsorbed to polystyrene tubes and converted to fibrin by thrombin. The result-

From the Harry Webster Thorp Laboratories, Division of Clinical Immunology, McGill University Clinic, Royal Victoria Hospital, Montreal, Quebec, Canada.

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Address for reprint requests: L. A. Moroz, M.D., Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1.

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ing ^{125}I -fibrin-polystyrene matrix provides the basis for a rapid, sensitive, and convenient assay of plasmin and of activators and inhibitors of this enzyme. In addition, the assay is amenable to similar study of other proteolytic enzymes which degrade fibrin.

MATERIALS AND METHODS

Reagents and materials were obtained from the following sources: ϵ -aminocaproic acid (EACA), bovine thrombin (400 U/mg), bovine fibrinogen (type I), myoglobin (whale skeletal muscle), cytochrome C (horse heart), horseradish peroxidase (type II), pronase (bacterial protease, type VI, *Streptomyces griseus*), and Tris(hydroxymethyl) aminomethane (Tris) from Sigma Chemical Company, St. Louis, Mo.; human fibrinogen from Connaught Medical Research Laboratories, Toronto, Canada; bovine serum albumin (BSA) from Pentex, Inc., Kankakee, Ill.; collagenase (clostridiopeptidase A), papain (*Papaya latex*, 20 U/mg), α -chymotrypsin (bovine pancreas, 50 U/mg), pepsin (swine stomach mucosa, 2800 U/mg), trypsin (bovine pancreas, 188 U/mg) from Worthington Biochemical Corporation, Freehold, N. J.; thrombin (bovine, topical), Parke, Davis & Company, Montreal, Canada; Varidase (streptokinase-streptodornase, for intramuscular use) from Lederle Products Department, Cyanamid of Canada Ltd., Montreal, Canada; Bio-Gel A 50 m and A 0.5 m from Bio-Rad Laboratories, Richmond, Calif.; Sephadex G-25 (coarse), Sepharose 4B, blue dextran 2000, aldolase from Pharmacia, Uppsala, Sweden; trans-4-amino-methylcyclohexanecarboxylic acid (AMCHA, tranexamic acid) from Aldrich Chemical Company, Milwaukee, Wis.; heat-inactivated fibrin plates (Enzo-diffusion plate kits) from Hyland Division, Travenol Laboratories, Costa Mesa, Calif.; disposable polystyrene culture tubes (Lab-Tek no. 4411, 12 \times 75 mm) from Lab-Tek Products, Miles Laboratories, Westmont, Ill. Urokinase (Abbott 38790) was a generous gift of Dr. J. Donahoe, Abbott Laboratories, North Chicago, Ill. Ragweed allergen Ra5 was kindly provided by Dr. L. Goodfriend.

Preparation of Human Plasminogen (PLG) and Plasmin (PL)

Plasminogen was prepared from normal human plasma by affinity chromatography on lysine-Sepharose 4B by the method of Deutsch and Mertz,⁹ followed by gel permeation on Bio-Gel A 50 m. It was quantitated by absorbancy at 280 nm, employing the extinction coefficient, $E_{1\text{cm}}^{1\%} = 17$.¹⁰ PLG was activated to PL by incubation with streptokinase (SK) in the form of Varidase. Maximal activation of PLG to PL was obtained by incubation of PLG, 25 $\mu\text{g}/\text{ml}$, and SK, 20 U/ml, for 30 min at 37° C in 0.015 M Tris, 0.15 M NaCl, pH 7.4 (Tris-NaCl buffer).

