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Adsorption of Plasminogen Activator to Fibrin

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The plasminogen activator formed by reaction between streptokinase and human proactivator is quantitatively adsorbed on human fibrin clots. Separation of fibrin from adsorbed activator was not accomplished, but dilute neutral solutions suitable for estimation of activator could be prepared. The proactivator obtained by heat inactivation of acid solutions of fibrin and adsorbed activator was separated from the fibrin. It was extremely labile in neutralized solutions.

According to present knowledge [1,2], the effect of streptokinase in the activation of fibrinolysis can be described by the following schemes:

$$\text{streptokinase} + \text{proactivator} \rightleftharpoons \text{activator}$$

$$\text{plasminogen} \xrightarrow{\text{activator}} \text{plasmin}$$

A purified preparation of the plasminogen activator would be of interest for many reasons. Its properties could be studied and it could be compared with other components of the fibrinolytic system. It would also be valuable in clinical research. Müllertz [3,4] has found the activator to be adsorbed on fibrin. The present work describes the preparation of acid solutions of fibrin containing adsorbed activator, and the estimation of the content of activator in such solutions. With the procedure described, the yield of activator was nearly quantitative.

MATERIALS AND METHODS

Barbital buffer. Diethyl barbiturate (Michaelis), pH 7.8, to which was added NaCl to give a final ionic strength of 0.15.

Phosphate buffer (Sørensen) pH 7.6.

Streptokinase. "Varidase" was kindly supplied by the Lederle Laboratories Division, American Cyanamid Company. The units are those stated on the packages and determined according to Christensen [5].

Proactivator. Human plasma obtained from out-dated citrated bank blood was used without further purification.

Thrombin. A preparation of bovine thrombin containing about 20 000 NIH units (National Institute of Health) per g was kindly supplied by Løvens kemiske Fabrik, Copenhagen.

Measuring methods: Activator and proactivator were determined by the modified lysis time method described by Lassen [6].

RESULTS

1. *Adsorption of activator to fibrin.* The following procedure was found to give the best yield:

Streptokinase dissolved in 1 ml of phosphate buffer, pH 7.6, 0.1 mg of thrombin dissolved in 50 μl of the same buffer, and 1 ml of human plasma were cooled in an ice bath and mixed in the order mentioned. In general, 5 000 units of streptokinase were used, since this amount was found to be nearly equivalent to the content of proactivator in 1 ml human plasma, and thus ensured the best utilization of both precursors. After standing for 4 h at 0° the clot was finely divided with a glass rod and collected by centrifuging for 10 min in a high speed centrifuge (20 000 rev/min) at 0°. The precipitate was washed thoroughly with 5 ml of icecold phosphate buffer and again centrifuged. This washing procedure was repeated twice, first with buffer as before, and then with 5 ml of icecold distilled water. Finally, the fibrin was dissolved at 0° in 2 ml 0.05 M acetic acid, and cleared by centrifuging. It is important to keep the temperature at 0°, since even at this temperature the activator was found to deteriorate in acid solutions (5 — 10 % were lost per hour and the temperature coefficient was high).

