THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 6, Issue of March 25, pp. 2912-2919, 1982
Printed in U.S.A.

Kinetics of the Activation of Plasminogen by Human Tissue Plasminogen Activator

ROLE OF FIBRIN*

(Received for publication, May 13, 1981, and in revised form, November 6, 1981)

Marc Hoylaerts, Dingeman C. Rijken‡, Henri R. Lijnen, and Désiré Collen

From the Center for Thrombosis and Vascular Research, Department of Medical Research, University of Leuven, Belgium

The kinetics of the activation of Glu-plasminogen and Lys-plasminogen (*P*) by a two-chain form of human tissue plasminogen activator (A) were studied in purified systems, and in the presence of fibrinogen (f) and of fibrin films (F) of increasing size and surface density. The activation in the purified systems followed Michaelis-Menten kinetics with a Michaelis constant of 65 μM and a catalytic rate constant of 0.06 s^{-1} for Glu-plasminogen as compared to 19 μM and 0.2 s^{-1} for Lys-plasminogen. In the presence of fibrinogen plots of $1/v$ *versus* 1/[P] or $1/v$ *versus* 1/[f] yielded straight lines with an apparent Michaelis constant at infinite [f] of 28 μM and a catalytic rate constant of 0.3 s^{-1} for Glu-plasminogen as compared to 1.8 μM and 0.3 s^{-1} for Lys-plasminogen. In the systems with fibrin, plasmin was estimated from the rate of release of ^{125}I from ^{125}I-labeled fibrin films. The initial rate of activation (v) was calculated and Lineweaver-Burk plots of $1/v$ *versus* 1/[P] or $1/v$ *versus* 1/[F] yielded straight lines. Activation occurred with an intrinsic Michaelis constant of 0.16 μM and a catalytic rate constant of 0.1 s^{-1} for Glu-plasminogen as compared to 0.02 μM and 0.2 s^{-1} for Lys-plasminogen.

The kinetic analysis suggested that the activation in the presence of fibrin occurs through binding of an activator molecule to the clot surface and subsequent addition of plasminogen (sequential ordered mechanism) to form a cyclic ternary complex. The low Michaelis constant in the presence of fibrin allows efficient plasminogen activation on a fibrin clot, while its high value in the absence of fibrin prevents efficient activation in plasma.

Activation of the fibrinolytic system in blood may occur via an extrinsic pathway in which the plasminogen activator is released from the vascular wall or via an intrinsic pathway in which all components are present in precursor form in blood (1).

Extrinsic plasminogen activators as occurring in blood, the vascular wall, or extracted from tissues are very similar or identical, but they differ from urokinase. In the present study, the former type of activator will be referred to as tissue plasminogen activator. In addition, we have recently purified a plasminogen activator from human melanoma cells in culture which appeared to be very similar or identical with the human uterine plasminogen activator (2). Tissue plasminogen activators may be obtained either as single chain or two-chain molecules depending on whether aprotinin is used during purification or not (2, 3).

Both tissue plasminogen activator and plasminogen interact with fibrin and these molecular interactions probably play an important role in the regulation of fibrinolysis. Preliminary data on the role of fibrin in the activation of Lys-plasminogen by tissue plasminogen activator have recently been reported. Wallén *et al.* (4) found that the Michaelis constant of the activation was 18 μM in the absence and 0.15 μM in the presence of fibrin, whereas the catalytic rate constant only increased from 0.15 to 0.3 s^{-1}. Binder and Spragg (5) on the other hand found that, in the presence of fibrin, the Michaelis constant remained unaltered but the V_{max} increased approximately 20-fold when Glu-plasminogen was used for activation.

The kinetic analysis of plasminogen activation in the presence of fibrin is complicated by the fact that fibrin both stimulates the activation step and acts as a substrate of the formed enzyme plasmin. In the present study, a procedure was elaborated in which these two phenomena could be dissociated in time and measured separately. This enabled us to study the role of fibrin on the activation of plasminogen by tissue-type plasminogen activators in more details.

MATERIALS AND METHODS

Plasminogen—Native plasminogen (NH_2-terminal glutamic acid, Glu-plasminogen) was prepared from human plasma by affinity chromatography on lysine-Sepharose (6) and gel filtration on Ultrogel AcA 44. Partially degraded plasminogen (Lys-plasminogen) was generated by limited proteolysis of Glu-plasminogen (6 mg/ml in 0.1 M NH_4HCO_3) by plasmin (molar ratio 1/100) for 30 min at 37 °C (7). Plasmin was inhibited by repetitive addition of the synthetic inhibitor Pyr-Glu-Phe-Lys-CH_2Cl[1] (8) to 10^{-4} M, which was subsequently removed by dialysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified protein showed virtually complete conversion of Glu-plasminogen to Lys-plasminogen, and end group analysis by Edman degradation only revealed lysine and valine as NH_2-ter-

* This work was supported by grants from the Onderzoeksfonds K. U. Leuven (project OT/VII/31), and the Fonds voor Geneeskundig Wetenschappelijk Onderzoek (project 3.0061.80). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a fellowship from the ZWO (Netherlands Organization for the Advancement of Pure Research) during this study.

[1] The abbreviations used are: Pyr-Glu-Phe-Lys-CH_2Cl, pyroglutamyl-phenylalanyl-lysyl-chloromethylketone, an irreversible inhibitor of plasmin; P, plasminogen; p, plasmin; F, fibrin; f, fibrinogen; A, tissue-type plasminogen activator, two-chain form; $K_{m,f}$, Michaelis constant of plasminogen activation by tissue plasminogen activator at infinite fibrinogen concentration; $K_{m,F}$, Michaelis constant of plasminogen activation by tissue plasminogen activator at infinite fibrin concentration; K_F, dissociation constant of *A-F* complex; I, total area of fibrin film interface; d_I, surface density of fibrin monomers in a fibrin film interface (in moles per cm^2); IU, international units; S_{50}, time required to dissolve 50% of a ^{125}I-fibrin film; S-2251, D-valyl-leucyl-lysyl-*p*-nitroanilide, a chromogenic substrate for plasmin.

minal amino acids. Low molecular weight plasminogen (triple loop number 5 of the plasmin A-chain and the intact plasmin B-chain, M_r = 39,000) was obtained by elastase digestion of plasminogen followed by gel filtration on Sephadex G-75 and affinity chromatography on lysine-Sepharose (9). The concentration of all types of plasminogen was measured spectrophotometrically using $A^{1\%}_{1\,cm}$ = 16.1 at 280 nm (10). Activation of plasminogen with streptokinase and active site titration of generated plasmin with *p*-nitrophenyl-*p'*-guanidinobenzoate (11) indicated that the activatibility was over 95% for Glu- and Lys-plasminogen, and over 80% for low molecular weight plasminogen.