Radioiodination of Fibrinogen

Human or bovine fibrinogen was dissolved in Tris-NaCl buffer, clarified by centrifugation (10,000 g, 10 min), and chromatographed on Bio-Gel A 0.5 m. The fibrinogen was then freed of contaminating PLG by passage through a lysine-Sepharose column.⁹ It was quantitated by absorbancy at 280 nm, using the extinction coefficient, $E_{1\text{cm}}^{1\%} = 13.6$.¹¹ Labeling with ^{125}I (New England Nuclear Corporation, Montreal, Canada) was performed by a modification of the chloramine T method,¹² with the following reaction conditions: 0.5 ml of fibrinogen solution (2.5–5.0 mg/ml) was reacted with 0.1 ml of chloramine T solution (4 mg/ml) and ^{125}I iodide (1–2 mCi in 0.1 ml) for 2 min at room temperature and the reaction terminated by addition of 0.4 ml of Na metabisulfite (2.5 mg/ml), followed by addition of 0.1 ml of carrier KI (10 mg/ml). All reactants were dissolved in 0.05 M phosphate buffer, pH 8.0, and Sephadex G-25 separation of ^{125}I -fibrinogen and free ^{125}I iodide was carried out in the same buffer. The Sephadex G-25 column was precoated in the usual manner with BSA, but radioactive fractions were collected into tubes without addition of carrier BSA.

The specific activity of the ^{125}I -fibrinogen prepared by this method was 1.5–1.7 $\mu\text{Ci}/\mu\text{g}$, and more than 83% of the radioactive fibrinogen was clottable upon addition of thrombin. It was immediately adjusted to a specific activity of 40,000–75,000 cpm/ μg by addition of unlabeled fibrinogen prepared in the same manner as that used for radioiodination and was stored at 4° C until used for coating assay tubes, generally the same day.

Preparation of ¹²⁵I-Fibrin-coated Tubes

Disposable polystyrene culture tubes were used. For coating, ¹²⁵I-fibrinogen solution was adjusted to 0.015 M in phosphate by addition of water, to provide an ionic strength (approximately 0.1) which would provide adequate binding to the tubes, as determined previously in our laboratory.⁸ Into each tube was pipetted 0.1 ml of the adjusted fibrinogen solution, representing 10 μg of ¹²⁵I-fibrinogen (400,000–750,000 cpm). The tubes were rotated in a tissue-culture rotator at room temperature and an angle of 45° for 3 hr. The unbound ¹²⁵I-fibrinogen was then aspirated by suction and replaced by 0.5 ml of BSA solution (10 mg/ml in Tris–NaCl buffer), to bind any polystyrene sites unoccupied by the ¹²⁵I-fibrinogen. The tubes were allowed to stand 20 min at room temperature, following which they were aspirated and washed four times with 4.5-ml aliquots (one tube volume) of Tris–NaCl buffer.

After aspiration to near dryness, 0.2 ml of Tris–NaCl buffer, containing 2 U of topical thrombin, was pipetted into each tube, and the tubes were incubated at 37°C for 5 min. This amount of thrombin was found, in preliminary experiments, to suffice for the clotting of 1 mg of fibrinogen. Thrombin was freed of plasminogen by passage through a lysine–Sepharose column⁹ before use.

After aspiration of the thrombin solution, tubes were washed twice with Tris–NaCl buffer (4.5-ml aliquots), filled with the same buffer containing 0.1% sodium azide, and stored at 4°C until required. Tubes prepared in this manner were useable for at least 4 wk after preparation. Immediately before use as assay tubes, the storage buffer was aspirated, the tubes were washed three times with Tris–NaCl buffer, and aspirated to near dryness before introduction of enzyme reaction mixtures.

Consistently low-background (no enzyme) control values were obtained by using an aspirator designed to enter tubes only as far as the bottom curvature (avoiding the ¹²⁵I-fibrin matrix) for all washing procedures, and by careful aspiration under gentle vacuum, using a Pasteur pipette, prior to introduction of test solutions.