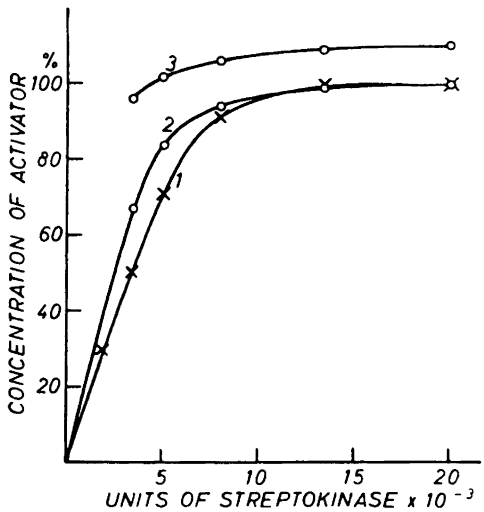

Fig. 1. Adsorption of activator to fibrin. 1 ml of streptokinase solutions in phosphate buffer, pH 7.6, containing the amounts of streptokinase stated on the abscissa were mixed with 50 μl thrombin solution at 0°. 1 ml plasma was then added and the clotted mixtures were stored overnight at 0°. Acid solutions of fibrin were then prepared as described in the text. The activator was estimated in the following solutions: Curve 2, 50 μl fibrin solution + 10 ml barbital buffer. Curve 3, 50 μl fibrin solution + 10 ml barbital buffer containing 500 units of streptokinase. Curve 1, the original mixtures of streptokinase and plasma were prepared without addition of thrombin and incubated for 10 min at room temperature. The activator was then estimated in 50 μl of these solutions diluted with 10 ml barbital buffer.

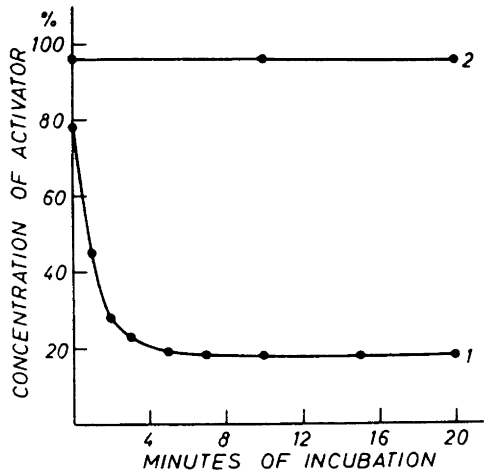

Fig. 2. Inactivation of activator at acid reaction. A solution of fibrin and activator in 0.05 M acetic acid, pH 3.5, was prepared as described. The solution was incubated at 37° and the activator (curve 1) and the sum of activator and proactivator (curve 2) were determined at different times. For the estimations of activator 50 μl of the acid solution were diluted with 10 ml of barbital buffer. For the estimations of activator + proactivator 250 units of streptokinase were added. 1 000 units of streptokinase dissolved in 1 ml of the heated solution of fibrin was found to be stable for 2 h at 37°.

After diluting 50 μl of the acid solution with 10 ml barbital buffer the activator could be measured as described [6]. This was possible since precipitation of fibrin is a very slow process at this high dilution. In four experiments in which 5 000 units of streptokinase per ml of plasma were used, the amounts of activator obtained constituted 110, 135, 140 and 150 %, respectively, of the amounts obtained when the same amount of streptokinase and proactivator (human plasma) were incubated for 10 min at room temperature. In all experiments the amount of activator which remained in serum constituted less than 0.5 %. The apparent excess over 100 % in "yields" can perhaps be ascribed to an influence of fibrin on the formation and stability of the activator.

In many experiments it was found more convenient to leave the clotted solutions overnight at 0°. This was investigated in the next experiment with different concentrations of streptokinase (Fig. 1, curve 2). Curve 1 shows the amounts of activator formed after incubation of the unclotted streptokinase-plasma mixtures for 10 min at room temperature. This curve is in full agreement with the results obtained by Müllertz [2] on the basis of which the reaction between streptokinase and proactivator was proposed to be stoichiometric. The similarity between curves 1 and 2 shows the adsorption of the activator to be nearly quantitative for all concentrations of streptokinase employed. Furthermore, the sum of activator and proactivator in the acid solutions of fibrin was estimated by adding an excess of streptokinase to the buffer used for the dilution (curve 3). The higher values obtained in curve 3 as compared

