The Effect of Urokinase and Fibrinolytic Euglobulins on Synthetic Amino Acid Esters

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The increasing interest and the accumulating knowledge of fibrinolysis has induced a search for more convenient and accurate methods for the measurement of this particular proteolytic phenomenon. Procedures allowing a more precise measurement of single phases of the complex process would be of great interest.

Fibrinolysis is induced by activating plasminogen, a precursor contained in most human body fluids, to plasmin, the active enzyme. The best known activating agent, until recently almost exclusively used, is streptokinase. Streptokinase acts probably through the intermediary of a pro-activator, also contained in most body fluids. It is also known that activating human 'kinases' exist, particularly in urine (Astrup & Sterndorff, 1952). Only recently has it been possible to isolate and purify this human kinase from urine and to make it available in quantities (Kaulla, 1954, 1956). The urinary material is called urokinase or urofibrinolysokinase. It enables the production of fibrinolysis with human activating agents instead of with the bacterial streptokinase.

In order to measure fibrinolytic processes more accurately, the fact has been exploited that proteolytic enzymes are capable of splitting synthetic amino acid esters. Purified plasmin preparations have been shown to split the synthetic amino acid esters (Troll, Sherry & Wachman, 1954; Troll & Sherry, 1955). However, Schultz, Moorman, Matoush & Lincoln (1957) have seen no difference in esterase activity as measured by the release of free amino acids from the esters when blood from patients with very marked spontaneous fibrinolytic activity was compared with non-fibrinolytic normal blood.

This paper presents the results from a study designed to investigate further the correlation of ester-splitting activity with the proteolytic breakdown of fibrin (fibrinolysis). The esterase activity of human urokinase preparations, fibrinolytic

human euglobulins and non-fibrinolytic euglobulins, alone and in the presence of plasma, were determined. The fibrinolytic euglobulins were obtained from plasma treated with human urine. When added before clotting they caused, in the concentration used, complete dissolution of a plasma clot in 20 min.-1 hr. The non-fibrinolytic euglobulins were obtained from untreated plasma. They did not cause appreciable lysis of a plasma clot on 24 hr. incubation.

Urokinase acts, not as a fibrinolytic enzyme itself, but as an activating agent towards plasminogen (which in our experiments is contained in the plasma), transforming it into plasmin (fibrinolytic enzyme). On the other hand, the fibrinolytic euglobulins contain active plasmin formed by activation of plasminogen during the treatment of the plasma from which they were derived with urine (see below).

Our results demonstrate a further lack of correlation between esterase activity and fibrinolytic activity when the activation of human-plasma plasminogen was brought about by human activators. This is in contrast with the results obtained after activation of plasma plasminogen by streptokinase. Both findings would allow for speculation whether the intermediate steps in the process of fibrinolysis could be correlated with esterase activity.

MATERIALS AND METHODS

Urokinase. Urokinase was prepared from fresh human urine according to the method described by Kaulla (1956) and stored under vacuum at 4°.

Euglobulins. For the preparation of fibrinolytic euglobulins, 1 vol. of outdated blood-bank plasma was added to 20 vol. of fresh human urine, and the mixture dialysed for 3 hr. against ice-cold running tap water. This treatment brings about the activation of plasminogen contained in the plasma to plasmin by the urine activator. The mixture was then saturated with CO₂. By this step the euglobulins were precipitated and most of the antifibrinolysin was removed. The plasmin was precipitated with the euglobulins (Kaulla & Taylor, 1957).

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The non-fibrinolytic euglobulins were prepared from plasma by diluting it with 20 vol. of distilled water and subsequently saturating with CO_2 , which precipitated them.

The precipitates obtained by these procedures were separated by centrifuging and dissolved in buffered saline [0.74M-sodium barbitol-acetate, pH 7.4, + 0.85% aq. NaCl (1:4, v/v)] to the original plasma volumes. The re-dissolved euglobulins were stored at -25° and thawed immediately before use.

Plasma. Only human plasma was used throughout the study. It was obtained from freshly drawn, citrated blood [1 vol. of 3.8% (w/v) sodium citrate (U.S.P.) to 4 vol. of blood] by centrifuging at 2000 rev./min. for 10 min.

