Diffusional transport of urokinase and acyl-urokinase into fibrin and plasma clots: time of contact and fibrinolytic response *in vitro*

L. I. Moukhametova, R. B. Aisina, and S. D. Varfolomeyev*

Department of Chemistry, M. V. Lomonosov Moscow State University, Leninskie Gory, 119899 Moscow, Russian Federation. Fax: +7 (095) 939 5417. E-mail: arb@enzyme.chem.msu.ru

The effect of short-time contacts (2—20 min) of fibrin and plasma clots with solutions of urokinase and acyl-urokinase in a buffer or human blood plasma on the degree and duration of fibrinolysis was studied *in vitro*. Both plasminogen activators readily diffuse into clots in these time intervals and initiate prolonged dose-dependent lysis of washed fibrin clots devoid of plasma inhibitors. In a plasma clot—plasma system containing inhibitors, the fibrinolytic action of urokinase that penetrated is rapidly suppressed. Acyl-urokinase that penetrated into plasma clots supports prolonged thrombolysis in the absence of an activator in the surrounding plasma due to its resistance to the action of inhibitors and slow reactivation ($k_{deac} = 6 \cdot 10^{-5}$ s^{-1}).

Key words: urokinase, acyl-urokinase, fibrin and plasma clots, diffusion, fibrinolysis kinetics.

Thrombolysis is a complex process, which involves the insoluble blood clot matrix (the solid phase) and the surrounding plasma (the liquid phase). Both phases contain the principal components of the fibrinolytic system, *viz.*, plasminogen, plasminogen activators, and their inhibitors. The fibrin network serves as an insoluble frame of a blood clot. Dissolution of this network under the action of plasmin is the final and key stage of thrombolysis. Fibrin of the clot not only serves as a substrate for plasmin but also plays an important role in the modulation of the functional properties of the components of fibrinolytic and antifibrinolytic systems.[1—6]

Due to the porous structure of the fibrin gel, mass exchange of proteins occurs between clots and plasma.[7] The penetration of plasminogen activators from the surrounding blood plasma into the clot is an important process because these activators are introduced into the bloodstream in the thrombolytic therapy. The mean pore diameter between fibrin filaments in blood clots is ~5 μm (Ref. 8) due to which proteins such as urokinase (54 kDa), tissue plasminogen activator (tPA, 72 kDa), and plasminogen (92 kDa) with the mean diameters of the molecules of 10 nm (Ref. 9) can penetrate into clots. It should be noted that the *in vivo* mass transfer of thrombolytic agents is accomplished by two ways. The penetration under the blood pressure, which accelerates lysis, occurs in the case of occluded blood clots.* The diffusion mass transfer prevails in the cases of non-occluded blood clots, venous blood clots, or residue of occluded blood clots subjected to partial lysis after reperfusion* where the blood pressure is small.[10]

The affinity of proteins for fibrin affects the mode of their penetration into a clot. Thus due to the high affinity for fibrin (K_d = 0.58 μmol L^{-1}),[12] tPA is concentrated in a thin surface layer of the clot rather than diffuses into the clot.[11] Plasminogen, which is a precursor of plasmin, is weakly bound to specific sites exposed on the intact fibrin** (K_d = 38 μmol L^{-1}).[13] However, this binding enhances the efficiency of plasminogen activation with its activators.[1,14,15] Due to limited splitting of fibrin with plasmin that formed, new binding sites appear. The latter exhibit high affinities for plasminogen and plasmin (K_d = 0.5 μmol L^{-1}) resulting in continuous accumulation of high concentrations of plasminogen, which is supplied by the surrounding plasma, on the clot surface.[3,15—17] Thus, plasminogen activators enhance the efficiency of binding of the substrate to the clot.[18,19]

Plasma inhibitors (type 1 and 2) of plasminogen activators (PAI-1 and PAI-2) and a plasmin inhibitor α_2-antiplasmin (α_2-AP) are also involved in regulation of thrombolysis.[20,21] High doses of the activators, which are introduced into the bloodstream in the therapy, activate plasminogen both in plasma and clots. Plasmin which is generated in plasma is rapidly neutralized with α_2-AP whose concentration (1 μmol L^{-1}) is comparable

* An occluded blood clot is a blood clot which completely blocks the vessel.

