Method of estimating the inhibitory effect of plasma or serum upon urokinase-activated fibrinolysis

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SYNOPSIS  Activator concentration in the range 50 to 100 Ploug units urokinase is directly proportional to the reciprocal of the lysis time both in the presence and absence of the plasma or serum inhibitors. Inhibitory activity can be estimated, therefore, by the difference in lysis time between control clots containing a buffer or euglobulin component and plasma or serum containing clots both activated by a common concentration of urokinase selected from within the critical range.

An experimental model for this purpose is described with an examination of the effects of each variable in this system and a reference curve in terms of a known inhibitor. In this study, it has been found that the effects of calcium are influenced by the plasminogen concentration and are biphasic in the presence of the serum inhibitors.

The complete fibrinolytic mechanism of blood includes an inhibitory system, and this paper presents one method of estimating the inhibitory activity of human plasma or serum upon a model fibrinolytic system in vitro.

Lysis of fibrin is ultimately due to the enzyme, plasmin, which is developed by blood activator, or other activators, such as streptokinase and urokinase, from an inactive plasma precursor, plasminogen. Sherry, Lindemeyer, Fletcher, and Alkjaersig (1959) state that plasminogen is adsorbed by fibrin in such a manner that plasmin developed within a clot is protected from the circulating and neutralizing antiplasmins which form the known inhibitory system.

The antiplasmins comprise two fractions: the first migrates electrophoretically with the alpha_4 globulins and combines slowly with plasmin in a manner which is temperature dependent and produces a stable complex; the second antiplasmin fraction migrates with the alpha_2 globulins and combines rapidly with plasmin in a manner which is independent of variation in temperature, but which produces a more readily dissociable complex (Norman, 1958; 1960).

Methods of estimating the inhibitory effect of diluted plasma or serum upon streptokinase- or urokinase-activated fibrinolysis have been described by Nilsson, Krook, Sternby, Söderberg, and Söderström (1961), Paraskevas, Nilsson, and Martinsson (1962) and O'Connell, Grossi, and Rousselot (1964).

This paper presents the results of a study which has established that, throughout a critical range of urokinase concentrations, activator concentration is directly proportional to the reciprocal of the lysis time both in the presence and absence of the inhibitors. In standard clot assays using urokinase, in which inhibitory activity is estimated by the difference in lysis time in the presence and absence of the inhibitors, the assay should therefore be activated by a urokinase concentration selected from within this critical range. An examination of the effect of each variable in such an experimental system, and a test for inhibitory activity based upon these relationships is also reported.

REAGENTS

The following were used:—

Fibrinogen, human, grade L (bottles of 1 g.), Kabi Pharmaceuticals Limited; plasminogen, human, grade B (120 casein units-Sgouris/vial), Kabi Pharmaceuticals Limited; plasmin, human, grade B (28 casein units-Sgouris/vial), Kabi Pharmaceuticals Limited; urokinase reference standard, human, (2,400 Ploug units/vial), Leo Pharmaceutical Products Limited; thrombin reagent, bovine (2,500 NIH units/vial), Leo Pharmaceutical Products Limited; Trasylol, bovine, proteinase inhibitor (25,000 Kallikrein inhibitor units/5 ml.), Farbenfabriken Bayer A.G. Veronal buffer, pH 7.4, 0.154 M., is made up of sodium diethyl barbiturate 11.745 g., sodium chloride 14.67 g., hydrochloric acid 0.1 N. 430 ml., and distilled water to 2 litres.

EXPERIMENTAL

The procedures used depended upon a comparison of standard clot lysis times in the presence of the plasma or
serum inhibitors, and similar lysis times in clots containing a control buffer component or a euglobulin component, from which the inhibitors are almost completely absent (Kowalski, Kopec, and Niewiarowski, 1959).

