Kinetics of Fibrin Clot Lysis

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The principles relating the lysis times of fibrin clots to their contents of fibrin, plasminogen and plasminogen-activator were investigated. Mathematical considerations suggested that the square of the lysis time should correlate linearly with the fibrin content, and inversely with the activator and the plasminogen contents of the system. Experimental studies, during which these parameters were independently varied, showed that the predicted relationships were valid for concentrations that gave clot-lysis times in the range normally used for studies of fibrinolysis.

The kinetics that govern the lysis of fibrin by plasmin have been investigated by Berg, Korsan-Bengtson & Ygge (1965). They employed a clot-lysis technique in which variable amounts of a plasminogen-streptokinase mixture were added to solutions of fibrinogen; thrombin was then added to bring about clotting and the time-intervals between clot formation and clot lysis were measured. From their results they inferred that, when the clotting time is small compared with the lysis time, fibrinolysis follows zero-order kinetics. The results in the present paper confirm their conclusion.

Plasminogen has a strong affinity for fibrinogen and probably also for fibrin and, unless special precautions are taken to exclude it, fibrinogen isolated from plasma contains substantial amounts of adsorbed plasminogen. Clots prepared from such fibrinogen, which are more akin to organic thrombi than those made from plasminogen-free fibrinogen, can be lysed by plasminogen-activators such as urokinase. In such fibrinolytic processes two reactions occur simultaneously: the conversion of plasminogen into plasmin by the activator, and the proteolytic breakdown of the fibrin molecules to the stage where the network becomes insufficiently rigid to maintain the structure of the clot.

So far the kinetics of this process do not seem to have been investigated, and the present paper is an attempt to correlate a theoretical treatment of the breakdown of fibrin clots by an activator with experimental evidence, including the effect of varying the concentration of the components of the fibrinolytic system on the clot-lysis time.

THEORETICAL TREATMENT

It is evident from the above that the fibrinolytic process involves two enzymic reactions occurring simultaneously:

If it is assumed that the first reaction follows Michaelis-Menten kinetics, then:

$$-\frac{dp}{dt} = \frac{kAp}{p + K_m}$$

where p is the concentration of substrate (plasminogen), A the concentration of activator, K_m the Michaelis constant and k the rate constant.

Integration of this expression, with the condition that $p = p_0$ (where p_0 is the initial concentration of plasminogen) when $t = 0$, gives the equation:

$$p_0 - p - K_m \ln \frac{p}{p_0} = Kat$$

and, since $p_0 - p = P$ (where P is the amount of plasmin formed at time t), this resolves to:

$$P - K_m \ln \left(1 - \frac{P}{p_0} \right) = Kat$$

Expansion of the logarithmic function, when $P < p_0$, results in the approximation:

$$P + \frac{K_m P^2}{2p_0} = Kat, \text{ or } P = \frac{kAp_0 t}{p_0 + K_m} \quad (1)$$

There is considerable evidence that, under clot-lysis conditions, the degradation of fibrin is of zero-order with respect to fibrin concentration. The supposition is justified (a) by the experimental finding that the rate of clot lysis by purified plasmin is linearly dependent on the amount of plasmin added, and (b) on the assumption that the dissolution of the visible clot represents only a fractional stage in the fibrinolytic process. In this case:

$$-\frac{dF}{dt} = k'P \quad (2)$$

where F is the concentration of fibrin molecules and of such breakdown products as contribute to the clot structure, and k' is the proteolytic rate constant.

Elimination of P from eqns. (1) and (2) gives:

$$-\frac{dF}{dt} = \frac{kk'Ap_0t}{p_0 + K_m}$$

which on integration leads to:

$$F_0 - F = \frac{kk'Ap_0t^2}{2(p_0 + K_m)}$$

If it is assumed that, at the clot-lysis time T , a fixed fraction of the plasmin-sensitive peptide bonds in each fibrin molecule has been hydrolysed, then $F = aF_0$ and:

$$(1-a)F_0 = \frac{kk'Ap_0T^2}{2(p_0 + K_m)} \quad (3)$$

or

$$T^2 \propto \frac{F_0}{A} \left(1 + \frac{K_m}{p_0}\right) \quad (4)$$

From eqn. (4) it is deduced that, if the initial concentrations of the reactants fibrin, urokinase and plasminogen are independently varied, linear plots of T^2 versus F_0 , $1/A$ or $1/p_0$ should be obtained.

EXPERIMENTAL

Urokinase, thrombin, plasminogen-rich fibrinogen and plasminogen-deficient fibrinogen were as defined by Gallimore & Shaw (1967). Plasmin was the 'insoluble' Kline-Fishman preparation described in the same paper.