* Reperfusion is recovery of the bloodstream after dissolution of the blood clot.

** Intact fibrin is fibrin which is not subjected to hydrolysis with plasmin.

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with that of plasminogen (2 μmol L^{-1}) in plasma,[21] whereas plasmin bound to fibrin is inhibited by α_2-AP 100 times more slowly.[20]

Unlike tPA, urokinase possesses no affinity for fibrin and hence it diffuses into pores of clots rather than is accumulated on the surface where the concentration of plasminogen is high.[11,22] This is one of the main explanations for the fact that the rate of tPA-induced plasminogen activation on fibrin is 1800 times higher than that in plasma, whereas the effect of fibrin on the activation of plasminogen by urokinase is moderate (eightfold increase).[23] As a result of neutralization with the inhibitors PAI-1 and PAI-2, urokinase is unstable in blood plasma *in vitro* ($\tau_{1/2stab}$ = 30 min) and is rapidly cleared from circulation *in vivo* ($\tau_{1/2clearance}$* = 10—16 min) due to the additional effect of the capture of the enzyme-inhibitor complex by liver parenchyma cells.[23—25] Previously, we have demonstrated that urokinase is protected from neutralization with specific plasma inhibitors due to reversible acylation of its active site. As a result, acyl-urokinase ($k_{deac} = 6 \cdot 10^{-5}$ s^{-1}) is much more stable in blood plasma and exerts lesser side effects on plasma proteins compared with native urokinase.[26,27]

In the present study, we examined the ability of urokinase and acyl-urokinase, which possess no affinity for fibrin, to diffuse into clots upon their short-time contact (in the absence of hydrostatic pressure) and initiate *in vitro* fibrinolysis. The effects of plasminogen and plasma inhibitors on the rate and duration of the fibrinolytic response were estimated.

Experimental

The study was carried out with the use of the following substances: high-molecular-weight two-chain urokinase from human urine (54 kDa) exhibiting the specific activity of 100000 IU mg^{-1} (Green Cross, Korea); bovine fibrinogen containing 64% of the coagulable protein and bovine thrombin (the Kaunas Plant of Bacterial Preparations, Lithuania); a pool of freshly frozen citrate human blood plasma (the Hematology Research Center of the Ministry of Public Health of the Russian Federation, Moscow); *p*-nitrophenyl *p*-guanidinobenzoate and Glp-Gly-Arg *p*-nitroanilide, S-2444 (Sigma, USA). Other reagents of "special purity grade" or "reagent grade" are domestically produced.

The amidase activity of urokinase was determined on a Philips spectrophotometer (England) at 405 nm by measuring the initial hydrolysis rate of a 0.4 *mM* solution of S-2444 in an 0.1 *M* phosphate buffer containing 0.15 *M* NaCl, pH 7.4 (buffer A).

Acyl-urokinase was prepared by treatment of a urokinase solution in a 0.05 *M* phosphate buffer at pH 7.4 (buffer B) with an excess of *p*-nitrophenyl *p*-guanidinobenzoate at 4 °C. The degree of acylation of the urokinase active site was established from a decrease in the amidase activity of the enzyme. The resulting guanidinobenzoyl-urokinase was purified from an excess of the titrant and the reaction products and then lyophilized.[27]

The kinetics of deacylation of acyl-urokinase was examined by incubation of 2 μ*M* acyl-urokinase in buffer A at 37 °C. The rate of reactivation of urokinase from acyl-urokinase was established by following the increase in the amidase activity of samples withdrawn during incubation.

