UROKINASE
AN ACTIVATOR OF PLASMINOGEN FROM HUMAN URINE

II. MECHANISM OF PLASMINOGEN ACTIVATION

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INTRODUCTION

In the preceding paper[1] the isolation of urokinase from human urine was described. This communication deals with the kinetics of the plasminogen activation process. Urokinase was found to transform plasminogen enzymically into plasmin by a first order reaction with pH optimum around 9. Besides plasminogenase activity, urokinase showed esterase activity towards lysine-ethylester and *p*-tosylarginine-methylester.

MATERIALS AND METHODS

Human plasminogen was prepared from human fraction III* by the method of KLINE[2].

p-Toluenesulfonyl-L-arginine-methylester, HCl (TAMe) was synthesized as described by TROLL, SHERRY AND WACHMANN[3]. The melting point was 145° C.

L-*Lysine-ethylester, 2HCl (LEe)* was synthesized according to WERBIN AND PALM[4]. The melting point was 144° C.

Bovine plasmin. Prepared by addition of urokinase (100 units/ml) to a 0.8% bovine fibrinogen solution[1]. After 20 min at room temperature, saturated ammonium sulphate was added to 0.25 saturation. The inactive precipitate was removed and the ammonium sulphate concentration increased to 0.30 saturation to precipitate the plasmin. The precipitate was collected, redissolved in water and lyophilized.

Plasminogen-deficient bovine fibrinogen was prepared as recommended by SHERRY[5] by subjecting fibrinogen precipitated from bovine plasma by 10% ethyl alcohol to the LAKI procedure[6]. Very inconsistent results were obtained. The plasminogen content was checked by measurements of the lysis time of a coagulum formed from 1 ml of this fibrinogen with addition of 100 units urokinase. If the prolongation of the lysis time was unsatisfactory, the Laki procedure was repeated. With addition of 100 units of urokinase, the lysis time of the particular preparation used in our experiments was 37 min, compared to a lysis time of about 4 min with the usual fibrinogen preparations.

Casein (Hammersten quality) Merck.

Heparin, commercially prepared by this factory. A preparation containing 100 i.units/mg was used, dissolved in 0.1 *M* phosphate buffer pH 7.2 (1 mg/ml).

Protamine sulphate, dissolved in 0.1 *M* phosphate buffer pH 7.2 (1 mg/ml).

Plasmin assay

Fibrinolytic assay of plasmin was performed as with the urokinase assay[1]. The incubation mixture consisted of 0.8 ml of 0.1 *M* phosphate buffer pH 7.2, 0.1 ml thrombin solution (100 NIH-units/ml), 0.1 ml of plasmin solution and 0.5 ml of 1.5% plasminogen-deficient fibrinogen. By plotting the reciprocal of the lysis time versus plasmin concentration, a straight-line relationship was obtained (Fig. 1) in accordance with the findings of FLETCHER[7].

* Obtained through the courtesy of the American Red Cross and supplied to us through E.R. Squibb and Sons.

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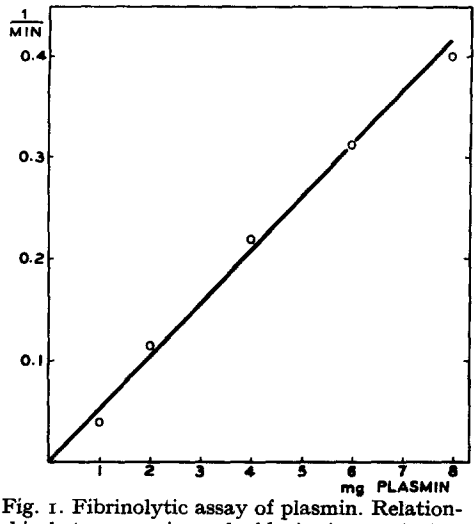

Fig. 1. Fibrinolytic assay of plasmin. Relationship between reciprocal of lysis time and plasmin concentration.