Fibrin clots were prepared in sodium phosphate buffer, pH 7.4 (0.1 M with respect to phosphate); unless otherwise stated the clots contained 1 mg. of fibrin and sufficient NaCl to bring the Cl⁻ ion concentration to 0.15 M. The total volume of each clot was 1 ml. They were prepared in an ice-water bath, clotted with 5 units of thrombin and transferred to a water bath at 37°. The lysis time was taken to be the interval between the addition of thrombin and the time when the air bubbles trapped in the fibrin network had risen to the surface.

In all essential respects the techniques were identical with those described by Gallimore & Shaw (1967).

RESULTS

When various amounts of plasmin were included in clots prepared from a constant amount of plasminogen-deficient fibrinogen, the results shown in Fig. 1 were obtained. It is evident that the reciprocal of the clot-lysis time is directly proportional to the plasmin concentration throughout the range of times normally used in clot-lysis experiments. Such a relationship is compatible with zero-order reaction kinetics, as are the results shown in Fig. 2; these indicate that linear relationships also apply when lysis time is plotted against substrate concentration for clots containing a single concentration of plasmin. This experiment

Fig. 1. Effect of plasmin concentration on the reciprocal lysis times of fibrin clots.

Fig. 2. Effect of substrate (fibrin) concentration on the lysis of fibrin clots by plasmin. ○, Clots containing 0.065 unit of plasmin; △, clots containing 0.086 unit of plasmin; □, clots containing 0.108 unit of plasmin.

was repeated at three plasmin concentrations and essentially linear plots were obtained with each.

When standard clots prepared from plasminogen-rich fibrinogen were incubated with various amounts of urokinase, it was found that the reciprocals of the squares of the clot-lysis times were proportional to the amount of urokinase present (Fig. 3), in accordance with the relationship (eqn. 4) derived in the Theoretical Treatment section. However, when the concentration of fibrin in the clots was varied and their lysis times were investigated with three concentrations of urokinase the results shown in Fig. 4 were obtained. It is evident that under these

Fig. 3. Effect of activator (urokinase) concentration on the lysis times of clots prepared from plasminogen-rich fibrinogen. Results are plotted as $1/T^2$ versus urokinase concentration.

Fig. 4. Effect of substrate (fibrin) concentration on the lysis by urokinase of clots prepared from plasminogen-rich fibrinogen. All clots contained the same ratio of fibrin to plasminogen: $\circ$, 4 units of urokinase; Δ , 6 units of urokinase; $\square$, 20 units of urokinase.

circumstances the lysis time of a fibrin clot is independent of its substrate (fibrin) content for a given amount of urokinase, and that the rate of fibrinolysis is governed by the plasminogen/fibrin ratio in the system rather than by the absolute concentration of plasminogen or fibrin.

Fig. 5. Effect of substrate (fibrin) concentration on the lysis times of fibrin clots. Each series of clots contained the same amount of plasminogen, introduced as plasminogen-rich fibrinogen: $\circ$, 75 units of plasminogen; ∇ , 100 units; $\blacksquare$, 125 units; Δ , 150 units; $\square$, 200 units. Results are plotted as T^2 versus fibrin concentration. All the clots contained the same amount of urokinase.

Table 1. Relationship between T^2/F_0 and $1/p_0$

Values are calculated from the slopes of the five plots in Fig. 5 and show that T^2/F_0 is proportional to $1/p_0$.

$10^3/p_0$ (units ⁻¹)	Slope (T^2/F_0)	$10^{-3} \times \text{Slope} \times p_0$
13.3	292	21.9
10.0	247	24.7
8.0	193	24.1
6.7	151	22.6
5.0	109	21.8

Fig. 5 demonstrates the influence of various concentrations of substrate on clot-lysis time when the amounts of plasminogen and of urokinase remain constant for each set of experiments. These conditions were achieved by adding different amounts of plasminogen-deficient fibrinogen to a constant (background) amount of plasminogen-rich fibrinogen. Five such sets of experiments were conducted each with a single concentration of plasminogen and, for each set, linear relationships were encountered between the squares of the lysis times and the fibrin content of the clots. As the plasminogen content of the plasminogen-rich fibrinogen could not be determined with confidence in absolute units, plasminogen concentrations are expressed in arbitrary units, the amount of plasminogen in 1 mg. of plasminogen-rich fibrinogen

Fig. 6. Effect of plasminogen content (p_0) on the lysis times (T) of fibrin clots; each series of clots contained the same amount of total fibrin: $\circ$, 4 mg. of fibrin; Δ , 3 mg. of fibrin; $\square$, 2 mg. of fibrin; ∇ , 1 mg. of fibrin. Results are plotted as $1/p_0$ versus T^2 . All the clots contained the same amount of urokinase.