Streptokinase. Varidase (Lederle Laboratories Division, American Cyanamid Co., New York, N.Y.) was used. The lyophilized preparation was dissolved in distilled water at a concentration of 10 000 units/ml. To each tube 0.5 ml. (5000 units) was added.

Synthetic amino acid esters. Lysine ethyl ester and toluene-*p*-sulphonyl- (tosyl)-arginine methyl ester (Mann Research Laboratories, New York, N.Y.) were each dissolved in distilled water at a concentration of 0.1M immediately before use.

Estimation of the esterase and fibrinolytic activities of urokinase. The lysine ethyl ester and tosylarginine methyl ester assays were performed in the manner described by Troll & Sherry (1955) with 40 and 60 min. incubation periods. A water blank was run to determine the amount of amino acid liberated by incubation without enzyme. Also, streptokinase, urokinase, a urokinase-streptokinase mixture, and plasma blanks were determined. An amount of urokinase (usually 0.5–2 mg. added before clotting) sufficient to cause lysis of a plasma clot in 20–120 min. at 37° was dissolved in 1 ml. of fresh plasma drawn from a healthy member of the laboratory. A sample of this mixture was diluted 1:4 with distilled water for the amino acid ester assays with and without the addition of streptokinase, and another sample was diluted 1:25 in 0.1M- CaCl_2 solution to bring about clotting in order to determine its effect on a fibrin clot after 45 min. and 24 hr. incubations. The amount of fibrin present after each period of incubation was estimated by its tyrosine content (Quick, 1951).

Determination of esterase and fibrinolytic activities of the euglobulin preparations. 1 ml. of either euglobulin preparation, equivalent to 1 ml. of the original plasma, was diluted 1:4 with distilled water and the esterase activity of the fibrinolytic as well as of the non-fibrinolytic euglobulins was determined with the two ester assays. To determine the effect of the euglobulins in the presence of plasma, 1 ml. of

either euglobulin was added to 1 ml. of fresh plasma and the mixture diluted to 5 ml. with distilled water for the ester assays, or to 25 ml. with 0.1M- CaCl_2 for the fibrin method. Both types of euglobulins, either alone or mixed with plasma, were run with and without the addition of streptokinase. At the same time the plasma alone was tested for esterase and fibrinolytic activity with and without addition of streptokinase.

In all esterase determinations, only the results of the 60 min. incubations are presented, since the statistical analysis showed comparable differences of values obtained with the various preparations for the 40 and 60 min. periods.

The significance of differences found with different systems was estimated by the use of Student's *t* test.

RESULTS

Comparison of the esterase and fibrinolytic activities of urokinase. When urokinase was added to plasma and the mixture was clotted, lysis of the fibrin clot occurred in 20–120 min. according to the strength of the urokinase preparation; no increase in esterase activity was observed on the addition of urokinase to plasma in the amino acid ester assays (Table 1). Streptokinase in plasma caused lysis in 1–2 hr., as did the combination of urokinase and streptokinase in plasma. There was no additive effect on fibrinolysis by the combination of these two plasminogen-activating agents. A significant increase in esterase activity was observed on the addition of streptokinase and streptokinase-urokinase combination to plasma in both ester assays. When streptokinase-urokinase-plasma values are compared with the streptokinase-plasma values, the increase in ester-splitting activity is significantly less in the lysine ethyl ester, but not in the tosylarginine methyl ester assay.

By comparing the 45 min. with the 24 hr. incubations for plasma alone in the fibrin method, a mean decrease of 2.7% of fibrin was observed. Plasma alone in the ester assays showed no significant increase in the amount of amino acid liberated over the water blank (Table 1). Also no increase of liberated amino acids was seen with the urokinase, streptokinase, and streptokinase-urokinase blanks.

Table 1. *Effect of urokinase on spontaneous and streptokinase-activated esterase activity in plasma*

Esterase activity is expressed as a mean increase in μ moles of acid liberated during 60 min. incubation. Numbers in parentheses indicate number of determinations. Tosylarginine and lysine figures are expressed as mean $\pm$ standard error.