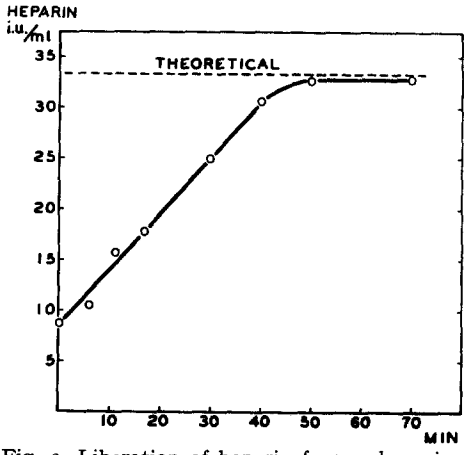

Fig. 2. Liberation of heparin from a heparin-protamin complex by plasmin. To a mixture of 1.0 ml heparin solution and 1.0 ml protamine sulphate solution was added 20 mg bovine plasmin dissolved in 1.0 ml 0.9% NaCl. The mixture was incubated at 37° C and 0.1 ml aliquots were removed at intervals. Heparin was present in excess, explaining the high zero time value. Plasmin itself does not interfere with the heparin determinations.

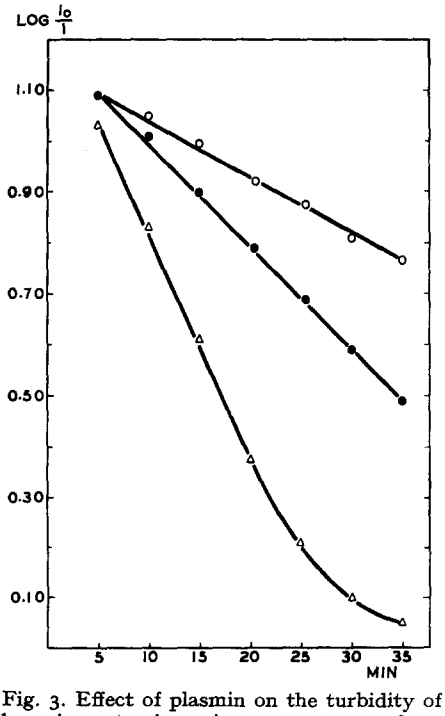

Fig. 3. Effect of plasmin on the turbidity of a heparin-protamine mixture. 100, 50 and 25 μl samples of a urokinase-activated plasminogen solution were added to the heparin-protamine mixture and incubated at 37° C. Optical density at 450 mμ is plotted against time of reaction. Density decrease per min: △—△ 0.040, ●—● 0.021, ○—○ 0.011.

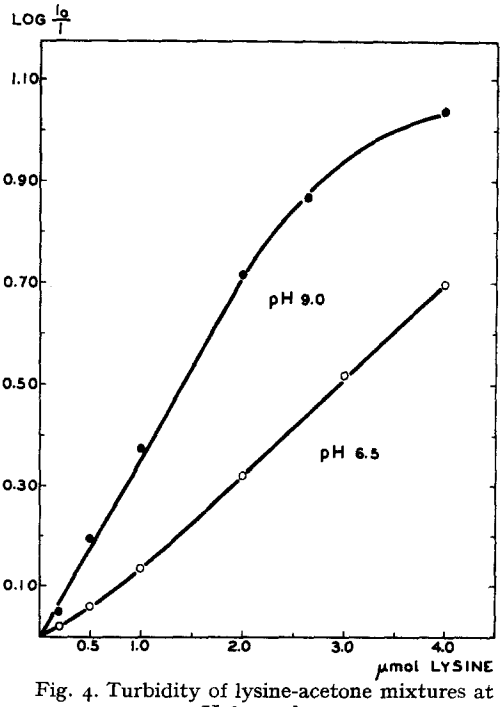

Fig. 4. Turbidity of lysine-acetone mixtures at pH 6.5 and 9.0.