Fibrinogen—Fibrinogen was prepared from freshly frozen blood bank plasma according to the method of Blombäck and Blombäck (12). The concentration was determined spectrophotometrically using $A^{1\%}_{1\,cm}$ = 15.1 at 280 nm and from its clottability (12). Fibrinogen contained less than 5% of higher molecular weight material as judged by gel filtration. ^{125}I-fibrinogen was obtained by labeling with Na^{125}I, using the chloramine-T method (13) in a reduced volume version (14).

Thrombin—Human thrombin was purified essentially as described by Fenton *et al.* (15). The concentration was determined spectrophotometrically using $A^{1\%}_{1\,cm}$ = 18.0 at 280 nm (15).

Human Tissue Plasminogen Activator—Tissue plasminogen activator was prepared from human uterine tissue as previously described (16) and melanoma plasminogen activator was isolated from the culture fluid of human melanoma cells (2). Both activators were purified without the addition of aprotinin and were obtained as two-chain molecules. Their activities were expressed in urokinase equivalent units by comparison of their fibrinolytic activity on plasminogen-enriched bovine fibrin films (17). Activities were converted to molar quantities using a conversion factor of 90,000 IU/mg and a molecular weight of 70,000 (2).

Other Reagents—Human plasmin, the plasmin substrate S-2251 (Coatest), streptokinase and tranexamic acid were gifts from Kabi AB, Stockholm, Sweden. Plasmin was titrated with *p*-nitrophenyl-*p'*-guanidinobenzoate prior to use (11). The plasmin inhibitor Pyr-Glu-Phe-Lys-CH_2Cl (8) was synthesized by UCB, Brussels, Belgium. *p*-Nitrophenyl-*p'*-guanidinobenzoate, zinc chloride, and gelatin were purchased from Merck, Darmstadt, Federal Republic of Germany, and Tween 80 was purchased from Baker. Sephadex products were from Pharmacia and Ultrogel AcA 22 and AcA 44 from LKB.

Kinetics of Plasminogen Activation in a Purified System—Different amounts of Glu-plasminogen (final concentration 3.3 to 100 μM) were incubated at 37 °C with uterine or melanoma tissue plasminogen activator (final concentration 150 IU/ml) in phosphate-buffered saline (0.01 M Na_2HPO_4; 0.14 M NaCl), pH 7.4, containing 0.25% gelatin and 0.006% Tween 80. At different time intervals (0 to 10 min), generated plasmin were measured spectrophotometrically with the synthetic substrate S-2251 after 20-fold dilution of the sample. Initial activation rates were obtained from plots of generated plasmin concentration versus activation time. Additionally, Lys-plasminogen (3.3 to 50 μM) was activated with tissue plasminogen activator (21 IU/ml) and the generated plasmin measured in the same way.

Kinetics of Plasminogen Activation in the Presence of Fibrinogen—Different amounts of plasminogen (final concentration 2.5 to 10 μM for Glu-plasminogen and 0.4 to 4 μM for Lys-plasminogen) were incubated at 37 °C in phosphate-buffered saline, pH 7.4, containing 0.25% gelatin and 0.006% Tween 80 with increasing amounts of fibrinogen (final concentration 0.25 to 4 μM) prior to addition of uterine tissue plasminogen activator (final concentration 21 IU/ml). Generated plasmin and initial rates of activation were determined as described above. Fibrinogen concentrations up to 0.2 μM did not compete noticeably with S-2251 for plasmin which was measured in 20-fold diluted samples.

Preparation of ^{125}I-Fibrin Films—^{125}I-Fibrin films were prepared essentially as previously described (18). ^{125}I-Fibrinogen was diluted with unlabeled fibrinogen in distilled water to final concentrations ranging from 0.06 to 1.6 μM and 4.10^6 cpm/ml. Of these solutions, 100 μl were introduced in each well of tissue culture plates with an inner diameter of 6 mm (Falcon) or 8 mm (home-made). Alternatively, 200 μl of 2-fold diluted fibrinogen solutions were introduced in tissue culture plates with an inner diameter of 12 mm (home-made) or 16 mm (Costar).

The plates were dried at 37 °C for 15 h and 100 μl (wells with 6 or 8 mm diameter) or 200 μl (wells with 12 or 16 mm diameter) of a solution containing 0.04 μg of thrombin/ml was added to the fibrinogen layer. After incubation for 4 h at 37 °C, the liquid was decanted and each well washed three times with 150 μl of phosphate-buffered saline, pH 7.4, containing 0.25% gelatin and 0.006% Tween 80. In this way, approximately 70% of the radioactivity applied to the wells was incorporated in the fibrin film. In order to calculate the total amount of fibrin in each well, an average clotting of 75% of the fibrinogen was assumed. The tissue culture plates were washed with distilled water and used immediately or stored at room temperature for up to 1 week. In the latter case, each well was washed twice again with water to remove small amounts of soluble radioactive material.

Digestion of ^{125}I-Fibrin Films by Plasmin—Fibrinolysis was measured following addition of 180 μl of plasmin (0.06 to 4 nM) in phosphate-buffered saline containing 0.25% gelatin and 0.006% Tween 80 and 20 μl of a mixture of tranexamic acid (final concentration 25 μM) and zinc chloride (final concentration 250 μM) to each fibrin film. The tissue culture plates were rotated at 37 °C, 10-μl samples were removed at fixed time intervals and released ^{125}I was counted in a Berthold Scintillation Counter BF 5300 (Benelux Analytical Instruments, Vilvoorde, Belgium). The cumulative released radioactivity was plotted against time and the time required to solubilize half of the radioactive fibrin (S_{50}) was determined.

Digestion of ^{125}I-Fibrin Films Following Plasminogen Activation—Plasminogen (40 to 1000 nM final concentration for Glu-plasminogen and 5 to 100 nM for Lys-plasminogen) and uterine or melanoma tissue plasminogen activator (0.1 IU/ml) in a final volume of 180 μl in phosphate-buffered saline containing 0.25% gelatin and 0.006% Tween 80 were incubated in the wells containing ^{125}I-fibrin (5.5 to 130 pmol of fibrin/well) and the culture plates were rotated at 37 °C. After fixed time intervals (0 to 30 min), 20 μl of a mixture of tranexamic acid (final concentration 25 μM) and zinc chloride (final concentration 250 μM) were added and the plates were rotated at 37 °C again. The rate of solubilization of ^{125}I-fibrin was then measured as described above.