The kinetics of fibrinolysis was studied by preparing columns of plasma and fibrin gels in standard Sali test tubes (d = 9.5 mm) according to the following procedure: a thrombin solution (20 μL) was added to plasma or a 8.8 μ*M* solution of fibrinogen in buffer A (0.6 mL) to the final concentration of 1 IU mL^{-1}; the mixture was shaken and left in the vertical position at 25 °C for 2 h. Then buffer A or human blood plasma (0.45 mL) was added to the resulting fibrin or plasma clots, respectively.

Fibrinolysis with continuous contact of the activator with clots. Solutions of urokinase or acyl-urokinase (50 μL) with different concentrations were added to the liquid phase over the clot. The kinetics of fibrinolysis was followed by monitoring the decrease in the height of the gel column (Δl) during incubation at 37 °C using a cathetometer.[28] The fibrinolysis rate was determined as the slope of the initial linear region of the kinetic curve ($\Delta l/\Delta t$). Each experiment was repeated in triplicate.

Fibrinolysis with short-time contact of the activator with clots. Solutions of the activator (0.5 mL) with different concentrations in a buffer or plasma were added to the fibrin or plasma clots, respectively, that were formed as described above. After incubation at 37 °C for 2, 10, or 20 min, the liquid phase containing the activator was removed and the clots were carefully washed with a tenfold volume of buffer A. Then the fresh buffer or plasma (0.5 mL) was added to the clots and the kinetics of fibrinolysis was measured as described above. In some runs, the clots were repeatedly washed after performing fibrinolysis for 90 or 150 min. Each experiment was repeated in triplicate.

Results and Discussion

As mentioned above, the lifetime of urokinase in the bloodstream is short ($\tau_{1/2clearance}$ = 10—16 min). A series of experiments was carried out with the aim of estimating the ability of urokinase or acyl-urokinase to penetrate into the inner cavities of the clots over a short period of their contact under the conditions of diffusional transport and for the purpose of evaluating the effects of plasminogen and plasma inhibitors on the degree and duration of fibrinolysis under the action of urokinase and acyl-urokinase that penetrated into the clots.

Lysis of fibrin clots immersed in a buffer. In the first series of experiments, the effects of continuous and short-time incubation of urokinase and acyl-urokinase with clots on the lysis rate were examined in the absence of plasma inhibitors. For this purpose, a fibrin gel containing plasminogen (0.25 μmol L^{-1}) was used as the solid phase and buffer A containing different concentrations of the activator was used as the liquid phase. The kinetics of fibrinolysis was measured either in the continuous presence of a solution of the activator over the clot or before and after the replacement of the latter by a fresh buffer after incubation for 2, 10, and 20 min.

The kinetics of lysis of fibrin clots during their continuous incubation with a solution of urokinase or acyl-urokinase in the buffer as a function of the dose is shown in Fig. 1. It can be seen that the linear rates of

* Clearance is the process of the removal of agents from the bloodstream.

fibrinolysis observed after the initial lag period are virtually identical for the equimolar concentrations of free and acylated urokinase, whereas the lag period is larger for acyl-urokinase. The presence of the lag period is associated with accumulation of the threshold concentration of plasmin in clots, which initiates the measurable fibrin lysis. The time of accumulation of this threshold concentration of plasmin is proportional to the initial rate of plasminogen activation, which, in turn, depends on its concentration in the clots as well as on the rate of diffusion into the gel and the catalytic efficiency of the plasminogen activator. The larger lag period for acyl-urokinase compared with that for urokinase is associated only with the rate of reactivation of the acyl activator because the concentrations of plasminogen in fibrin clots as well as the rates of diffusion of urokinase and acyl-urokinase into the gel from their solutions with the equimolar contents are identical. This was confirmed by the fact that the lag period was substantially reduced with the use of the same concentration of another specimen of acyl-urokinase (trimethylammoniocinnamoyl-urokinase) whose reactivation rate was an order of magnitude higher than that of acyl-urokinase under study (the data are not presented here). The rates of the mass transfer of urokinase and acyl-urokinase into the gel increased as their concentrations in the liquid phase were increased resulting in a reduction of the lag period and an increase in the linear rate of fibrin lysis (see Fig. 1). In spite of the comparable rates of linear lysis of fibrin clots with equimolar concentrations of two activators, the degree of lysis under the action of acyl-urokinase after 4 h is substantially smaller than that obtained under the action of urokinase due to the larger lag period (Table 1). In a control experiment (the clot was incubated in buffer A), no noticeable fibrinolysis was observed.