Assay Procedure

Fibrinolytic assays were performed in the ¹²⁵I-fibrin-coated tubes by addition of 0.1 ml of activated PL or other enzyme and 0.1 ml of Tris–NaCl buffer with or without inhibitors or activators to be tested. After incubation, assays were terminated by rapid, vigorous addition to each tube, by automatic pipettor, of two 1.0-ml aliquots of Tris–NaCl buffer, following which the tube contents were quantitatively transferred to new, uncoated tubes and counted in a Packard gamma scintillation spectrometer. Appropriate control tubes were included in all experiments and these blank values subtracted from test values.

Fibrin Plate Assays

Series of 2-mm wells were cut in the fibrin plates, 0.005 ml of appropriate reaction mixtures introduced, and the plates incubated at 37°C for 6 hr in a humidified incubator, following which the diameters of zones of lysis surrounding the sample wells were measured under magnification.

RESULTS

Properties of the ¹²⁵I-Fibrin-Polystyrene Matrix

The binding of ¹²⁵I-fibrinogen to the polystyrene tubes was reproducible, in quantities adequate for the intended use, and resistant to nonenzymatic removal by both gentle and rather drastic conditions. At room temperature, maximal binding occurred within, 3 hr, at which time 15%–23% of the ¹²⁵I-fibrinogen (1.5–2.3 μg per tube) was bound. Binding within each batch of tubes prepared was consistent. For example, in one batch exposed to 10 μg ¹²⁵I-fibrinogen (500,000 cpm) per tube, mean binding in ten randomly selected tubes was 75,000 cpm ± 8500 (SD), representing 1.5 μg ± 0.17 (SD) of radiolabeled fibrinogen.

The calculated surface area of the polystyrene tube involved in the binding phase was 2.2 sq cm (2.2 × 10¹⁶ sq Å). Assuming a molecular weight for

fibrinogen of 341,000 daltons,¹³ molecular dimensions of $400 \times 65 \text{ \AA}$,¹⁴ and monolayer binding, this area would accommodate the amounts bound (1.5–2.3 μg) provided that at least 80% of the molecules were aligned with their long axis perpendicular to the polystyrene surface.

Subsequent treatment of the tubes with thrombin resulted in release of 2%–3% of the polystyrene-bound radioactivity (1500–2250 cpm), consistent with the absence of tyrosine residues in fibrinopeptides A and B liberated from human fibrinogen on clotting by thrombin.¹⁵ Vigorous washing with Tris–NaCl buffer released less than 0.8% of the bound radioactivity, and nonspecific release on incubation with buffer at 37°C for 30 min, the time subsequently chosen for routine assays, resulted in consistent release of less than 1200 cpm from tubes coated with 100,000 cpm of ¹²⁵I-fibrin. More drastic measures, such as washing with 1 N HCl or 1 N NaOH, released 2.3% and 12% of the radioactivity, respectively.

Tubes were incubated (37°C, 30 min) with streptokinase (SK) or urokinase (UK) at concentrations of 500 U/ml and 430 CTA U/ml, respectively. Release of radioactivity was consistently less than 1000 cpm, indicating the absence of contaminating plasminogen (PLG) in the polystyrene-bound ¹²⁵I-fibrinogen and the lack of proteolytic attack of the enzymes on ¹²⁵I-fibrinogen at these concentrations, which were greatly in excess of the concentrations of these two activators subsequently studied (see below).

Assay of Plasmin

To determine the suitability of the radiofibrin–polystyrene matrix as a substrate for plasmin (PL), PL was generated from PLG by SK, and release of radioactive products from the matrix was determined. A time curve, with a PL concentration of 2.5 $\mu\text{g}/\text{ml}$ (0.5 μg per tube), is shown in Fig. 1. Release is maximal after incubation for 1 hr. The amount of ¹²⁵I-labeled product released was limited to a maximum of 40% of the bound ¹²⁵I-fibrin, even after 24 hr of incubation with PL. However, as indicated below, greater release was obtained with other proteolytic enzymes.