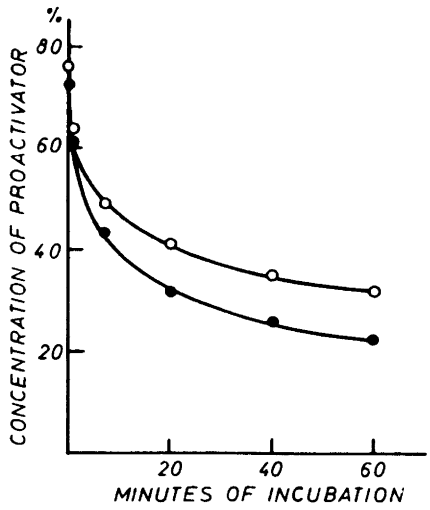

Fig. 3. Lability of proactivator prepared from two different solutions of fibrin plus activator. These solutions were stored overnight at −20°, thawed and centrifuged. The supernatants were neutralized at room temperature with equal volumes of 0.2 M Na_2HPO_4 and the proactivator was estimated at different time intervals.

with curve 2 indicate the presence of an excess of proactivator in the acid solutions of fibrin.

Attempts at separating fibrin and activator have been performed as follows: Precipitation with sodium chloride, or with ethanol at low temperature, or with sodium tungstate, or by neutralization in the presence of phosphate buffer at different concentrations and in the presence of L-lysine ethylester, benzoyl-L-arginine ethylester, amino acids, proteins (including casein, gelatin, Witte peptone, or bovine fibrin hydrolysed with trypsin or chymotrypsin), nucleic acid, sodium sulphosalicylate or sodium tungstate. The best yield (5 %) was obtained with bovine fibrin hydrolysed by trypsin. By precipitation at neutral reaction most of the activator was recovered in the fibrin, while the precipitations at acid reactions were accompanied by deterioration of activator. Furthermore, selective adsorption to barium sulphate and Amberlite IRC 50 from the acid solutions was studied, but these procedures caused either inactivation or irreversible adsorption of activator. An attempt to hydrolyse fibrin in acid solution with pepsin resulted in rapid inactivation of activator. Fibrin made by thrombin coagulation of the citrated plasma used was found to be insoluble in dilute acetic acid. An attempt was made to produce insoluble fibrin from the mixture of plasma and streptokinase by centrifuging immediately after formation of the clot, but some of the fibrin dissolved and the yield of activator was low.

2. *Inactivation of activator in acid solutions.* Troll and Sherry [1] inactivated the activator at acid pH under rather drastic conditions (100° for 15 min at pH 2). They found that most of the original amount of proactivator was

still present in the heated solutions, since reactivation could be obtained by addition of streptokinase to the neutralized solutions. They assumed the acid inactivation to result from destruction of streptokinase and concluded that the activator was formed through a reversible reaction between streptokinase and proactivator:

$$\text{Streptokinase} + \text{proactivator} \rightleftharpoons \text{activator}$$

The inactivation at acid reaction was explained by a displacement of this reaction towards the left, due to removal of streptokinase. The activator present in the acid solution of fibrin (pH about 3.5) was found to be extremely labile at 37°, as well as at room temperature, although complete inactivation could never be achieved (Fig. 2, curve 1). During inactivation equivalent amounts of proactivator were apparently formed (Fig. 2, curve 2). These results are in accordance with Troll and Sherry [1]. However, streptokinase was found to be stable under the experimental conditions (legend to Fig. 2). The assumption that the inactivation of activator at acid reaction is caused by destruction of the orginal streptokinase therefore appears questionable.

3. *Separation of proactivator and fibrin.* Several attempts to isolate the proactivator produced by acid inactivation have been made. Of many experiments the following procedure was found to give the nearest approach to a separation: Acid solutions of fibrin and adsorbed activator were prepared as described. The solutions were placed overnight at —20° and thawed the next day. This procedure caused irreversible precipitation of the fibrin. The centrifuged solutions contained very little activator and about 75 % of the theoretical amount of proactivator. However, proactivator produced in this manner was found to be extremely labile in the neutralized solutions (Fig. 3). In this respect this "proactivator" apparently differs from the genuine proactivator.

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