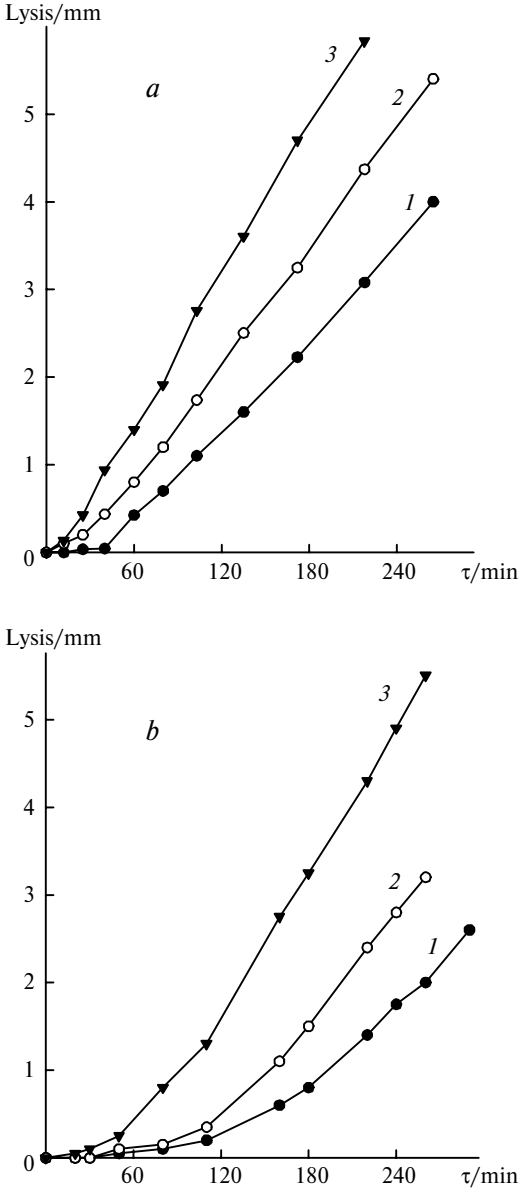

Fig. 1. Kinetics of lysis of fibrin clots upon their continuous incubation with solutions of urokinase (*a*) and acyl-urokinase (*b*) in a buffer. The concentrations of the activators: 100 (*1*), 250 (*2*), and 500 IU mL^{-1} (*3*).

The kinetics of lysis of fibrin clots after their short-time incubation with a solution of urokinase or acyl-urokinase in the buffer, washing, and addition of the fresh buffer is shown in Fig. 2. The results obtained indicate that even the 2—3 min-long contact is sufficient for a portion of the activator to penetrate into the gel and activate plasminogen incorporated into the gel, thus causing lysis of fibrin in the absence of the activator in the liquid phase. The amount of the activator, which had penetrated into the gel, increased as the duration of the contact was increased to 20 min resulting in shortening of the lag period and an increase in

Table 1. Degrees of lysis of fibrin clots and plasma clots in a buffer and plasma, respectively, upon continuous incubation for 4 h and short-time incubation with different concentrations of urokinase (UK) and acyl-urokinase (acyl-UK) (37 °C)