Except when otherwise stated, a 2 ml clot volume was used comprising 0·6 ml. (5 mg.) human fibrinogen; 0·1 ml. (2·5 c.u.) human plasminogen; 1 ml. plasma or serum, or resuspended euglobulin derived from the same plasma or serum, or veronal buffer, pH 7·4, as a control; 0·1 ml. (0·296 mg.) calcium chloride dried solution; 0·1 ml. (50 Ploug units) urokinase and 0·1 ml. (5 units) thrombin. The concentration of calcium chloride was 0·001 M., therefore, in the final clot volume.

Veronal buffer, pH 7·4, was used as the diluent for all of the reagents, which were retained in an ice water bath at 4°C. in which each standard clot was prepared.

Lysis times were commenced as the urokinase was added and gels then formed as rapidly as possible by the addition of thrombin. Each assay was retained in the ice water bath at 4°C. for exactly five minutes from the commencement of lysis time, before being transferred to a water bath at 37°C. to permit lysis to occur.

The end point was recorded when air bubbles trapped in the gel were suddenly released and the opaque gel became totally transparent. In addition, the development of the sol phase was obvious and associated with the formation of a curved meniscus in place of the irregular gel surface.

PREPARATION OF EUGLOBULIN Resuspended euglobulin was obtained from plasma derived from blood decalcified with 10% of its own volume of 2% potassium oxalate. Glass contact was not avoided, but the temperature was maintained at 4°C. throughout and the blood centrifuged at 3,000 r.p.m. for 20 minutes at 4°C. The euglobulins were precipitated from the aspirated plasma, diluted with 19 times its own volume of ice-cold distilled water, by diffusing carbon dioxide over the surface of the solution for three minutes (von Kaul, 1963). This procedure produced a pH of 5·3 to 5·35 and the euglobulin precipitate was recovered by centrifugation at 2,000 r.p.m. for 10 minutes at 4°C. The supernatant was discarded and the euglobulins resuspended in a volume of ice-cold veronal buffer, pH 7·4, equivalent in volume to the plasma from which they were derived.

Resuspended euglobulin was prepared in a similar manner from serum obtained from blood clotted in glass containers for two hours at 4°C.

RESULTS

PRELIMINARY ESTIMATION OF THE OPTIMAL PLASMINOGEN REQUIREMENT Serial concentrations of from 0·0024 to 4·8 c.u. plasminogen in 0·1 ml. volumes were assayed in control buffer containing clots activated by 200 Ploug units urokinase, which was the maximum activator concentration used in these experiments.

The results are illustrated in Figure 1. A plasminogen concentration of 2·5 c.u. was found to be optimal; greater concentrations did not further accelerate the rate of lysis, and lesser concentrations resulted in prolonged lysis times progressively dependent upon the increasing plasminogen deficiency.

ASSAY OF INHIBITORY EFFECT ON UROKINASE-ACTIVATED FIBRINOLYSIS Standard clots were prepared containing 1 ml. plasma, 1 ml. resuspended euglobulin derived from the same plasma, or 1 ml. veronal buffer, pH 7·4, as a control. Similar groups of three such clots were prepared for each urokinase concentration examined between the range 10 to 200 Ploug units. This assay was repeated using serum and euglobulin derived from serum and, on other occasions, plasma and serum each with and without 2·5 c.u. plasminogen. Finally, the effect of the known proteinase inhibitor, Trasylol (50 k.i.u./ml.), was examined in the system after preliminary experiments to determine a concentration which produced lysis times comparable with those of pooled plasma or serum.

Typical results with plasma or serum are illustrated on double logarithmic paper in Fig. 2; between the urokinase concentrations 50 to 100 Ploug units only, the relationship between activator concentration and lysis time in the control, euglobulin, and plasma or serum-containing tubes has always been found to be linear. Within this range only,
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activator concentration is, therefore, directly proportional to the reciprocal of the lysis time both in the presence and absence of the inhibitors. Thus, in estimating inhibitory activity by the difference in lysis time in the presence and absence of the inhibitors, it is desirable that the clots should be activated by a common concentration of activator selected from the range in which these simultaneous relationships persist.