	Tosylarginine	Lysine	Clot lysis time
Water (13)	0.180 $\pm$ 0.022	0.018 $\pm$ 0.005	—
Streptokinase (13)	0.125 $\pm$ 0.029	0.020 $\pm$ 0.007	—
Urokinase (4)	0.210 $\pm$ 0.075	0	—
Urokinase + streptokinase (4)	0.172 $\pm$ 0.021	0	—
Plasma (12)	0.206 $\pm$ 0.020	0.022 $\pm$ 0.006	> 24 hr.
Plasma + urokinase (12)	0.206 $\pm$ 0.046	0.023 $\pm$ 0.007	20–120 min.
Plasma + streptokinase (12)	0.580 $\pm$ 0.029	0.405 $\pm$ 0.040	60–120 min.
Plasma + urokinase + streptokinase (12)	0.540 $\pm$ 0.055	0.292 $\pm$ 0.030	60–120 min.

Table 2. *Ester-splitting activity of fibrinolytic and non-fibrinolytic euglobulins used alone and with plasma*

Results are expressed as in Table 1.

	Tosylarginine	Lysine	Clot lysis time
Fibrinolytic euglobulin (9)	0.383 $\pm$ 0.048	0.018 $\pm$ 0.009	—
Non-fibrinolytic euglobulin (9)	0.193 $\pm$ 0.031	0.028 $\pm$ 0.007	—
Fibrinolytic euglobulin + streptokinase (9)	0.430 $\pm$ 0.019	0.112 $\pm$ 0.025	—
Non-fibrinolytic euglobulin + streptokinase (9)	0.514 $\pm$ 0.046	0.216 $\pm$ 0.034	—
Plasma (12)	0.262 $\pm$ 0.033	0.029 $\pm$ 0.008	> 24 hr.
Plasma + fibrinolytic euglobulin (12)	0.246 $\pm$ 0.027	0.031 $\pm$ 0.009	20–50 min.
Plasma + non-fibrinolytic euglobulin (12)	0.132 $\pm$ 0.025	0.036 $\pm$ 0.009	> 24 hr.
Plasma + streptokinase (12)	0.644 $\pm$ 0.047	0.380 $\pm$ 0.022	60–120 min.
Plasma + fibrinolytic euglobulin + streptokinase (12)	0.677 $\pm$ 0.035	0.418 $\pm$ 0.022	20–60 min.
Plasma + non-fibrinolytic euglobulin + streptokinase (12)	0.836 $\pm$ 0.043	0.710 $\pm$ 0.049	20–60 min.

Enzyme activity of the euglobulin preparations in absence of plasma. The fibrinolytic euglobulins had a marked enzyme activity in the tosylarginine methyl ester assay which is not significantly increased by the addition of streptokinase (Table 2). The fibrinolytic euglobulins showed no activity in the lysine ethyl ester assay, but a significant increase is observed with the addition of streptokinase to them.

The non-fibrinolytic euglobulins exhibited no enzyme activity in either assay. The addition of streptokinase to the non-fibrinolytic euglobulins resulted in an increase of the ester-splitting activity significantly higher than the values obtained with the addition of streptokinase to the fibrinolytic euglobulins in the lysine ethyl ester assay, but not significantly higher in the tosylarginine methyl ester assay.

Effect of the euglobulin preparation in the presence of plasma. When the fibrinolytic euglobulins were added to plasma and the fibrin method was used, they caused a complete lysis of the fibrin clot in 20–50 min. In contrast with this, a mean decrease of only 29.4 % of fibrin occurred in 24 hr. with the non-fibrinolytic euglobulins and a decrease of 7.9 % with plasma alone. When either of the euglobulins was added together with streptokinase to plasma, complete lysis of the fibrin clot occurred in 20–60 min.

The esterase activity of plasma to which fibrinolytic euglobulins were added was no different from that of plasma alone, though they induced strong fibrinolysis. The non-fibrinolytic euglobulins caused a significant decrease in the tosylarginine methyl ester assay, but there was no change in the lysine ethyl ester assay. With both esters, the increase in activity of fibrinolytic euglobulins in combination with streptokinase was not significantly higher than that with streptokinase alone. The increase in activity of non-fibrinolytic euglobulins together with streptokinase over that with streptokinase alone was highly significant (Table 2).

DISCUSSION

Little correlation could be found between the fibrinolysis-inducing effect of urokinase and fibrinolytic euglobulins and the plasma-esterase activity. The esterase activity and the fibrinolysis phenomenon are possibly two concomitant aspects of plasmin. A previous investigation has shown a lack of correlation between the two (Schultz *et al.* 1957) in the presence of plasma. Lysis of a plasma clot was readily induced by the addition of fibrinolytic euglobulins, urokinase or streptokinase; an increase in enzyme activity, as measured by the splitting of synthetic amino acid esters, was demonstrated here only on the addition of streptokinase.