Table 2. Relationship between $T^2 \times p_0$ and F_0

Values are calculated from the slopes of the four plots in Fig. 6 and show that $T^2 \times p_0$ is proportional to F_0 .

F_0 (mg.)	Slope ($10^{-3} \times T^2 \times p_0$)	Slope/ F_0
4	52.2	13.05
3	42.6	14.20
2	29.7	14.85
1	15.4	15.40

14.38

being assigned a value of 100 units. The slopes of the five curves are given in Table 1.

Finally, Fig. 6 shows the relationship between the square of the clot-lysis time and the reciprocal of the plasminogen content for a group of fibrin clots all containing the same amount of substrate and of urokinase. The results, like those shown in Fig. 5, were obtained by investigating the lysis of clots prepared from mixtures of plasminogen-rich and plasminogen-deficient fibrinogen, all of which contained the same amount of urokinase; four series of experiments were performed in which the total clot fibrin contents were 1, 2, 3 and 4 mg. The slopes of these curves are given in Table 2.

The results in Figs. 5 and 6 satisfactorily confirm the theoretical predictions that the squares of the lysis times of fibrin clots incorporating plasminogen

and urokinase are linearly related to their content of fibrin, and to the reciprocal of their content of plasminogen.

DISCUSSION

The results illustrated in Figs. 1 and 2 confirm the conclusions of Berg *et al.* (1965) that, under fibrin-clot conditions, fibrinolysis by plasmin is governed by zero-order kinetics, i.e. $-dF/dt = k'P$. Such a relationship implies the existence of a large excess of substrate throughout the reaction and is compatible with the view that the events up to clot lysis represent only a fraction of the total proteolytic sequence. Also implicit in this relationship is the view that, at the time of clot lysis, only a very small fraction of the original fibrin molecules, and such of their fission products as are capable of supporting gel structure, are still present in the system. The assumption that zero-order kinetics were applicable to the second (proteolytic) reaction, when clot lysis was initiated by urokinase in clots prepared from plasminogen-rich fibrinogen, resulted in a substantial simplification of the theoretical treatment.

The satisfactory agreement between the theoretically predicted relationships and those demonstrated experimentally, in the plots of A versus $1/T^2$ (Fig. 3), of F_0 versus T^2 (Fig. 5) and of $(1/p_0)$ versus T^2 (Fig. 6), constitutes convincing evidence that the assumptions made in the Theoretical Treatment section are substantially correct. Moreover, the results in Fig. 4, which show that when F_0/p_0 is constant the lysis times of fibrin clots containing the same amount of activator are independent of their content of substrate, are compatible with the relationship in eqn. (4), if the second term of the expression $(F_0 K_m/p_0 A)$ is large compared with the first term (F_0/A) , i.e. provided that K_m/p_0 is large. The K_m value for the activation reaction is not at present known, but it is very probable that this condition applies.

When the relationships implicit in eqn. (3) are considered in more detail, certain other features emerge. It is clear for example that when $T^2 = 0$, i.e. at the intercepts of the graphs of T^2 versus $1/p_0$ with the abscissa (Fig. 6), $1/p_0 = -1/K_m$, if A and F_0 are constant and if $(1 - \alpha)$ is not zero. Also, the slopes of the individual plots with different values of F_0 and A should be proportional to F_0/A ; in the particular circumstances of Fig. 6, where A is constant for all points, the slopes of each curve should be proportional to F_0 and should intersect the ordinate ($1/p_0 = 0$) at points proportional to the fibrin content. Further, in Fig. 5, where F_0 is plotted against T^2 , the slope of each curve (A being constant) should be proportional to $1/p_0$.

With regard to Figs. 5 and 6 it appears from the values calculated for the slopes (see the Results

section) that the predicted relationships are in reasonable, though not exact, agreement with the experimental results. However, when the relationship involving the Michaelis constant, K_m , for the activation reaction is considered, it is obvious that an anomaly exists, for the intersections of the plots in Fig. 6 with the abscissa vary with their fibrin content and are positive in $1/p_0$. They thus lead to negative values for K_m and are clearly invalid.

Figs. 5 and 6 suggest that the observed values for the lysis times are consistently low. The deficiency appears to be constant in terms of T^2 ,

and to have a value of approx. 100 min.². No satisfactory explanation for this discrepancy has so far been found.

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