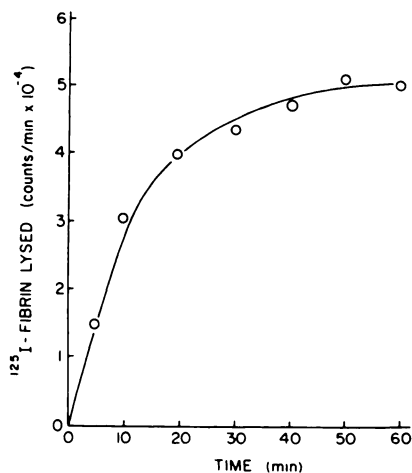


Fig. 1. Lysis of ¹²⁵I-fibrin by plasmin. Plasmin (6 μg PL in 0.2 ml Tris–NaCl buffer) was incubated in assay tubes coated with 2.3 μg ¹²⁵I-fibrin (bovine; 120,000 cpm).

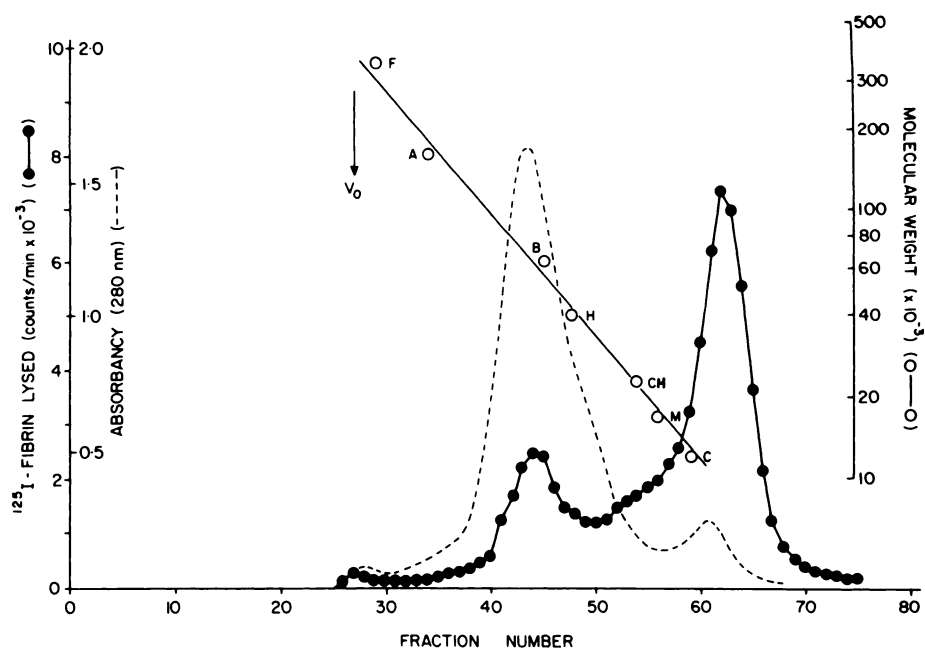


Fig. 2. Gel permeation of reaction products released from 125 I-fibrin by plasmin. Radioactive products of PL digestion at 37°C for 30 min were chromatographed on a column (1.5×87 cm) of Bio Gel A 0.5 m in Tris-NaCl buffer, and 2.0 ml fractions were collected and counted. Calibration was with the following markers: V_0 , void volume, blue dextran 2000; F, human fibrinogen (341,000); A, aldolase (158,000); B, BSA (65,000); H, horseradish peroxidase (40,000); CH, chymotrypsin, (23,000); M, myoglobin (17,000); C, cytochrome C (13,000). Ragweed allergen Ra 5 (4900) eluted at tube 62. Recovery of radioactivity applied to the column (82,000 cpm) was 100%. The absorbancy profile at 280 nm represents the degradation products obtained by PL lysis (37°C , 60 min) of a nonradioactive clot formed by action of 50 U of thrombin on 22 mg of purified human fibrinogen.