Concentration of the activator /IU mL^{-1}	Time of contact /min	Degree of lysis after 4 h (%)			
		Fibrin clots		Plasma clots	
		UK	acyl-UK	UK	acyl-UK
100	20	24.2	—	—	—
	Continuous	57.9	29.0	15.8	26.6
250	20	33.0	—	—	—
	Continuous	79.2	48.5	36.7	43.3
500	2	20.0	12.0	—	—
	10	40.0	35.0	—	—
	20	51.0	43.0	11.6	23.3
	Continuous	>100	82.0	45.0	66.0
650	Continuous	—	—	53.3	76.7
1000	20	—	—	20.8	36.7
	Continuous	—	—	83.3	88.3
1500	20	—	—	26.7	55.0
	Continuous	—	—	100	>100

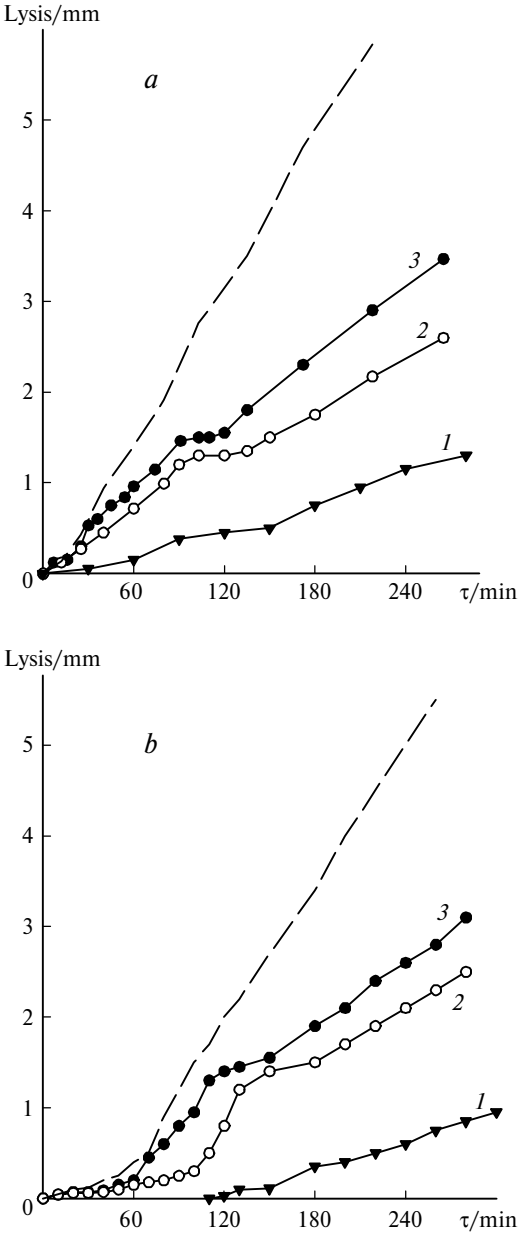

Fig. 2. Effect of the duration of the preliminary contact of fibrin clots with urokinase (*a*) and acyl-urokinase (*b*) (500 IU mL^{-1}) in a buffer on the fibrinolytic response. The time of contact was 2 (*1*), 10 (*2*), and 20 min (*3*). The lysis curves upon continuous incubation of the activators with fibrin clots are shown by dashed lines.

the fibrinolysis rate. In this case, both urokinase and acyl-urokinase, which had penetrated into the gel, caused rather deep lysis of fibrin clots in 4 h in the absence of inhibitors in the system (see Table 1).