In some instances, minimal residual inhibitory activity was retained by the euglobulin component. This activity has never exceeded 6% of that of the parent plasma or serum when both the euglobulin and parent substance were activated by 50 Ploug units urokinase. In these instances, the relationship between activator concentration 50 to 100 Ploug units and euglobulin lysis time remained linear. The negligible difference between lysis time in the euglobulin and control clots justifies the use of control clots only in estimating, by comparison, the inhibitory activity of biological fluids.

In a similar manner, the relationship between the lysis time of plasma and serum, each with and without 2-5 c.u. plasminogen, has been found to be linear between the range of urokinase concentrations 50 to 100 Ploug units, although lysis is grossly delayed by omission of plasminogen enrichment. Oxalated plasma and simultaneously prepared serum have been found to have identical inhibitory activity, but a variable loss of inhibitory activity occurs in some instances during the preparation of serum. This loss is not prevented by maintaining a temperature of 4°C. throughout and is not dependent upon contact with glass surfaces. In view of these findings, it is suggested that studies such as those described should preferably be undertaken on plasma. Lysis times in plasma are not materially affected by preparation from blood anticoagulated with 2%, 4%, or 6% potassium oxalate solutions.

Finally, as shown in Fig. 3, similar linear relationships between urokinase concentration and lysis time in the presence and absence of inhibitory activity were found when a suitable concentration of Trasylol was substituted for plasma or serum in the experimental model described. These relationships have, therefore, been duplicated with a known proteinase inhibitor.

SENSITIVITY OF THE ASSAY TO A KNOWN INHIBITOR

Serial concentrations of 1·5 to 5,000 k.i.u. Trasylol, in 1 ml. volumes, were examined in comparison with control buffer-containing clots, both being activated by 50 Ploug units urokinase.

The sensitivity of the assay was such that 3 k.i.u. Trasylol caused an inhibitory effect lasting 15 sec. The results obtained with Trasylol concentrations of 30 to 70 k.i.u. are illustrated in Fig. 4, as they form a reference curve extending throughout the range of inhibitory effects produced by normal human plasma or serum.

From Fig. 4 it is evident that when the inhibitory effect is slight an increase in this effect represents a considerable increase in inhibitor concentration. Conversely, when the inhibitory effect is great, an equivalent increase in this effect represents only a slight increase in inhibitor concentration.

EFFECT OF SUBSTRATE CONCENTRATION

Concentrations of 0·5 to 15 mg. human fibrinogen, in 0·6 ml. volumes, were examined in buffer, euglobulin, and serum-containing clots activated with 75 Ploug units urokinase.
FIG. 4. Trasylol reference curve: serial concentrations of Trasylol in clots activated by 50 Ploug units urokinase. Inhibitory effect = lysis time in Trasylol-containing clots minus lysis time in control buffer-containing clots.

Fig. 5. Effect of added fibrinogen: serial concentrations of added fibrinogen in control and euglobulin clots (coincident at left) and serum-containing clots (at right). Each clot activated with 75 Ploug units urokinase.

The results are illustrated in Fig. 5 and establish that relatively great increases in substrate concentration cause comparatively little prolongation of lysis time.

EFFECT OF DILUTING PLASMA OR SERUM  Plasma and serum in dilutions to form 50% to 1.5% of the final clot volume were compared with control clots, 50 Ploug units urokinase being used throughout.

As shown in Fig. 6, dilution progressively reduced the inhibitory effect which remained measurable with 5% plasma or serum, but was insignificant with 2.5% serum.