Proteolytic assay of plasmin. BRUNFELDT AND POULSEN[8] demonstrated the protaminase activity of plasmin by the splitting of an insulin-protamine complex. In analogy with this observation the proteolytic activity of plasmin was assayed by the splitting of a heparin-protamine complex. The reaction was followed by measuring the liberated heparin (Fig. 2), using the method of KJEMS AND WAGNER[9] or, more conveniently, by following the decrease in turbidity as measured by the change in optical density of the heparin-protamine mixture in the Beckman DU spectrophotometer at 450 mμ. Equal volumes of heparin and protamine solutions were mixed, forming a very stable turbid liquid. 3.0 ml were used for the assay in a 1.0 cm Beckman cell, with addition of 0.1 ml of plasmin solution. Since no temperature-compensating device for the Beckman DU spectrophotometer was at our disposal, the cells were maintained in a warm air oven at 37° C between the turbidity readings. Fig. 3 shows the straight line decrease in turbidity with time and the proportionality between plasmin concentration and slope. The sensitivity of this method is of the same order of magnitude as that of the fibrinolytic assay. The reproducibility of the substrate and the ease of measurements suggest its use in other proteolytic enzyme studies.

TAMe esterase activity was followed as indicated by TROLL, SHERRY AND WACHMANN[3].

LEe esterase activity. The liberated lysine was determined by mixing 0.1 ml aliquots of the reaction mixture with 3.0 ml acetone and measuring the turbidity formed in the Beckman DU spectrophotometer at 450 mμ about 2 min after mixing. Fig. 4 shows the turbidity produced by mixing 0.1 ml samples of L-lysine solutions buffered at pH 6.5 and 9.0 with 3.0 ml acetone.

RESULTS

Plasminogen activation

Since no means were found to remove specifically, or inactivate, urokinase in the presence of plasmin, only fast and sensitive methods were used to study the kinetics of the activation process. Thus we minimized the influence of continued plasminogen activation during the plasmin determinations. Preliminary studies showed clearly the high lability of the formed plasmin, even at 0° C. As reported by KLINE[10] the plasmin formed from plasminogen by streptokinase activation is protected by casein or other plasmin substrates. This was found also in the case of urokinase; all activation experiments were therefore performed in the presence of casein.

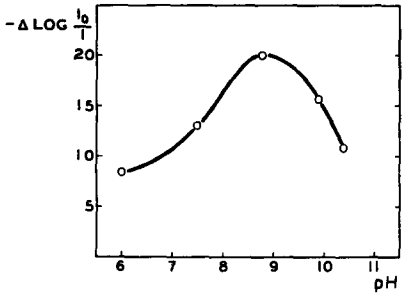

Fig. 5. Effect of pH on urokinase activation of plasminogen. Plasminogen was incubated for 10 min with 100 units per ml of urokinase in buffers at various pH values in the presence of 0.5% casein. Plasmin activity was measured by the decrease per min in optical density in the proteolytic assay.

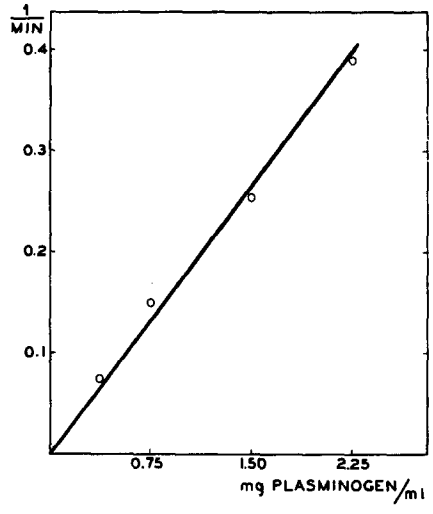

Fig. 6. Effect of plasminogen concentration on activation. Various amounts of human plasminogen in 0.25 *M* Tris buffer pH 9.0 with 0.5% casein and 150 units urokinase per ml were incubated at 23° C for 10 min, and 0.1 ml samples were removed and tested for fibrinolytic activity. The curve shows relationship between reciprocal of lysis time and plasminogen concentration.

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The pH optimum of the activation with urokinase is about pH 9, as shown in Fig. 5.