Unlike linear lysis, which was observed after the lag period upon continuous incubation of fibrin clots with the activators (see Fig. 1), the kinetic curves of lysis of the clots obtained after their short-time incubation with urokinase or acyl-urokinase have a pronounced plateau after the large initial lag period and subsequent acceleration of the reaction (see Fig. 2). The fibrinolysis rate after the plateau is lower than that before the plateau. To reveal the reasons for this effect, we carried out additional experiments. The curves of lysis of fibrin clots incubated with different concentrations of urokinase (100, 250, and 500 IU mL^{-1}) for 20 min were analogous to curves *1*—*3* in Fig. 2, *a* (data are not shown here). When fibrin clots which have been washed after incubation with a solution of urokinase (500 IU mL^{-1}) for 20 min were incubated in the buffer and repeatedly washed after either 90 min (curve *1*) or 150 min (curve *2*), lysis in the fresh buffer persisted, but a new plateau appeared (Fig. 3). Such prolonged fibrinolysis, which was observed in the absence of plasma inhibitors even after repeated washing of fibrin clots, demonstrates that urokinase penetrates rather deeply into the gel upon the contact for 20 min. Although urokinase, unlike tPA, is not adsorbed on the clot surface, its concentration in the inner cavities of the clot decreases from the surface toward the center of the clot due to diffusion, whereas the concentration of plasminogen that is incorporated is constant throughout the fibrin gel. Hence, the rate of activation of plasminogen and, consequently, the lysis rate are maximum in the surface layer of the gel. In the course of lysis with plasmin, fibrin fibers in this layer become thinner, their mobility increases, and mobile agglomerates linked to the immobile fibrin network appear.[11] Apparently, washing of the gel leads to the enhancement of the mobility of agglomerates due to which plasminogen molecules included in these agglomerates become more accessible to the action of urokinase molecules located in this layer. Once the highly mobile layer is completely dissolved with the transfer of the plasmin and urokinase molecules, which are located in this layer, into the solution (thus exposing the less mobile layer with a lower urokinase content), the lysis rate falls (the plateau) until the threshold concentration of plasmin is achieved in this layer. Then lysis proceeds at a constant

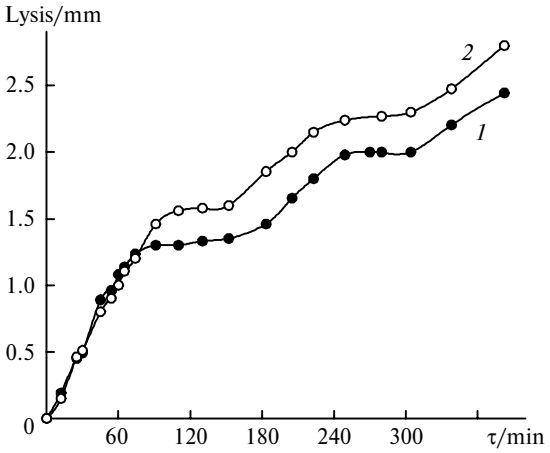

Fig. 3. Effect of repeated washing of clots incubated with a solution of urokinase in a buffer (500 IU mL^{-1}) for 20 min. The time of washing was 20 and 90 min (*1*), 20 and 150 min (*2*).

rate in the absence of repeated washings, which is apparently associated with gradual diffusion of urokinase into deeper layers and the local redistribution of plasmin exhibiting high affinity for partially degraded fibrin[15] between the liquid and solid phases within the clot layer being dissolved.

Lysis of plasma clots immersed in plasma. The kinetics of lysis of plasma clots upon their continuous incubation with solutions of urokinase or acyl-urokinase with different concentrations in plasma is shown in Fig. 4. Under these conditions, spontaneous lysis of plasma clots is immeasurable. From comparison of curves *1—3* in Figs. 1 and 4, it can be seen that the lag periods and the rates of lysis of plasma clots are smaller than the corresponding values for fibrin clots. After the lag period, the rate of lysis of plasma clots with urokinase gradually decreases, whereas linear lysis is observed in the case of acyl-urokinase up to the concentrations of 650 IU mL^{-1} (Fig. 4). After 4 h, the degree of lysis of plasma clots with both activators is lower than that of fibrin clots (see Table 1) due to the differences both in the activators and the compositions of the solid and liquid phases of two systems. Unlike the fibrin clot immersed in buffer A, the plasma clot and the surrounding plasma contain plasminogen and inhibitors of urokinase and plasmin (PAI-1 and α_2-AP, respectively). On the one hand, the higher concentration of plasminogen in plasma clots (2 μmol L^{-1}) and its continuous sorption from the plasma on the surface subjected to lysis lead to an increase in the rate of lysis with the activators. On the other hand, urokinase and plasmin inhibitors suppress lysis, α_2-AP playing a significant role. Thus we found that the incorporation of α_2-AP (0.8 μmol L^{-1}) into fibrin clots has no substantial effect on the lag period of urokinase fibrinolysis but decreases the rate of fibrinolysis both with urokinase (500 IU mL^{-1}) and plasmin (0.3 μmol L^{-1}) by a factor of approximately 2.5—3 (data are not shown here). In addition, neutralization of urokinase with the inhibitor PAI-1 makes a noticeable contribution to inhibition of lysis of plasma clots under the action of urokinase. Acyl-urokinase, which was protected from the action of PAI-1 before reactivation, caused more rapid and deeper lysis of plasma clots compared with urokinase (see Fig. 4 and Table 1).