RESULTS

Kinetics of Plasminogen Activation in a Purified System

The initial rates of plasminogen activation in the absence of fibrinogen or fibrin were determined with the chromogenic substrate S-2251. Plots of $1/v$ *versus* $1/[P]$ (Fig. 1) obeyed Michaelis-Menten kinetics with a Michaelis constant K_m = 65 μM and k_{cat} = 0.06 s^{-1} for Glu-plasminogen but K_m = 19 μM and k_{cat} = 0.2 s^{-1} for Lys-plasminogen (Table I). The second order rate constants k_{cat}/K_m, a measure for the catalytic efficiency and for the substrate specificity of the enzyme, were found to be 0.001 $\mu M^{-1}s^{-1}$ for Glu-plasminogen and 0.011 $\mu M^{-1}s^{-1}$ for Lys-plasminogen.

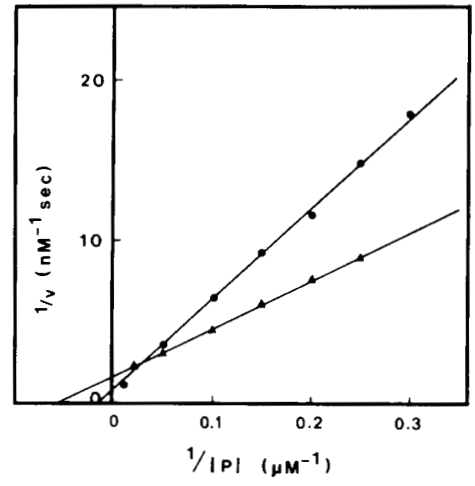


FIG. 1. **Plasminogen activation in a purified system.** Lineweaver-Burk plots of the activation of Glu-plasminogen (3.3 to 100 μM) by 150 IU/ml of uterine plasminogen activator (●) and of Lys-plasminogen (4 to 50 μM) by 21 IU/ml of uterine plasminogen activator (▲). The activation was carried out in phosphate-buffered saline, pH 7.4, containing 0.25% gelatin and 0.006% Tween 80.

TABLE I

Kinetic parameters of plasminogen activation by two-chain uterine tissue plasminogen activator

Similar results were obtained with tissue plasminogen activator obtained from human uterine tissue or from a human melanoma cell culture.

Complex	Kinetic parameters	Glu-plasminogen	Lys-plasminogen
$A \underset{}{\overset{K_m}{\rightleftharpoons}} A-P \xrightarrow{k_{cat}}$	K_m (μM)	65	19
	k_{cat} (s^{-1})	0.06	0.2
	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.001	0.011
$A \underset{[f]=\infty}{\overset{K_{m,f}}{\rightleftharpoons}} A-P \xrightarrow{k'_{cat}}$	$K_{m,f}$ (μM)	28	1.8
	k'_{cat} (s^{-1})	0.3	0.3
	$k'_{cat}/K_{m,f}$ ($\mu M^{-1}s^{-1}$)	0.011	0.17
$A \overset{K_F}{\rightleftharpoons} A-F \overset{K_{m,F}}{\rightleftharpoons} A(-P)(-F) \xrightarrow{k''_{cat}}$	K_F (μM)	0.14	0.14
	$K_{m,F}$ (μM)	0.16	0.02
	k''_{cat} (s^{-1})	0.1	0.2
	$k''_{cat}/K_{m,F}$ ($\mu M^{-1}s^{-1}$)	0.63	10

Kinetics of Plasminogen Activation in the Presence of Fibrinogen

Addition of fibrinogen slightly increased the activation rate of Glu-plasminogen of Lys-plasminogen by tissue plasminogen activator. Plots of $1/v$ *versus* $1/[P]$ or $1/v$ *versus* $1/[f]$ were linear for all fibrinogen concentrations (0.25 to 4 μM) as shown for Glu-plasminogen in Fig. 2. A further increase of the fibrinogen concentration (4 to 20 μM) did not cause significant additional increase of the activation rate. The Michaelis constants and the catalytic rate constants of plasminogen activation at infinite [*f*] for Glu-plasminogen ($K_{m,f}$ = 28 μM, k'_{cat} = 0.3 s^{-1}) and Lys-plasminogen ($K_{m,f}$ = 1.8 μM and k'_{cat} = 0.3 s^{-1}) indicate that at infinite [f] tissue plasminogen activator has a 15-fold higher affinity for Lys-plasminogen than for Glu-plasminogen, but that both substrates are activated at the same rate. The second order rate constants $k'_{cat}/K_{m,f}$ (0.011 $\mu M^{-1}s^{-1}$ for Glu-plasminogen and 0.17 $\mu M^{-1}s^{-1}$ for Lys-plasminogen) also reflect the higher substrate specificity for Lys-plasminogen than for Glu-plasminogen.

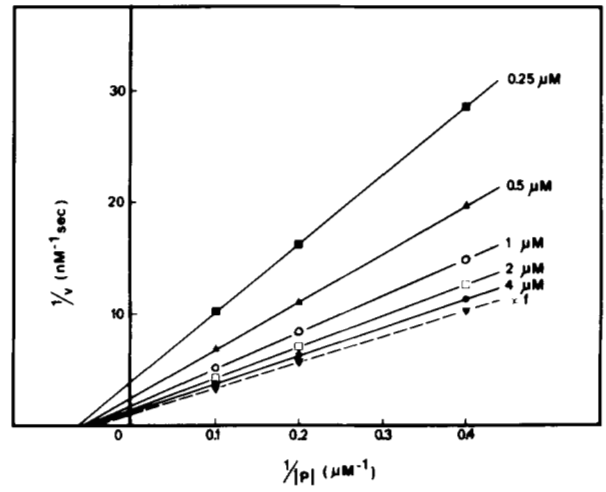
Saturation of the high affinity lysine binding site of plasminogen with tranexamic acid (0 to 20 μM) did not influence the activation rate of Glu- or Lys-plasminogen by uterine plasminogen activator. These findings indicate that the enhancing effect of fibrinogen on the activation rate is not mediated through interactions with the high affinity lysine binding site of plasminogen. Addition of fibrinogen caused a similar increase in the activation rate of low molecular weight plasminogen by tissue plasminogen activator (not shown).

Kinetics of Plasminogen Activation in the Presence of Fibrin

The activation of plasminogen by tissue plasminogen activator in the presence of fibrin was measured using ^{125}I-fibrin films as described under "Materials and Methods."

Digestion of ^{125}I-Fibrin by Plasmin—Plots of the cumulative release of ^{125}I from ^{125}I-fibrin films following addition of plasmin yield sigmoidal solubilization curves (Fig. 3*a*). The time to dissolve half of the ^{125}I-fibrin (S_{50}) was found to be an adequate parameter to describe the solubilization rate and plots of S_{50} against the logarithm of the plasmin concentration resulted in a curvilinear relationship (Fig. 3*b*). S_{50} is only slightly influenced by the addition of 25 μM tranexamic acid and 250 μM zinc chloride, which strongly inhibit plasminogen activation (see below).