EFFECT OF CALCIUM CONCENTRATION Three tenfold dilutions of calcium chloride dried (Analar—75% CaCl₂) were prepared, commencing with a solution of 2.96 g./10 ml. Quantities of each of these dilutions of 0.1 ml. were included in serum and control clots to provide final concentrations of from 0.1 to 0.0001 M calcium chloride in each 2 ml. clot volume. The clots were activated with 50 Ploug units urokinase and the examination was repeated with each clot containing 2.5, 1.25, 0.625, and 0.3125 c.u. plasminogen. Finally, the tests were repeated throughout with the calcium chloride dilutions prepared in a solution of magnesium chloride, such that

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the final divalent metal ion concentration in each clot was constant at 0.1 M, but the calcium chloride concentrations varied from 0.1 to 0.001M as before.

The results are tabulated, the omitted values being intermediate both in terms of calcium concentration and effect.

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tr>
<td>EFFECT OF CALCIUM IN THE ABSENCE (CONTROL CLOTS) AND PRESENCE OF THE INHIBITORS (SERUM CLOTS)</td>
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<tr>
<td>Serum Inhibitors</td>
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When the divalent metal ion concentration was constant, this concentration in successive clots was that of the serial dilutions of calcium only. When the divalent metal ion concentration was constant, it was produced in successive clots by serial dilutions of calcium chloride solution with magnesium chloride solution in such a manner that the ionic concentration of calcium varied as before, but the total metal ion concentration was constant at 0.1 M.

EFFECT OF THROMBIN CONCENTRATION Tenfold dilutions of from 1,000 to 1 units thrombin, in 0.1 ml volumes, were included in control, euglobulin, and serum clots activated by 50 Ploug units urokinase.

One unit was inadequate to cause clotting. Lysis times were not materially affected by thrombin concentrations between 5 and 100 units. Lysis in the presence of serum was accelerated by 1,000 units thrombin.

THE TEST OF INHIBITORY EFFECT The results of the previous experiments permitted estimation of the inhibitory effect by comparison of lysis times in control and plasma-containing clots comprising 0.6 ml (5 mg) human fibrinogen, 1 ml veronal buffer, pH 7.4, or 1 ml plasma, 0.1 ml (2.5 c.u.) plasminogen, 0.1 ml (0.296 mg) calcium chloride dried, 0.1 ml (50 Ploug units) urokinase, and 0.1 ml (5 units) thrombin.

Using the technique described, the inhibitory effect of plasma was estimated in 30 male subjects aged 40 to 60 years and free from overt evidence of disease. The mean plasma inhibitory effect was 18.23 ± 6.37 (range 9.50 to 35.75) minutes.

DISCUSSION The effect of the inhibitors can be removed by diluting blood (Macfarlane, 1937) but, in the experimental system described, 5% plasma or serum retained measurable inhibitory activity. Such a dilution approximated to 10% whole blood, as is present in the dilute blood clot lysis time technique of Fearnley, Balmforth, and Fearnley (1957). This finding suggests that, when activator activity is minimal and the dilute blood clot lysis time is prolonged, the result may be disproportionately influenced by residual inhibitory activity.

Calcium is known to accelerate lysis in the absence of the inhibitors and to inhibit lysis in the presence of the inhibitors (Bruce, 1964). In this study, it has been confirmed that increasing calcium concentrations accelerate lysis in the absence of the inhibitors, but it has been found that this effect is greater when lysis proceeds more slowly due to a relative plasminogen deficiency; that this effect is independent of variations in ionic concentration is suggested by the findings when magnesium ions were substituted for calcium ions in the experimental model.

The effects of increasing concentrations of calcium in the presence of the inhibitors have been found to be complex. When lysis proceeds rapidly due to optimal plasminogen concentrations, calcium inhibits lysis. However, this effect is eliminated by maintaining a constant total divalent metal ion concentration with magnesium. When lysis proceeds more slowly due to a relative plasminogen deficiency, the effect of calcium in the presence of the inhibitors is reversed and lysis is accelerated. This latter effect appears to be independent of ionic concentration. Thus, the effect of calcium in the presence of the inhibitors is biphasic and directly or indirectly influenced by the concentration of plasminogen. Magnesium prolongs lysis time, especially when plasminogen is deficient, and thereby relatively enhances the acceleratory effects of calcium.

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