The lack of increase in esterase activity on the addition of urokinase to plasma possibly might be due to the well-known presence of plasmatc inhibitors (Bastian, Hill & Ercoli, 1956). The decrease in activity on the addition of urokinase together with streptokinase compared with that obtained on the addition of streptokinase alone could be explained either by a competitive effect of the urokinase (which is added to the plasma before streptokinase) on plasminogen to make it no longer available for streptokinase activation, or by the presence of an inhibitory substance for esterase activity of plasmin in the urokinase preparation. A proteolytic inhibitor which will inhibit human plasma enzyme activated with streptokinase has been purified from human urine (Shulman, 1955).

An activation scheme for plasmin has been presented by Müllertz (1955) and Astrup (1956), in which a proactivator is converted by streptokinase into an activator, which in turn activates plasminogen to plasmin. However, no conclusive information is available as to whether this activation scheme holds true for the activation of plasminogen brought about by human 'kinases', or whether the activation induced by them follows another pathway. Troll & Sherry (1955) have concluded that the activator has the ability to split the lysine esters,

whereas plasmin splits both the arginine and lysine esters.

The results presented in this paper indicate that the fibrinolytic euglobulin preparations contain plasmin which splits the tosylarginine ester but not the lysine ester; they contain little plasminogen, since there is no increase in the splitting of the arginine esters with the addition of streptokinase; and they contain some proactivator, as is shown by the increased breakdown of lysine esters after addition of streptokinase. The non-fibrinolytic euglobulin contains little plasmin but considerable amounts of proactivator and plasminogen, which can be converted into activator and plasmin respectively, as is shown by the marked increase in esterase activity in both assays on the addition of streptokinase.

When fibrinolytic euglobulins are added to plasma, the lack of increase of esterase activity could be caused by the inhibitors of the plasma which have been shown to inhibit ester-splitting activity of trypsin (Bastian *et al.* 1956). The significant inhibition of the plasma-esterase activity by the non-fibrinolytic euglobulins could be due to the presence in the euglobulins of inhibitors which might have been eliminated from the fibrinolytic euglobulins during their preparation. Such inhibitors have been demonstrated in precipitated euglobulins (Müllertz, 1953). The significant increase of esterase observed when streptokinase is added to the non-fibrinolytic euglobulins in plasma can be explained by the presence of plasminogen and proactivator in the euglobulin preparation.

SUMMARY

1. Little correlation could be found between the fibrinolysis-inducing effect of urokinase and fibrino-

lytic euglobulins as shown by lysis of plasma clots and the plasma-enzyme activity measured by the splitting of synthetic amino acid esters.

2. When urokinase was added to plasma there was no increase in esterase activity, but a marked fibrinolytic activity was observed.

3. Fibrinolytic euglobulins used alone showed an activity towards tosylarginine methyl ester, but not towards lysine ethyl ester. There was no increase in activity when the fibrinolytic euglobulins were added to plasma, but a strong fibrinolysis was obtained.

4. It is concluded that the synthetic-substrate methods are not applicable as tests for fibrinolytic activity in the presence of plasma. Possible reasons for the discrepancies are discussed.

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Studies on the Aspartic Decarboxylase of *Nocardia globerula*

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Specific decarboxylases have been described for several amino acids and have proved useful for the estimation of these amino acids in mixtures (Gale, 1957). The enzyme system from *Clostridium welchii* is much more active on glutamic acid than aspartic acid. The determination of aspartic acid with this preparation depends on the difference between the volume of carbon dioxide produced from glutamic acid plus aspartic acid, and that produced from glutamic acid alone when the aspartic decarboxylase is inhibited (Meister, Sober & Tice, 1951). The

present communication describes a preparation specific for the decarboxylation of L-aspartic acid and completely inactive on all other amino acids tested.

MATERIALS AND METHODS

Organism. The organism was isolated from soil and corresponded closely in properties with *Nocardia globerula* (Gray, 1928). A strain of this organism has been deposited with the National Collection of Industrial Bacteria, Teddington, Middlesex, under the no. 8852. During these studies the organism was maintained on agar slopes