Digestion of ^{125}I-Fibrin following Plasminogen Activation—Addition of mixtures of plasminogen and uterine plasminogen activator to the wells yielded sigmoidal solubilization curves as illustrated in Fig. 4*a* (*squares*) resulting from plasminogen activation and digestion of fibrin by generated plas-



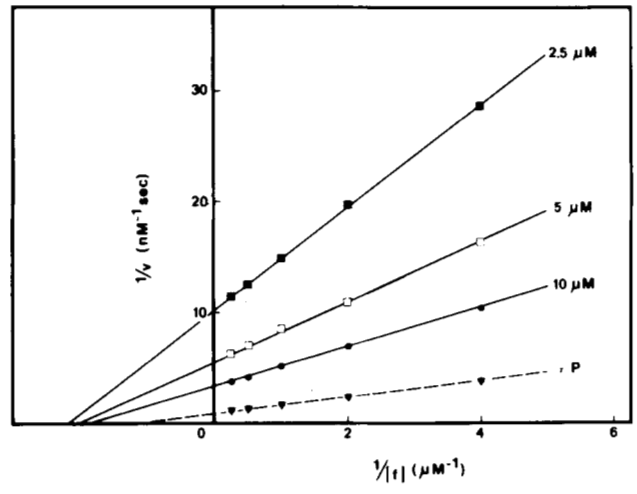


FIG. 2. **Plasminogen activation in the presence of fibrinogen.** *a*, Lineweaver-Burk plots of $1/v$ *versus* $1/[P]$ for the activation of Glu-plasminogen (2.5 to 10 μM) by uterine plasminogen activator (21 IU/ml) in the presence of increasing fibrinogen concentrations (0.25 to 4 μM). The *dotted line* represents $1/v$ at infinite fibrinogen concentration (data obtained from ordinate intercepts in Fig. 2*b*). *b*, Lineweaver-Burk plots of $1/v$ *versus* $1/[f]$ obtained by replotting the data of *a* for increasing plasminogen concentrations. The *dotted line* represents $1/v$ at infinite plasminogen concentration (data obtained from ordinate intercepts in *a*).

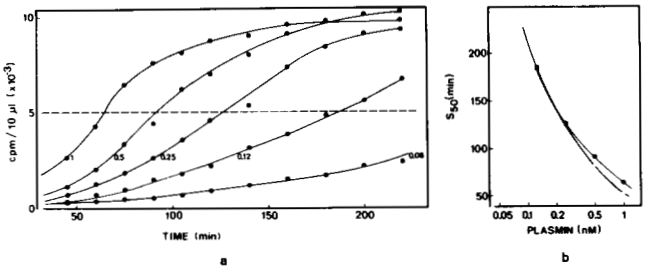


FIG. 3. **Solubilization of ^{125}I-fibrin by plasmin.** *a*, cumulative release of ^{125}I upon introduction of 180 µl of plasmin (0.06 to 1 nM) in 6-mm wells containing ^{125}I-fibrin films (30 pmol/well) and 20 µl of a mixture of tranexamic acid (final concentration 25 µM) and zinc chloride (final concentration 250 µM) in phosphate-buffered saline, pH 7.4, containing 0.25% gelatin and 0.006% Tween 80. The *dotted line* indicate 50% solubilization of ^{125}I-fibrin, from which S_{50}, the time to solubilize 50% of the fibrin, is determined. *b*, corresponding plots of S_{50} against plasmin concentration in the presence (●) and absence (○) of 25 µM tranexamic acid and 250 µM zinc chloride.

min. Addition of 25 µM tranexamic acid and 250 µM zinc chloride which do not inhibit plasmin significantly, resulted in a marked prolongation of S_{50} (Fig. 4*a*, 0′). Since tranexamic acid displaces plasminogen from the fibrin surface (19) and zinc chloride appeared to inhibit tissue plasminogen activator reversibly, these effects can be explained by inhibition of the fibrin-mediated activation of plasminogen. Incubation of plasminogen and uterine plasminogen activator prior to addition of the mixture of zinc chloride and tranexamic acid indeed resulted in a progressive shortening of the S_{50} values (Fig. 4*a*). Optimal inhibition of plasminogen activation without significant plasmin inhibition was obtained with final concentrations of 25 µM tranexamic acid and 250 µM zinc chloride. However, at these concentrations, some residual plasminogen activation still occurred (Fig. 4*a*, 0′).

The accumulated plasmin level at the time of addition of the zinc chloride-tranexamic acid mixture, was calculated from the observed S_{50}, using conversion curves as shown in Fig. 3*b*, assuming that plasmin would have been generated at time zero. This approximation appears to be justified by the fact that the activation time (15 to 20 min) is relatively short compared to the time required to solubilize 50% of the fibrin. Plots of generated plasmin against the activation time (before blocking the activation process), resulted in a concave curve (Fig. 4*b*, *open circles*). After correction for residual activation by subtracting the base-line curve (0′ in Fig. 4*a*) from each solubilization curve, a straight line through the origin is obtained (Fig. 4*b*, *closed circles*). At sufficiently long activation times (15 to 25 min) prior to addition of tranexamic acid and zinc chloride, residual activation is negligible compared to the extent of activation before their addition, and therefore, the concentration of generated plasmin can be determined without correction for residual activation. The concentration of plasmin generated at fixed activation times was proportional to the activator concentration used (not shown). From Fig. 4*b* it appears that the rate of plasmin generation represents the initial velocity (v) for the activation of plasminogen by tissue plasminogen activator; this is in line with the observation that the concentrations of generated plasmin in the present experiments are very small (less than 1%) compared to the original plasminogen and fibrin concentrations.

In order to study the kinetics of the activation, different plasminogen concentrations (40 to 1,000 nM) were incubated with identical fibrin films and activated for 15 or 20 min with uterine plasminogen activator (0.1 IU/ml) prior to addition of 25 µM tranexamic acid and 250 µM $ZnCl_2$. The solubilization rate of fibrin by generated plasmin was measured as described above (Fig. 5). S_{50} values were converted to plasmin concentrations using calibration curves similar to those shown in Fig. 3*b*, obtained under the same experimental conditions and initial rates of plasmin formation (v) were calculated.