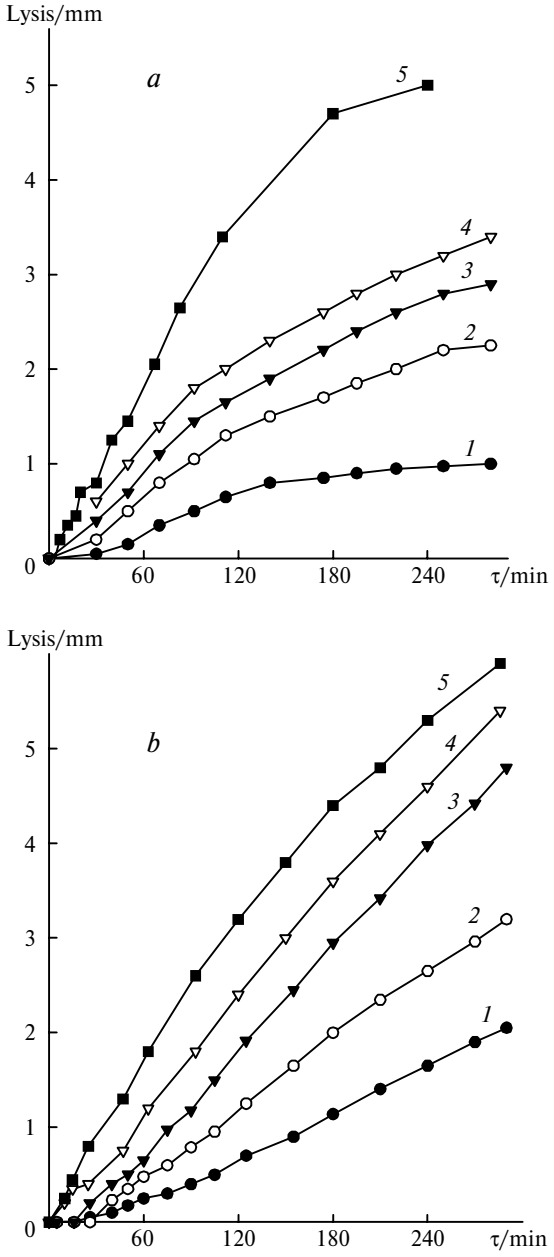

Fig. 4. Kinetics of lysis of plasma clots upon their continuous incubation with solutions of urokinase (*a*) and acyl-urokinase (*b*) in blood plasma. The concentrations of the activators were 100 (*1*), 250 (*2*), 500 (*3*), 650 (*4*), and 1000 IU mL^{-1} (*5*).

