PLASMIN DETERMINATION BASED ON RATE OF LYSIS OF FIBRIN GEL

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The importance of the early detection of deviations from the normal plasma plasminogen level in various situations and diseases has necessitated the development of quantitative methods of plasminogen assay. Many methods based on hydrolysis of various synthetic and protein substrates by plasmin already exist. The most specific are those in which fibrin, the physiological substrate of plasmin, is used [4, 6, 7, 14]. Each of these methods has disadvantages. Highly sensitive methods using fibrin containing a radioactive [3] or fluorescent label [11], and also the method of titration of amino groups released during lysis of fibrin [2] are without the majority of these defects. However, their use is restricted by the difficulty of preparing labeled fibrin clots and the need for special equipment and standards. These methods are based on recording soluble fibrinolysis products, which may be heterogeneous both for size of the polypeptides and for content of label. The true velocity of fibrinolysis can be determined by direct recording of the change in weight (or volume) of the fibrin clot. A method of measuring the kinetics of solution of gelatin gel by proteinases, based on the rate of change of volume of the solid substrate, has been described [1]. We adopted this principle as the basis and developed a simple and highly sensitive method of measuring the velocity of lysis of fibrin gel by plasmin.

EXPERIMENTAL METHOD

Plasminogen was isolated from human blood plasma by affinity chromatography on Lys-sepharose 4B [5]. Plasmin was obtained by activation of plasminogen by streptokinase (Streptase, West Germany) in 0.05 M Tris-HCl buffer, containing 0.02 M L-lysine, pH 7.4, at 37°C, for 30-40 min, with streptokinase in the ratio of 500 U to 1 mg plasminogen. Completeness of activation was verified spectrophotometrically by measuring the rate of hydrolysis of N-D-valyl-L-leucyl-L-lysine p-nitroanilide (S-2251, "Cabi Diagnostica," Sweden) [15]. The caseinolytic activity of plasmin, determined by the method in [12], was 14-16 C.U./mg protein. Fibrinogen, containing 84% of coagulable protein, was obtained from bovine plasma [9]. Bovine thrombin (Kaunas Bacterial Preparations Factory) contained 1000 U of activity per 0.2 g dry weight of the preparation. The protein content in the plasminogen and fibrinogen preparations was determined by Lowry's method [8]. The fibrinolytic activity of plasmin on standard fibrin plates was determined by the known method [4].

To determine the rate of lysis of fibrin a column of fibrin gel was formed in test tubes (d = 8 mm): 0.9 ml of 3% fibrinogen solution in 0.15 M NaCl at 37°C was treated with 0.2 ml of thrombin solution (4 mg/ml), and the mixture was shaken and allowed to stand in the vertical position at room temperature for 1.5 h. The columns of gel thus obtained were used immediately or kept overnight at 4°C. To 0.9 ml of the gel 1 ml of 0.1 M Tris-HCl buffer containing 0.02 M L-lysine and various concentrations of plasmin were added and the rate of fibrinolysis was monitored at 25 and 37°C by studying changes in the height of the gel column by means of a KM-6 cathetometer.

EXPERIMENTAL RESULTS

As was shown in [1] the rate of change of weight of a solid substrate, if the concentration of the substrate in the solid phase (c) and the area of contact of the phases (s) are constant, can be represented in the form

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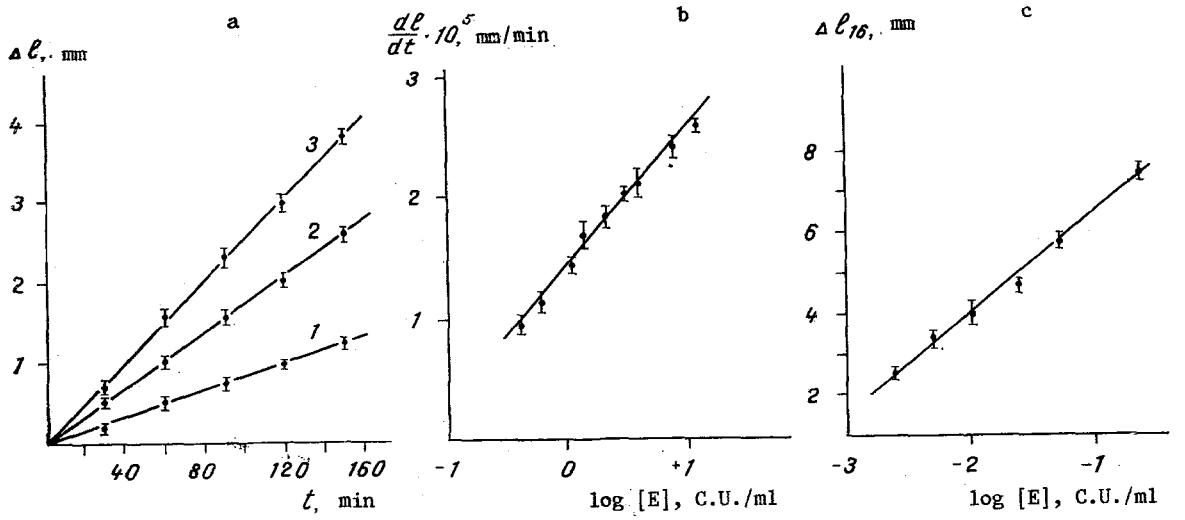