Fig. 6 shows the proportionality between plasminogen concentration and the plasmin formed after 10 min incubation with 150 units/ml of urokinase at 23° C. These conditions of time, concentration and temperature bring about an almost complete transformation of plasminogen into plasmin.

The kinetics of activation was studied by the fibrinolytic assay at pH 9.0 and 23° C in the presence of 0.5% casein. Mixtures of 0.3 ml plasminogen solution (5 mg/ml), 0.3 ml of 0.5 *M* tris(hydroxymethyl)aminomethane (Tris) buffer pH 9.0 and 0.1 ml of 4% casein solution in Tris buffer were incubated with amounts of urokinase varying from 6.7 units/ml to 145 units/ml. Samples were removed at intervals for plasmin determinations. Maximal activation was established with 1000 units/ml of urokinase and the plasmin activity obtained was used as a measure in the total amount of plasminogen.

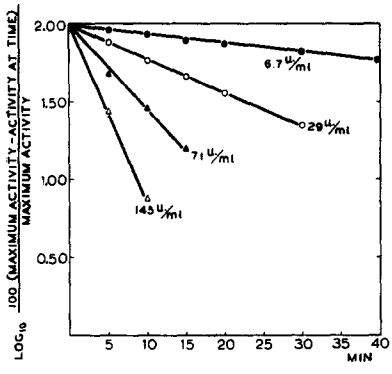

Fig. 7. Kinetics of plasminogen activation by urokinase at 23° C.

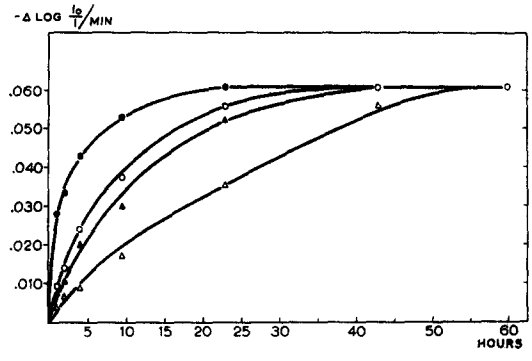

Fig. 8. Activation of plasminogen at 0–4° C measured by the proteolytic assay. ●—● 125 units urokinase/ml, ○—○ 25 units urokinase/ml, ▲—▲ 16 units urokinase/ml, △—△ 6 units urokinase/ml. Abscissa: incubation time of plasminogen–urokinase mixtures. Ordinate: Turbidity decrease per min.

The results of the experiments are shown in Fig. 7 where the logarithm of the ratio of the amount of plasminogen not activated at any time (expressed as maximum plasmin activity–plasmin activity) to the total amounts of plasminogen (expressed as maximum plasmin activity) is plotted against the time. The resulting curves are all straight lines, showing that the reaction is monomolecular. The slopes of the curves are proportional to the urokinase concentration (Table I), as would be expected from the formula for a first order enzymic reaction:

$$\log \frac{c_0 - c_t}{c_0} = -k\, c_E\, t$$

Where c_0 is the amount of plasminogen at time 0, c_t the amount of plasmin activity at time t, and c_E the urokinase concentration. $k\, c_E$ is the observed numerical slope, k', at any urokinase concentration. The velocity constant at unity, k, is found to be $76 \cdot 10^{-5}$ per minute per unit urokinase per ml.

Although evidence of an enzymic process was thus obtained, the casein concentration was too low to stabilize plasmin completely. The same plasmin level was not attained in all samples regardless of the urokinase concentration. Activation

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TABLE I

VELOCITY CONSTANT AT 23° C OF UROKINASE-CATALYZED PLASMINOGEN ACTIVATION

Urokinase		*k'*	*k*
units/ml	*μg/ml*	*l/min*	*l/min/unit/ml*
6.7	0.9	0.005	$75 \cdot 10^{-5}$
29	3.9	0.022	$76 \cdot 10^{-5}$
71	9.5	0.054	$76 \cdot 10^{-5}$
145	19.5	0.114	$78 \cdot 10^{-5}$

experiments were therefore performed with the casein concentration increased to 2.5% and at an incubation temperature between 0 and 4° C.