The products released by PL were fractionated on Bio-Gel A 0.5 m, using appropriate molecular weight markers (Fig. 2). There was no significant radioactivity in fractions corresponding to a molecular weight greater than 100,000 daltons. Specifically, the void volume and the fibrinogen region (molecular weight 341,000) contained only traces of radioactive material, indicating the absence of fibrin monomer (molecular weight, 330,000) or larger complexes in the reaction products. The elution volume of approximately 75% of the radioactive material (fractions 55–70) corresponded to molecular weights less than 17,000, while the remainder (fractions 40–50) corresponded to the approximate molecular-weight range of 40,000–90,000. The first peak (fractions 40–50) encompassed the theoretical elution volumes for fragments D (90,000) and E (50,000),¹⁶ while the second peak (fractions 55–70) corresponded to the molecular-weight range for the A, B, and C polypeptides and smaller fragments.¹⁷

The applicability of the method to the quantitation of PL was compared with a method in common use, the fibrin-plate technique (Fig. 3 and 4). With fibrin plates (Fig. 3), the semilogarithmic plot of PL concentration versus diameter of lysis was linear to a PL concentration of $20 \mu\text{g}/\text{ml}$. The ordinate is intentionally drawn at a diameter of 2 mm, the dimensions of the sample well, since this is the

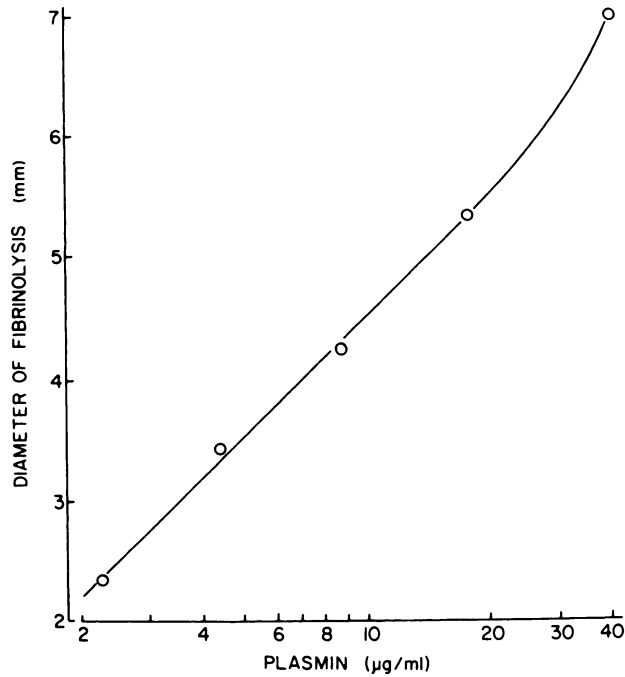


Fig. 3. Plasmin standard curve by the fibrin-plate method. Semilogarithmic plot of plasmin concentration (ordinate) versus diameter of plate lysis (abscissa) measured after incubation for 6 hr at 37°C. The ordinate is drawn at a diameter of 2 mm, the dimensions of the sample wells in the fibrin plates.

operational zero point for this type of assay. The lowest PL concentration detected ($2.3 \mu\text{g/ml}$) is at the lower limit of sensitivity of this method; with a well diameter of 2 mm, this value represents an increase in diameter of only 0.1–0.15 mm beyond the well margin, a measurement inconsistent with reproducible determinations.