The influence of fibrin on the kinetic parameters was studied by varying either the surface density or the size of the fibrin films. In the former type of experiments, films containing increasing amounts of fibrin (5.5 to 130 pmol of fibrin/well) were produced in wells with 6 mm internal diameter. In these experiments, the total fibrin surface remained constant while the number of molecules per unit of area (surface density) (20) increased. Plots of $1/v$ *versus* $1/[P]$ were found to be linear for each set of fibrin films (Fig. 6*a*). The latter

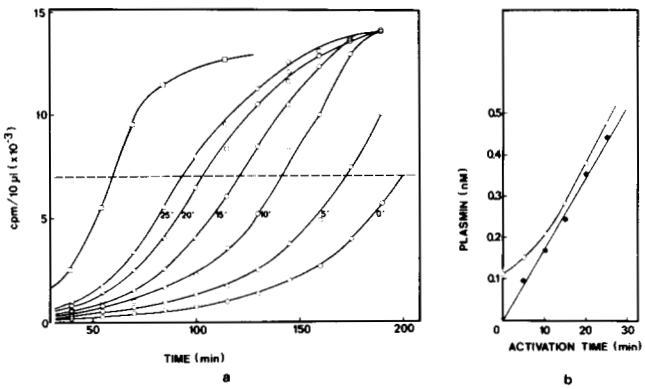


FIG. 4. **Solubilization of ^{125}I-fibrin by mixtures of Glu-plasminogen and uterine plasminogen activator.** *a*, cumulative release of ^{125}I upon incubation of Glu-plasminogen (200 nM) and uterine plasminogen activator (0.1 IU/ml) in 180 µl of phosphate-buffered saline, pH 7.4, containing 0.25% gelatin and 0.006% Tween 80, in 6-mm wells containing ^{125}I-fibrin films (30 pmol/well). At different time intervals (0 to 25 min after the start of incubation), 20 µl of a mixture of tranexamic acid (final concentration 25 µM) and zinc chloride (final concentration 250 µM) were added. The *open squares* represent the release of ^{125}I during incubation in the absence of tranexamic acid and zinc chloride. *b*, plot of the concentration of generated plasmin (obtained from the S_{50} values in *a* and the calibration curve in Fig. 3*b*) *versus* the incubation time prior to the addition of tranexamic acid and zinc chloride (○). The *solid circles* represent the same data after correction for residual activation in the presence of tranexamic acid and zinc chloride (0′ line in *a*).

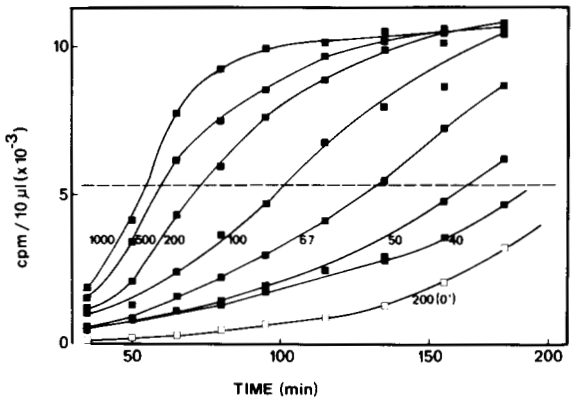
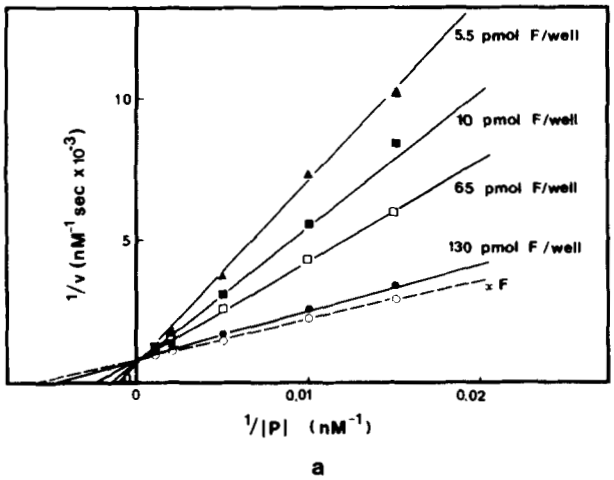


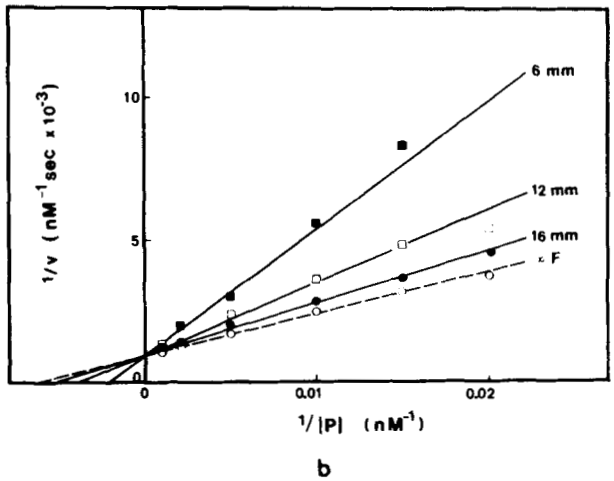
FIG. 5. **Influence of plasminogen concentration on the solubilization rate of ^{125}I-fibrin.** Cumulative release of ^{125}I after incubation of Glu-plasminogen (40 to 1000 nM as indicated) and uterine plasminogen activator (0.1 IU/ml) in 6-mm wells containing ^{125}I-fibrin films (18 pmol/well) in phosphate-buffered saline, pH 7.4, containing 0.25% gelatin and 0.006% Tween 80. Tranexamic acid (final concentration 25 µM) and zinc chloride (final concentration 250 µM) were added after 20 min incubation (■); residual activation of Glu-plasminogen upon addition of the inhibitor at zero time is shown for a plasminogen concentration of 200 nM (□).

types of experiments were carried out at a relatively low but constant surface density (32 pmol of fibrin monomer/cm^2 well surface) but at increasing total surface, by preforming the fibrin in wells of increasing diameter (6 to 16 mm). Again linear plots were obtained for $1/v$ *versus* 1/[P] (Fig. 6*b*). All lines of Fig. 6, *a* and *b*, intersected on the ordinate (same maximal velocity). In addition, it appeared that the Michaelis constants at infinite fibrin concentration (∞F in Fig. 6 *a* and *b*) are approximately 0.16 μM in both cases. These findings suggest that the activation rate is only dependent on the total amount of fibrin available and not on either the size or the surface density of the fibrin film.