Mixtures of 0.4 ml plasminogen solution (5 mg/ml) and 0.4 ml 5% casein in 0.2 *M* Tris buffer pH 7.9 were incubated with varying amounts of urokinase in the cold room. Aliquots (0.1 ml) were removed at intervals and the plasmin activity determined by the rate of decrease in optical density in the heparin-protamine assay. As seen in Fig. 8, urokinase acts like a typical enzyme with the ultimate amount of plasmin formed being independent of the urokinase concentration.

Esterase activity of urokinase

TROLL AND SHERRY[11] found that LEe is hydrolyzed by the plasminogen activator formed by the interaction of streptokinase and an activator precursor in human blood. Since this activator reacts enzymically with plasminogen[11,14] it was obvious that urokinase should be tested for esterase activity. Indeed, it was found that LEe was split by urokinase. Mixtures of 0.8 ml of 0.1 *M* imidazole buffer pH 6.5 and 0.2 ml of 0.2 *M* LEe in water were incubated at 37° C with 50 μl, 25 μl and 10 μl of a urokinase solution containing 90,000 units/ml. Aliquots were removed at intervals for determination of the liberated lysine. In analogous experiments 0.5 *M* Tris buffer pH 9.0 and a urokinase solution with 80,000 units/ml were used. The hydrolysis of LEe was found to follow a first order reaction at pH 6.5 and a zero order reaction at pH 9.0 (Fig. 9), indicating a distinct change in the Michaelis constant in this range. In both cases the hydrolysis of the ester was negligible in controls without urokinase.

Since plasmin can also split TAMe[3,11], this ester was tested with urokinase and indeed found to be hydrolyzed, as shown in Fig. 10.

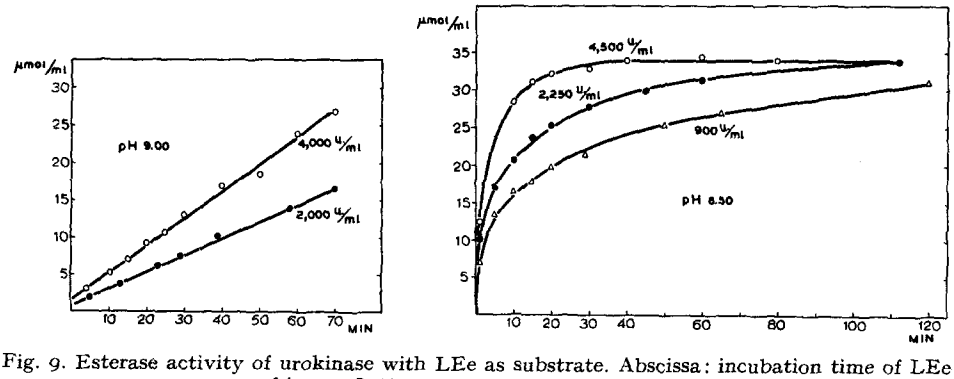

Fig. 9. Esterase activity of urokinase with LEe as substrate. Abscissa: incubation time of LEe urokinase; Ordinate: amount of lysine liberated.

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High urokinase concentrations were necessary for hydrolysis of the esters. Inhibition experiments were performed to determine whether the esterase and plasminogenase activity were due to a common site on the enzyme or to impurities.

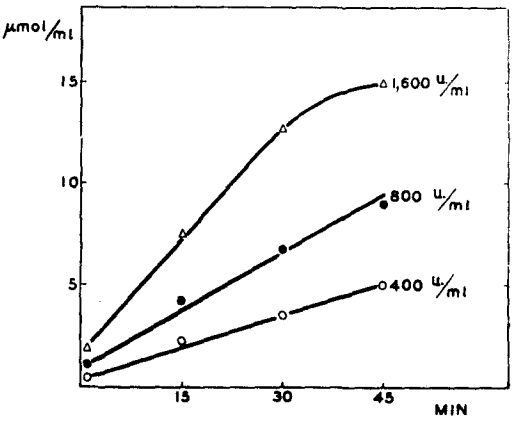

Fig. 10. Hydrolysis (at 37° C) of 0.02 *M* TAMe in 0.25 *M* Tris buffer pH 9.0 with varying amounts of urokinase.