Fig. 1. Lysis of fibrin clot by plasmin. a) Change in height of fibrin gel column with time at 25°C and pH 7.4 in presence of plasmin: 1) 0.12, 2) 1.2, and 3) 12 C.U./ml; b) dependence of velocity of fibrinolysis on plasmin concentration under the same conditions; c) dependence of change in height of fibrin gel column during 16 h at 37°C on plasmin concentration. Mean results of five experiments shown.

$$V^M = \frac{dM}{dt} = c \cdot s \cdot \frac{dl}{dt}, \tag{1}$$

where M = c·s·l is the mass of substrate, and l the height of the gel column.

In that case the change in height of the gel column with time (dl/dt) determines the rate of solution of the solid substrate by weight, expressed as a ratio of its concentration in the gel and the phase separation area:

$$V^l = \frac{dl}{dt} = \frac{V^M}{c \cdot s} = k \cdot V^M \tag{2}$$

where k = 1/c·s.

Thus the rate of change of height of the gel column is directly proportional to the rate of utilization of the substrate.

During the investigation the rate of fibrinolysis was accordingly studied by recording changes in height of the gel column in the presence of the enzyme in the fluid above the gel, which was vigorously mixed (on a shaker) to remove difficulties in the reaction between soluble enzyme and solid substrate due to external diffusion.

In the experiments of series 1 lysis of a fibrin clot by plasmin obtained by activation of purified plasminogen by streptokinase was investigated. The initial regions of the graphs showing the change in height of the gel column (Δl as a function of time), in the presence of various plasmin concentrations at 25°C, are given in Fig. 1a. The tangents of the angles of slope (dl/dt) of these graphs, corresponding to velocities of the fibrinolyis reaction according to equation (2), increase with an increase in enzyme concentration. In the control experiments, in the absence of fibrin, no change was observed in the height of the gel column, which was measured with an accuracy of 0.01 mm. Dependence of the velocity of the fibrinolysis reaction on plasmin concentration between semilogarithmic coordinates is shown in Fig. 1b. It remains linear during a change in enzyme concentration by more than an order of magnitude; the lower limit of sensitivity for plasmin, moreover, is about 50 μg/ml.

To determine lower concentrations of plasmin fibrinolysis was carried out at 37°C. In this case the fibrinolytic activity of plasmin was taken to be the change in height of the gel column during incubation for 16 h (Δl_{16}). As will be clear from Fig. 1c, dependence of Δl_{16} on enzyme concentration in semilogarithmic coordinates also was linear during a change

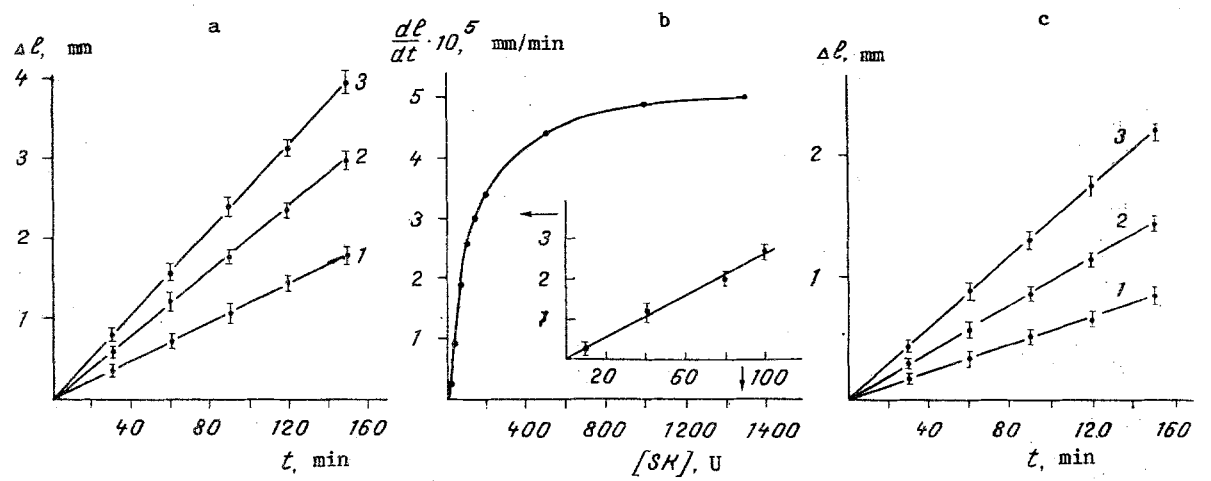

Fig. 2. Dependence of change in height of gel column on time. a) Change in height of fibrin gel column with time at 37°C and pH 7.4 in presence of streptokinase-activated plasmin; 1) 40, 2) 80, and 3) 100 U activator/1 ml plasma. Mean values of three experiments shown; b) dependence of velocity of fibrinolysis, by activated plasma on streptokinase concentration under the same conditions. Mean value of two experiments. Inset shows linear region of this dependence: c) change in height of gel column as a function of time in presence of different volumes of activated plasma: 1) 0.1; 2) 0.2, and 3) 0.3 ml plasma in 1 ml of liquid above the gel (c). Mean results of three experiments.