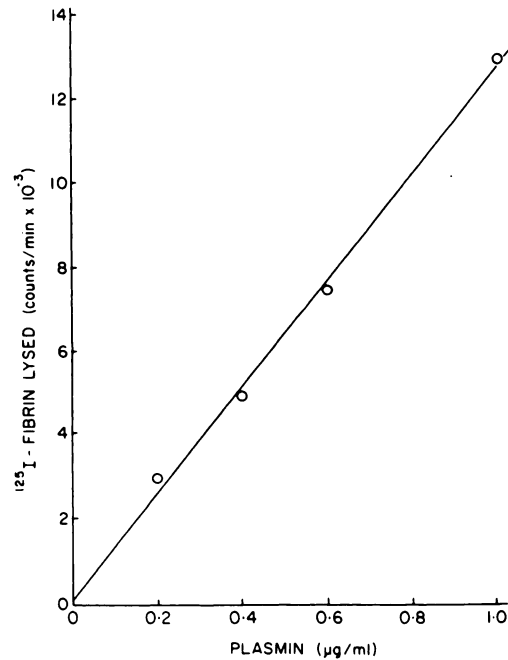


Fig. 4. Plasmin standard curve by the ^{125}I -fibrin tube method. Tubes were coated with $1.5 \mu\text{g}$ of ^{125}I -fibrin (human), representing 112,500 cpm.

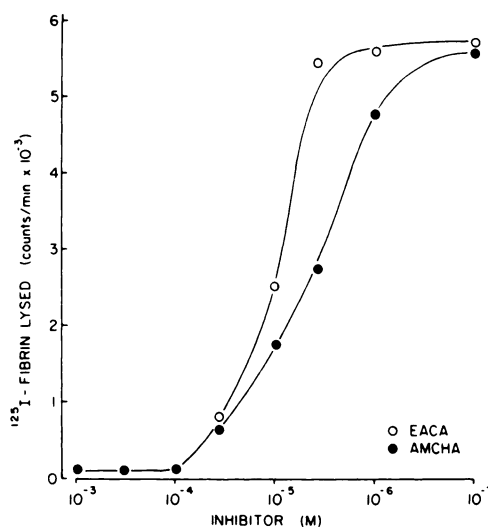


Fig. 5. Effect of EACA and AMCHA on lysis of ^{125}I -fibrin by plasmin. Reaction mixtures contained $0.1 \mu\text{g}$ PL in 0.2 ml Tris-NaCl buffer, with EACA or AMCHA at concentrations indicated, and incubation was at 37°C for 30 min. Tubes were coated with $1.5 \mu\text{g}$ ^{125}I -fibrin (human; 60,000 cpm).

With the ^{125}I -fibrin-tube method, on the other hand, it is possible to consistently detect $0.2 \mu\text{g}$ PL per ml ($0.04 \mu\text{g}$ per tube), representing the lysis of approximately 36 ng ^{125}I -fibrin, a sensitivity approximately tenfold greater than that achievable in the fibrin-plate assay. As shown in Fig. 4, the assay is linear for PL concentrations from 0.2 to $1 \mu\text{g}/\text{ml}$. Because of the consistent control (no enzyme) values obtained ($1147 \text{ cpm} \pm 90 \text{ SD}$ equivalent to lysis of $15.2 \text{ ng} \pm 1.2 \text{ SD}$ of ^{125}I -fibrin), this concentration of PL is consistently detectable.

Effects of Inhibitors of Plasmin Activity

The effects of two known inhibitors of plasmin function, EACA (epsilon amino caproic acid) and AMCHA (amino methyl cyclohexane carboxylic acid) were examined by studying their effects on release of radioactive products by maximally activated plasmin (Fig. 5). Both curves are similar, with complete inhibition of PL activity by EACA and AMCHA at 10^{-4} M , and 50% inhibition (PL concentration, $0.5 \mu\text{g}/\text{ml}$) at approximately 10^{-5} M for EACA and $3 \times 10^{-6} \text{ M}$ for AMCHA.

Assay of Plasminogen Activators

The generation of PL by incubation of PLG with varying concentrations of SK and UK, commonly used PLG activators, is shown in Figs. 6 and 7. The detection of the activating effect of $0.02 \text{ U}/\text{ml}$ of SK or $0.04 \text{ CTA U}/\text{ml}$ of UK represents a 25-fold greater sensitivity for detection of activating activity by these factors than we have been able to achieve with the fibrin-plate method, where the lower limit of sensitivity is approximately $0.5 \text{ U}/\text{ml}$ for both.