Inhibition of plasmin formation by LEe and TAMe

Addition of LEe to a urokinase-activated plasminogen solution in Tris buffer pH 9.0 with 2.5% casein results in the formation of a precipitate. This precipitate is caused by the interaction of casein and the lysine formed from LEe by plasmin hydrolysis. If LEe is added to the plasminogen solution before or at the same time as urokinase, no precipitate is seen; thus no lysine is formed and the inhibition of plasmin formation is demonstrated. Quantitative experiments confirmed this finding and showed the inhibition of plasmin formation by both LEe and TAMe.

The data in Table II show the strong inhibition of the activation process and the much weaker inhibition of the plasmin action in the activated samples, thus tracing the plasminogenase and esterase activities to the same active groups.

TABLE II

INHIBITION OF PLASMIN FORMATION BY LEe AND TAMe

		Inhibition %	
	Concn.	*Ester added*	
		before urokinase	*after urokinase*
LEe	0.06 *M*	75	10
TAMe	0.015 *M*	100	15

Mixtures of 0.4 ml plasminogen solution (5 mg/ml), 0.4 ml 5% casein in 0.5 *M* tris buffer pH 9.0 and 0.01 ml urokinase solution (10,000 units/ml) were incubated for 10 min at 24° C and then 0.05 ml of 1.0 *M* LEe or 0.25 *M* TAMe was added. In other samples the esters were added before addition of urokinase and incubation.

Plasmin activity was determined in the heparin-protamine system. Results are expressed as % inhibition compared to non-inhibited controls.

Proteolytic activity of urokinase

High concentrations of urokinase degrade a heparin-protamine complex. The decrease in optical density demonstrated a straight line relationship with time. Proportionality between the rate of decrase in optical density and urokinase concentration was observed. With a urokinase concentration of 6,000 units/ml the decrease in optical density was found to be 0.010 per min.

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DISCUSSION

Urokinase is the first physiological plasminogen activator which has been isolated in highly purified form. In accordance with the results of SGOURIS, TAYLOR AND MCCALL[12] and SHERRY AND ALKJAERSIG[13], the experiments reported confirm that urokinase transforms plasminogen into plasmin by an enzymic reaction following the kinetics of a first order reaction. MÜLLERTZ[14] and TROLL AND SHERRY[11] showed the enzymic nature of the plasminogen activator formed in human blood by the action of streptokinase on an activator precursor. Fibrinolytic systems, very much resembling that in blood, are found in most body secretions such as tears[15], saliva[16] and milk[17]. The streptokinase-sensitive precursor is dominant in these cases and only small amounts of free activator are found. The reverse is the case with urine where the free activator is present in large amounts. This poses the question of the identity of urokinase with the blood activator and the possible transformation of the precursor in the kidneys. The blood activator was reported to shown esterase activity only towards LEe[11]. We found urokinase to show esterase activity towards both LEe and TAMe. This, together with the stability of urokinase in the acid range[1], seems to indicate that urokinase is different from the acid labile blood activator[14]. However, the apparent acid instability of urokinase when measured in urine[1] demonstrates the difficulties in comparing impure and purified systems. Final proof must await further studies with purified preparations.

SUMMARY

1. The transformation of plasminogen into plasmin by urokinase was shown to be a first order enzymic reaction, with pH optimum around pH 9. Plasmin formation was followed by fibrinolytic and proteolytic measurements.

The proteolytic action of plasmin was determined by the degradation of a heparin-protamine complex.

2. Urokinase was found to possess esterase activity towards L-lysine ethyl ester and *p*-toluenesulfonyl-L-arginine methyl ester. The plasminogen activation, in turn, is inhibited by these esters. It is concluded that the plasminogenase and esterase activities are due to the same active sites on the enzyme.

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