The kinetics of lysis of plasma clots, which was measured after their incubation with urokinase or acyl-urokinase (500 or 1000 IU mL^{-1}) for 20 min, washing, and introduction of the fresh plasma, is shown in Fig. 5. It can be seen that lysis under the action of urokinase, which had penetrated into clots in 20 min, rapidly

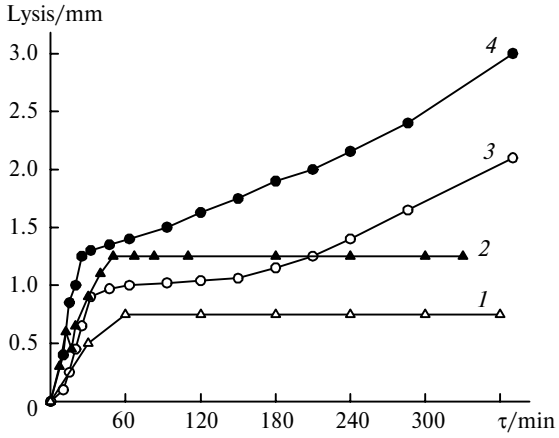

Fig. 5. Lysis of plasma clots after their incubation with urokinase (*1*, *2*) and acyl-urokinase (*3*, *4*) (the concentrations were 500 IU mL^{-1} (*1*, *3*) and 1000 IU mL^{-1} (*2*, *4*)) during 20 min in plasma, washing, and addition of fresh plasma.

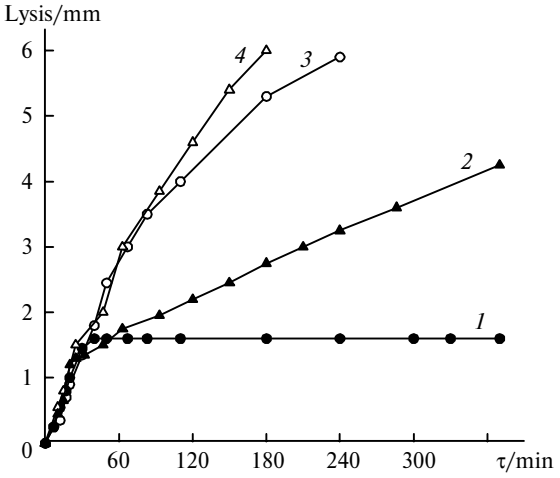

Fig. 6. Comparison of the kinetics of lysis of plasma clots after their incubation for 20 min (*1*, *2*) and upon continuous incubation (*3*, *4*) with urokinase (*1*, *3*) and acyl-urokinase (*2*, *4*) (1500 IU mL^{-1}).

ceased when the degree of lysis reached 12 and 21%, respectively, whereas acyl-urokinase, which had penetrated into the gel, maintained prolonged lysis of plasma clots after the second lag period (the plateau). Due to the contributions of partial local inhibition of plasmin that formed with α_2-antiplasmin and inhibition of urokinase, which was slowly released from acyl-urokinase, with PAI-1 in the layer subjected to lysis, the plateau was larger and the rate of lysis of plasma clots was lower than those of fibrin clots (curve *3* in Fig. 5 and curve *3* in Fig. 2, *b*). This lag period occurred, apparently, for the same reason for which the lag period appeared upon washing of fibrin clots. The difference in the efficiency of lysis of plasma clots under the action of acyl-urokinase and urokinase, which had penetrated into gels in 20 min, increased as the concentrations of the activators in plasma were increased to 1500 IU mL^{-1} (Fig. 6). After incubation of plasma clots with acyl-urokinase (1500 IU mL^{-1}) for 20 min, the degree of lysis of these clots (4 h) reached 55% and lysis lasted for 6 h, whereas lysis in the case of urokinase was terminated after 40 min with the degree of lysis of 26.7% (see Table 1).

Thus, urokinase and acyl-urokinase can diffuse rather deeply into inner cavities of fibrin and plasma clots even upon incubation for 2—20 min. Both activators which had penetrated into clots initiate noticeable and prolonged lysis of fibrin clots containing plasminogen. The fibrinolytic effect of urokinase which had penetrated into the plasma clot is rapidly suppressed due to neutralization with specific plasma inhibitors. It was demonstrated for the first time that acyl-urokinase, which penetrates into clots and is being slowly reactivated, can maintain prolonged thrombolysis in the presence of plasma inhibitors as well. The results of the present study show that reversible acylation of the urokinase active site enhances the fibrinolytic efficiency of the enzyme.

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