Fibrinolysis by Other Proteolytic Enzymes

Plasmin and other proteolytic enzymes were tested at the same concentration (on a weight basis) (Fig. 8). Trypsin, chymotrypsin, papain, pronase, and collagenase, which are known to cleave fibrin, were active in the assay, indicating the feasibility of this method for assay of other proteolytic enzymes in addition to plasmin.

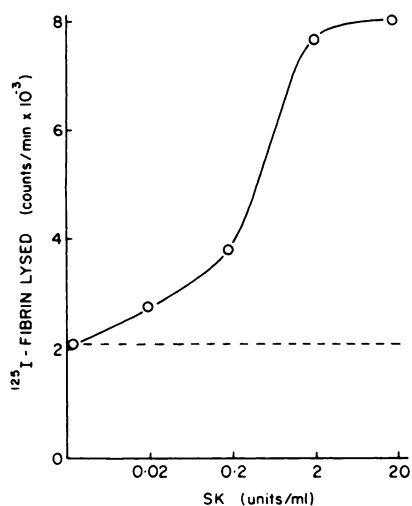


Fig. 6. Activation of plasminogen to plasmin by streptokinase. Increasing concentrations of SK were incubated (37°C, 30 min) with 6 µg PLG in Tris-NaCl buffer (total volume, 0.2 ml) in tubes coated with 1.5 µg ¹²⁵I-fibrin (human; 60,000 cpm). Radioactivity released by SK alone, at a concentration of 20 U/ml, was 321 cpm. Lysis due to prior spontaneous activation of the PL preparation used is indicated by interrupted horizontal line (approximately 25% of maximal release on streptokinase activation).

Fig. 7. Activation of plasminogen to plasmin by urokinase. Increasing concentrations of urokinase were incubated with 1.25 µg PLG for 30 min at 37°C (final volume, 0.2 ml). Tubes were coated with 1.5 µg ¹²⁵I-fibrin (human; 112,500 cpm).

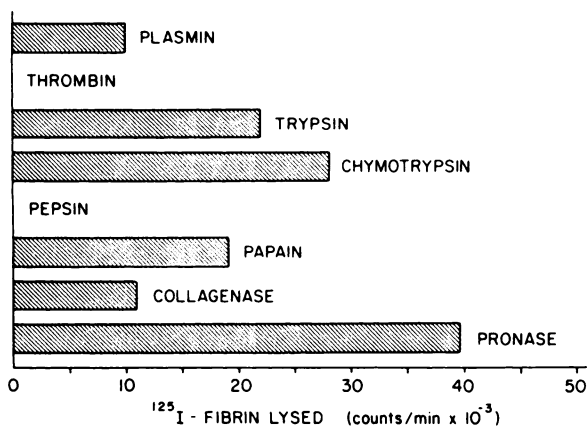
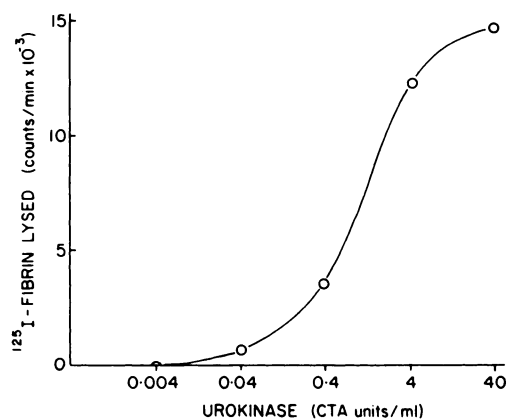


Fig. 8. Lysis of ¹²⁵I-fibrin by plasmin and other proteolytic enzymes. Enzyme (2 µg in 0.2 ml Tris-NaCl buffer containing 0.01 M CaCl₂ and 0.001 M MgCl₂) was incubated (30 min, 37°C) in tubes coated with 1.5 µg ¹²⁵I-fibrin (human; 60,000 cpm).