The changes in apparent Michaelis constant $K_{m,app}$ as a function of increasing amounts of fibrin per well, obtained upon activation of Glu-plasminogen with uterine plasminogen activator, is illustrated in Fig. 7. These $K_{m,app}$ values were determined from experiments similar to those illustrated in Fig. 6*a*, using either wells with an internal diameter of 6 mm



a



b

FIG. 6. **Lineweaver-Burk plots of plasminogen activation on ^{125}I-fibrin films.** *a*, activation of Glu-plasminogen (67 to 1,000 nM) by uterine plasminogen activator (0.1 IU/ml) in 6-mm wells (200 μl volume) in the presence of increasing amounts of fibrin (5.5 to 130 pmol/well). The *dotted line* represents $1/v$ at infinite amounts of fibrin (data obtained from ordinate intercepts in plots similar to those shown in Fig. 8*b*). *b*, activation of Glu-plasminogen (50 to 1,000 nM) by uterine plasminogen activator (0.1 IU/ml) in wells of increasing diameter (6 to 16 mm) with a constant surface density of 32 pmol of fibrin/cm^2 well surface (200 μl volume). The *dotted line* represents $1/v$ at infinite fibrin surface (data points obtained from ordinate intercepts in plots similar to those shown in Fig. 8*b*).

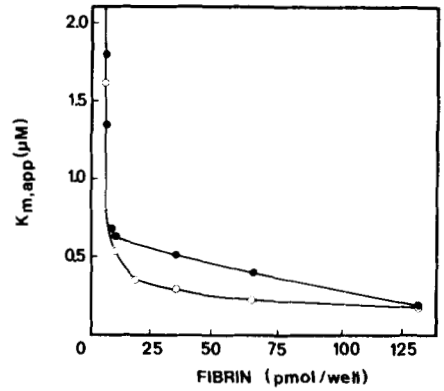


FIG. 7. **Apparent Michaelis constant ($K_{m,app}$) as a function of the amount of fibrin per well.** Glu-plasminogen (67 to 1000 nM) was activated with uterine plasminogen activator (0.1 IU/ml) in 6-mm wells (●) and in 12-mm wells (○).

or 12 mm. At fibrin amounts of 130 pmol/well, very similar $K_{m,app}$ values are obtained in both systems. At lower amounts of fibrin per well, however, the $K_{m,app}$ is lower in the 12-mm wells compared to the 6-mm ones. This is probably due to insufficient availability of fibrin molecules for the activation process in the 6-mm wells with a consequent overestimation of the effective fibrin concentration. The $K_{m,app}$, however, remains small for amounts of fibrin down to 8 pmol/well, but at smaller amounts, $K_{m,app}$ increases very rapidly.

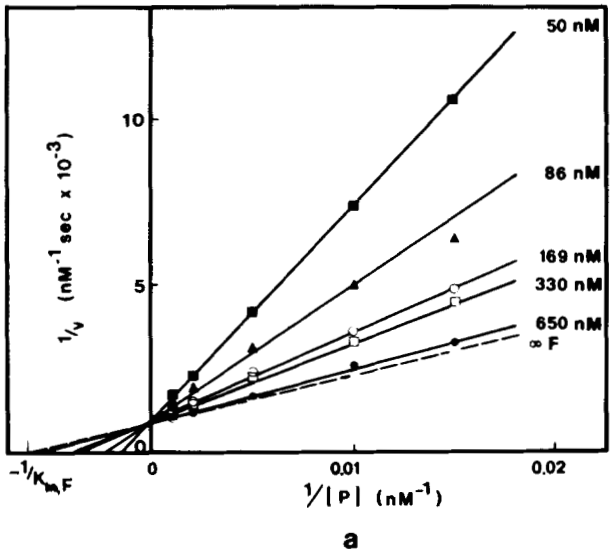
Quantitation of Solid Phase Fibrin—Since both plasminogen and fibrin interact with tissue plasminogen activator the activation process is formally analogous to a two-substrate enzyme system (21, 22). The mechanism of such reactions can be elucidated by measuring the influence on the reaction rate of varying concentrations of one substrate at constant concentration of the other. This requires an adequate expression of the fibrin concentration.

From Figs. 6*a* and 7, it appears that the apparent kinetic parameters of the plasminogen activation depend both on the amount and the availability of fibrin molecules. The active fibrin concentration which appears to be a function of its surface density, can be expressed as follows (20):

$$[F] = d_I \times \frac{I}{V}$$

with d_I, surface density of fibrin monomer (moles per interface area); I, total area of the fibrin film interface (possibly larger than the well surface); V, total volume of the supernatant.

In this formula, [F] is expressed in moles per liter. At sufficiently low surface densities, all fibrin monomers are available for interaction with soluble components (which diffuse freely) and the active fibrin concentration is equal to the formal concentration expressed in moles per volume. Fig. 8 illustrates plots of $1/v$ *versus* 1/[P] (Fig. 8*a*) and of $1/v$ *versus* 1/[F] (Fig. 8*b*), derived from experiments in which [F] is expressed in molar concentrations. These experiments were carried out with Glu-plasminogen in wells with 12 mm internal diameter. Straight intersecting lines are obtained in both plots. The ordinate intercepts in Fig. 8*b* represent the initial rates of plasmin formation at infinite fibrin concentration and are replotted in Fig. 8*a* (∞F). The abscissa intercept of this replot yields a Michaelis constant ($K_{m,F}$ = 0.16 μM) of plasminogen activation at infinite fibrin concentration (at which all plasminogen activator is assumed to be bound to fibrin), whereas the ordinate intercept allows the determination of the catalytic rate constant (k''_{cat} = 0.1 s^{-1}) for Glu-plasminogen activation by fibrin-bound tissue plasminogen activator (see below). In a similar way, the kinetic parameters for Lys-



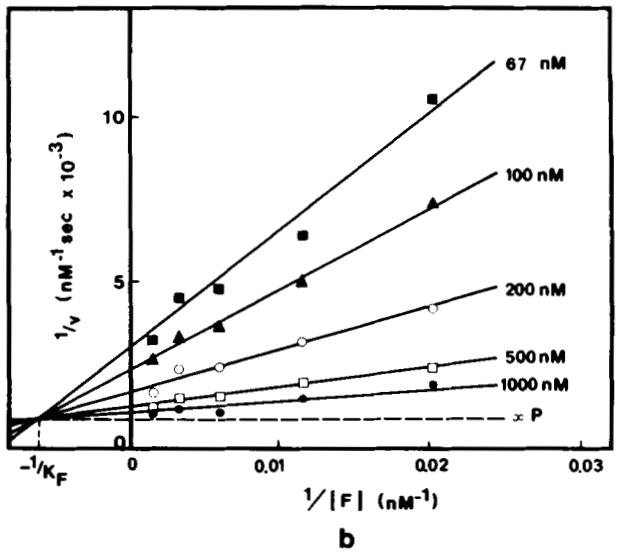


FIG. 8. **Plasminogen activation in the presence of fibrin.** *a*, Lineweaver-Burk plots of $1/v$ *versus* $1/[P]$ for the activation of Glu-plasminogen (67 to 1,000 nM) by uterine plasminogen activator (0.1 IU/ml) in the presence of increasing fibrin concentrations (50 to 650 nM) carried out in 12-mm wells. The *dotted line* represents $1/v$ at infinite fibrin concentration (data obtained from ordinate intercepts in *b*). *b*, Lineweaver-Burk plots of $1/v$ *versus* $1/[F]$ obtained by replotting the data of *a* for increasing plasminogen concentrations. The dotted ine represents $1/v$ at infinite plasminogen concentration (ordinate intercept of *a*). Similar results were obtained when using tissue plasminogen activator purified from a melanoma cell culture.