in enzyme concentration by two orders of magnitude, with a lower limit of sensitivity for plasmin of about 0.5 μg/ml. Incidentally, as regards sensitivity to plasmin, the suggested method (about 0.5 μg/ml) is superior to the fibrin plates method (about 2.5 μg/ml), but very slightly inferior to one of the most sensitive methods, using polystyrene tubes, coated with a film of ^{125}I-labeled fibrin (about 0.2 μg/ml) [15]. The range of plasmin concentrations detectable by this method is very wide (from about 0.5 to about 1000 μg/ml), whereas in methods described in the literature [2, 6, 10, 11, 14] dependence of fibrinolytic activity on plasmin concentration is linear over a range of change of the latter by one order of magnitude. Thus the method enables high plasmin concentrations (about 50-1000 μg/ml) to be detected at 25°C quickly (in 1-2.5 h), whereas to determine low concentrations of the enzyme (about 0.5-50 μg/ml) the temperature has to be raised to 37°C and the reaction time to 16 h. Concentration regions are given above and the conditions are described under which plasmin is determined with the greatest accuracy. These regions may overlap: for example, the sensitivity of the method at 25°C can be increased to 10 μg/ml with some loss of accuracy.

In the second series of experiments the possibility of using the method to determine the potential fibrinolytic activity of human blood plasma was tested. For this purpose 1 ml of plasma was activated in the presence of various concentrations of streptokinase (18 min, 37°C), and 0.2 ml of this mixture was added to 0.8 ml of 0.1 M Tris-HCl buffer, pH 7.4, with which the gel was equilibrated, and the kinetics of fibrinolysis was studied for 2.5 h at 37°C. A graph showing dependence of the change in height of the gel column on time in the presence of plasma, activated by various concentrations of streptokinase, is given in Fig. 2a. It will be clear from this figure that the tangent of the angle of slope of these graphs rises with an increase in concentration of the activator. Dependence of the velocity of fibrinolysis on streptokinase concentration is shown in Fig. 2b. The velocity of fibrinolysis was directly proportional to the streptokinase concentration up to 100 U of activator to 1 ml of plasma (see the inset to Fig. 2b). Deviations from linearity at higher concentrations of the activator are evidently connected with plasminogen exhaustion. In control experiments using buffer instead of plasma, different concentrations of streptokinase were shown not to cause any appreciable changes in height of the gel column during 2.5 at 37°C, for the main mass of the contaminating plasminogen was removed from the bovine fibrinogen during purification by the method in [9]. Under the experimental conditions, plasma not activated by streptokinase likewise caused no change in height of the fibrin gel column. Dependence of the change in height of the gel column on time in the presence of different volumes of plasma, activated by a constant concentration of streptokinase (40 U to 1 ml plasma), is shown in Fig. 2c. The

tangents of the angles of slope of these graphs are directly proportional to the volume of plasma in the liquid above the gel.

The method of determination of the fibrinolytic activity of plasmin relative to the change in height of the fibrin gel column is simple, well reproducible, and highly sensitive. Unlike methods involving the use of radioactively or fluorescently labeled fibrin, in the method described above there is no background noise. By means of the method it is possible to study the kinetics of fibrinolysis and to determine plasmin in concentrations of above 50 μg/ml for 1-2.5 h at 25°C. The sensitivity of the method at 37°C is 0.5 μg/ml. The method is also suitable for quantitative assay of plasminogen in human plasma, which may be used to verify the potential fibrinolytic activity of plasmin during thrombolytic treatment.

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FLUORESCENCE OF LENTICULAR HOMOGENATES FROM Cat^{Fr} MICE WITH HEREDITARY CATARACT

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Cataract, or opacity of the lens, is the commonest cause of blindness in the population [3]. Familiar remedies against cataract, including Vitaiodurol, Sencatalin, and Catachrome, only delay the development of opacity of the lens, and in by no means every patient: one-third of patients do not respond to treatment [6]. This state of affairs has led to a search for new remedies [11], but this is made more difficult by the fact that the pathogenesis of the disease has not been adequately studied. Cataracts of different origin probably have common stages of their pathogenesis. The development of senile and hereditary cataract has been shown to be accompanied by activation of free radical lipid oxidation (FRLO) [7, 8, 13]. Changes in morphology [1, 2] and ionic homeostasis of the lens [4] and in the electrophoretic properties of the crystallins [9] have been demonstrated for mice of the Cat^{Fr} line. Fluorescence of lipid extracts of opaque lenses have been studied in only one investigation [5].

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