DISCUSSION

There is need for assay methods of high sensitivity which monitor the proteolytic activity of plasmin on its major physiologic substrate, fibrin, permit fluid-phase interaction between plasmin and its activators or inhibitors, and allow convenient separation and quantitation of the reaction products. The solid-phase radiofibrin procedure described here fulfills these requirements. It reproduces certain established features of plasmin function (effect of inhibitors, streptokinase and urokinase activation). It is technically convenient: assay tubes are readily prepared in large numbers (batches of 1500 tubes have been processed in 1 day) and have a relatively long shelf-life (at least 4 wk at 4°C with azide as preservative). Individual assays may be completed within $\frac{1}{2}$ hr, which is a marked improvement on the minimum time (6 hr) required by the fibrin-plate method, and reaction products are subsequently removed and counted in a matter of minutes.

The method is highly sensitive (at least tenfold greater than the fibrin-plate method) for measurement of plasmin activity and is consequently ideally suited for monitoring plasmin and, indirectly, for rapid screening of activators or inhibitors of plasmin function. The latter application is indicated by its high sensitivity for the effects of both streptokinase and urokinase, relative to the fibrin-plate method, and by the confirmation of the inhibitory effects on plasmin function of EACA and AMCHA previously described.¹⁸⁻²¹ In terms of lysis of ¹²⁵I-fibrin, the low control or background levels achievable (15.2 ng \pm 1.2 SD ¹²⁵I-fibrin lysed) permits a sensitivity (30-50 ng fibrin) which is greater than published values (1000-5000 ng) for fibrin-clot methods involving radioiodine or fluorochrome-labeled substrates.²⁻⁴

For physiologic clot formation, fibrin formed by the action of thrombin on fibrinogen must be further stabilized by the action of factor XIII (plasma transglutaminase).¹⁷ Plasmin digestion of this cross-linked form of fibrin yields fibrin degradation products which include fragments D, E, and smaller peptides and amino acids. In contrast to the monomeric fragment D (molecular weight, 90,000) resulting from plasmin digestion of non-cross-linked fibrin, lysis of cross-linked fibrin yields a disulfide-bonded, dimeric form of fragment D (molecular weight, 170,000).²² The absence of material corresponding to this molecular weight in the plasmin digest of the solid-phase ¹²⁵I-fibrin or the control (nonradioactive, non-solid-phase) fibrin clot (Fig. 2) indicates that the fibrin involved in the present assay method is probably of the non-cross-linked variety. It would be of interest to determine if treatment of the polystyrene-bound ¹²⁵I-fibrin with purified factor XIII would alter the profile of degradation products obtained. Presumptive evidence for cross-linking of fibrin would be easily obtained by detection of radioactive fragments consistent in size with the fragment D dimer in that region of the elution profile (tubes 30-33) corresponding to a molecular weight of 170,000.

The method is not immediately amenable to kinetic analyses requiring variations in substrate concentration, a type of study which has been done to date with other methods, such as esterolytic assays, employing low-molecular-weight, soluble substrates, or fibrinolytic assays employing microdispersed forms of fibrin.²¹ It may be feasible, however, accurately to prepare tubes coated

with varying amounts of radiolabeled fibrin, making such analyses possible. These shortcomings do not detract from the usefulness of the assay for the purposes outlined, i.e., study of activators and inhibitors of plasmin function. We have found the method, as presently constituted, particularly useful for monitoring partially purified chromatographic fractions prepared during the isolation of activator activities from plasma and tissues for which the fibrin-plate method, widely used for this purpose,²³ lacks sufficient sensitivity and facility.

The significant proteolysis of the solid-phase ¹²⁵I-fibrin by other enzymes together with the almost universal firm binding of proteins to polystyrene at low ionic strength indicate the feasibility of using the present substrate for the assay of these enzymes or, alternatively, of developing similarly sensitive and rapid methods employing other radioiodinated protein substrates for their assay.

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