plasminogen activation were found to be $K_{m,F} = 0.02$ μM and $k''_{cat} = 0.2\ s^{-1}$ (Table I). From the intersection point in Fig. 8*b*, the dissociation constant of the fibrin-tissue plasminogen activator complex (see below) is calculated to be $K_F = 0.14$ μM. The second order rate constants $k''_{cat}/K_{m,F}$ (0.63 $\mu M^{-1}s^{-1}$ for Glu-plasminogen and 10 $\mu M^{-1}s^{-1}$ for Lys-plasminogen) show that plasminogen activation is markedly enhanced in the presence of fibrin.

The assumption that the fibrin concentration may adequately be described by its formal concentration (in moles per volume) is substantiated by the linear relationships observed in Fig. 8*b* which were obtained from experiments performed in 12-mm wells. Plots of $1/v$ *versus* $1/[F]$ derived from similar activation studies performed in 6-mm wells, however, showed deviations from linearity (not shown). In the latter experiments, straight lines were only obtained at formal fibrin concentrations below 0.1 μM corresponding to a surface density of about 70 pmol/cm^2 well surface. These findings support the assumption that in the 6-mm wells not all of the fibrin binding sites remain available for interaction with plasminogen and tissue plasminogen activator when the fibrin surface density increases above 70 pmol/cm^2.

Replots of $1/v$ *versus* $1/[F]$ of data such as shown in Fig. 6*a* (for [F] smaller than 0.1 μM, which corresponds to 70 pmol/cm^2 or 20 pmol/well) and Fig. 6*b* (constant surface density of 32 pmol/cm^2 well surface) yielded very similar slopes and intercepts. The dotted lines in Fig. 6, *a* and *b*, are replots of the ordinate intercepts of the corresponding $1/v$ *versus* $1/[F]$ plots and yield almost the same abscissa intercepts as shown in Table I. The ordinate intercepts of Figs. 6, *a* and *b*, and 8*a* (V_{max}) are identical and are independent of the fibrin concentration (horizontal line in Fig. 8*b* (∞P)). This finding gives further support to the hypothesis that the active fibrin concentration at low surface density is adequately described by its formal concentration.

DISCUSSION

Several investigators have shown that the activation of plasminogen by the "tissue-vascular-blood"-type plasminogen activator is enhanced in the presence of fibrin (4, 5, 23, 24). The present study deals with a kinetic analysis of the activation of human plasminogen by human uterine tissue plasminogen activator and by a tissue-type plasminogen activator from a human melanoma cell culture. The activation mechanism was studied in purified systems, in the presence of fibrinogen and in the presence of fibrin.

In the purified systems, plasminogen activation obeyed classical Michaelis-Menten kinetics. Lys-plasminogen appeared to be somewhat more readily activated by tissue plasminogen activator than Glu-plasminogen as evidenced by a 3-fold lower Michaelis constant and a 3-fold higher catalytic rate constant (Table I). Addition of tranexamic acid up to 10 μM, which saturates the high affinity lysine binding site of plasminogen did not influence the activation rate.

Addition of fibrinogen to mixtures of plasminogen and plasminogen activator results in a small concentration-dependent increase of the activation rate. The apparent Michaelis constant for Glu-plasminogen activation in the presence of fibrinogen (∞f) is 28 μM, which is much higher than the plasma plasminogen concentration (about 1.5 μM). This indicates that tissue plasminogen activator will not efficiently convert plasminogen to plasmin in plasma. It seems unlikely that the increased activation rate is due to the presence of fibrin monomers in the fibrinogen preparation, since no further change of K_m was observed above fibrinogen concentrations of 4 μM. Furthermore, there was no effect of tranexamic acid (up to 20 μM) on the activation rate in the presence of fibrinogen, whereas this substance strongly interferes with the effect of fibrin on the activation rate.

The kinetic analysis of plasminogen activation in the presence of fibrin is complicated by the fact that fibrin both stimulates the activation and acts as a substrate for the formed plasmin. In addition, since fibrin occurs as a solid phase, heterogeneous catalysis occurs which hampers the direct application of Michaelis-Menten kinetics. The problem that fibrin both stimulates plasminogen activation and acts as a substrate for generated plasmin was circumvented by separating these two processes in time. The activation process could indeed be largely arrested by addition of a mixture of tranexamic acid and zinc chloride at concentrations which did

not significantly influence the activity of plasmin. Under the experimental conditions used (activation time up to 20 min), the concentration of formed plasmin was directly proportional to the activation time and to the concentration of activator and did not exceed 1% of the initial plasminogen concentration. All these findings indicate that initial activation rates were measured.

Since $[F] = d_I \times I/V$, the fibrin concentration [F] (moles per volume) can be changed (at constant volume) either by varying the surface density d_I at constant surface or by varying the interface surface I at constant surface density d_I. Both approaches were evaluated, and plots of $1/v$ *versus* 1/[P] yielded straight lines in both cases, with identical ordinate intercepts (V_{max}) and Michaelis constants at infinite fibrin concentration. These experiments suggest that no allosteric interactions exist between fibrin molecules in the clot surface, and that the stimulation of plasminogen activation depends only on the amount of available fibrin binding sites. These observations also indicated that the activation mechanism was not by interaction of tissue plasminogen activator with fibrin-bound plasminogen. The solid phase fibrin concentration was formally expressed in terms of its volume concentration. At surface densities of less than 70 pmol of fibrin monomer/cm^2 well surface plots of $1/v$ *versus* 1/[F] were linear, but at higher surface densities, a deviation from linearity was observed, probably due to overestimation of the "active" fibrin concentration.

Evaluation of the kinetic data reveals that all lines intercept on the ordinate at the same V_{max} (Fig. 8*a*), indicating that the reaction rate is independent of [F] at infinite [P]. These findings suggest that the reaction mechanism is predominantly ordered and sequential (21) via the pathway:

$$A + F \underset{}{\overset{K_F}{\rightleftharpoons}} A\!\!\setminus\!\!F \quad + P \overset{K_{m,F}}{\rightleftharpoons} A\!\!\setminus\!\!F\!\!-\!\!P$$

Since saturation of the high affinity lysine-binding site of plasminogen with tranexamic acid (19) or histidine-rich glycoprotein (25) abolishes or greatly diminishes the activation of plasminogen by fibrin-bound activator, it is very likely that in the ternary complex plasminogen is bound to fibrin via its high affinity lysine binding site, and that no active ternary complex can be formed when this interaction is blocked. The conversion of fibrinogen to fibrin would thus result in an increase in its affinity for plasminogen, as evidenced by the drop in Michaelis constant from 28 μM to 0.16 μM for Glu-plasminogen activation as a result of a specific interaction between fibrin and plasminogen.

Since, in direct binding studies, it has been shown that only a small amount of native plasminogen binds to fibrin (26), the free plasminogen concentration is essentially equal to the total concentration. Moreover, since plots of $1/v$ *versus* 1/[P] are linear, it is very likely that the free plasminogen is the actual substrate for A\F, rather than fibrin-bound plasminogen. Plots of $1/v$ *versus* 1/[P] have the same maximal velocity, independent of [F] (Fig. 8), suggesting that at infinite [P] the equilibrium A + F ⇄ A\F is pulled to the right. Hence, at infinite [P], the concentration of A\F/P equals the total tissue plasminogen activator concentration, independent of [F]. At infinite [F] $1/v$ *versus* 1/[P] yields an abscissa intercept $-1/K_{m,F}$, which represents the intrinsic Michaelis constant for fibrin dependent activation of plasminogen.

Plasminogen is converted to plasmin with very similar rate constants in purified systems and in the presence of fibrinogen or fibrin. This suggests that fibrin binds the activator and that consecutively plasminogen is added to form a cyclic ternary complex, thereby increasing the local plasminogen concentration and reducing the apparent Michaelis constant through formation of a fibrin bridge between enzyme and substrate, without significantly influencing the catalytic efficiency of the enzyme. In this respect, the mechanism of the activation of plasminogen is analogous to that proposed for the divalent metal ion activation of D-xylose isomerase (27) and L-arabinose isomerase (28) in which cyclic metal ion bridges are formed between the enzyme and its substrate, according to an ordered addition of metal (Mn^{2+}) and substrate to the enzyme.

This mechanism for plasminogen activation and the derived kinetic parameters show that the fibrinolytic process is triggered by fibrin. Indeed the second order rate constant in plasma ($k'_{cat}/K_{m,f}$) is 0.01 $\mu M^{-1}s^{-1}$ and plasminogen will not be activated efficiently. On the clot surface however the second order rate constant ($k''_{cat}/K_{m,F}$) is 0.63 $\mu M^{-1}s^{-1}$ and plasminogen activation becomes efficient.

The kinetic parameters for the activation of Glu-plasminogen by melanoma plasminogen activator and by uterine plasminogen activator in purified systems as well as in the presence of fibrin were found to be very similar, which agrees with the proposed identity of both types of activator (2).

REFERENCES

1. Collen, D. (1980) *Thromb. Haemostasis* **43,** 77–89
2. Rijken, D. C., and Collen, D. (1981) *J. Biol. Chem.* **256,** 7035–7041
3. Wallén, P., Rånby, M., Bergsdorf, N., and Kok, P. (1981) in *Progress in Fibrinolysis* (Davidson, J. F., Nilsson, I. M., and Åstedt, B., eds) Vol. 5, pp. 16–23, Churchill Livingstone, Edinburgh
4. Wallén, P., and Rånby, M. (1980) *XXVIIIth Annual Colloquium on Protides Biological Fluids, Brussels,* Abstr. 67
5. Binder, B. R., and Spragg, J. (1980) in *Protides of the Biological Fluids* (Peeters, H., ed) Vol. 28, pp. 391–394, Pergamon Press, Oxford
6. Deutsch, D. G., and Mertz, E. T. (1970) *Science* **170,** 1095–1096
7. Claeys, H., Molla, A., and Verstraete, M. (1973) *Thromb. Res.* **3,** 515–523
8. Collen, D., Lijnen, H. R., De Cock, F., Durieux, J. P., and Loffet, A. (1980) *Biochim. Biophys. Acta* **615,** 158–166
9. Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., and Magnusson, S. (1977) in *Progress in Chemical Fibrinolysis and Thrombolysis* (Davidson, J. F., Rowan, R. M., Samama, M. M., and Desnoyers, P. C., eds) Vol. 3, p. 191–209, Raven Press, New York
10. Wallén, P., and Wiman, B. (1970) *Biochim. Biophys. Acta* **221,** 20–30
11. Chase, T. Jr., and Shaw, E. (1970) *Methods Enzymol.* **9,** 20–27
12. Blombäck, B., and Blombäck, M. (1956) *Ark. Kemi* **10,** 415–443
13. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89,** 114–123
14. Edgington, T. S., Plow, E. F., Chavkin, C. I., De Meer, D. H., and Nakamura, R. M. (1976) *Bull. Cancer (Paris)* **63,** 673
15. Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., and Finlayson, J. S. (1977) *J. Biol. Chem.* **252,** 3587–3598
16. Rijken, D. C., Wijngaards, G., Zaal-de Jong, M., and Welbergen, J. (1979) *Biochim. Biophys. Acta* **580,** 140–153
17. Astrup, T., and Müllertz, S. (1952) *Arch. Biochem. Biophys.* **40,** 346–351
18. Unkeless, J. C., Tobia, A., Ossowski, L., Quigley, J. P., Rifkin, D. B., and Reich, E. (1973) *J. Exp. Med.* **137,** 85–111
19. Hoylaerts, M., Lijnen, H. R., and Collen, D. (1981) *Biochim. Biophys. Acta* **673,** 75–85

20. Verger, R., Mieras, M. C. E., and de Haas, G. H. (1973) *J. Biol. Chem.* **248,** 4023–4034
21. Cleland, W. W. (1970) in *The Enzymes—Kinetics and Mechanism* (Boyer, P. D., ed) Vol. 2, Academic Press, New York
22. Bright, H. J. (1965) *J. Biol. Chem.* **240,** 1198–1210
23. Camiolo, S. M., Thorsen, S., and Astrup, T. (1971) *Proc. Soc. Exp. Biol. Med.* **138,** 277–280
24. Gurewich, V., Hyde, E., and Lipinski, B. (1975) *Blood* **46,** 555–565
25. Lijnen, H. R., Hoylaerts, M., and Collen, D. (1980) *J. Biol. Chem.* **255,** 10214–10222
26. Rákóczi, I., Wiman, B., and Collen, D. (1978) *Biochem. Biophys. Acta* **540,** 295–300
27. Mildvan, A. S., and Rose, I. A. (1969) *Fed. Proc.* **28,** 534
28. Nakamatu, T., and Yamanaka, K. (1969) *Biochim. Biophys. Acta* **178,** 156–165