Technical Papers

Clotting Time and Reaction Velocity in the Interaction of Bovine Fibrinogen and Thrombin

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The clotting time and coagulation time end points of different techniques vary. The first appearance of compactible fibrin in an agitated solution (*1, 2*), the formation of a visible fibrin web in a quiescent solution (*3*), or the formation of a gel sufficiently rigid to resist deformation on tilting (*4–6*), has been used. Clotting times (*7, 8*) have been employed to characterize the development of gel structure, to compare clinically important agents, and frequently to establish the level of activity in an unknown solution. In the latter case, the unknown concentration is adjusted to the point where, when a small volume is added to a solution containing other components in reproducible concentration, pH, etc., the end point is obtained in a stated period of time. The necessity of controlling adequately all known variables has been emphasized (*2, 9*). The assumption has been made (*10*) that clotting time is related inversely to the reaction velocity.

It has been our experience that the quantity of fibrin and potential fibrin[1] necessary to establish a clotting time based on the first appearance of fibrin strands, or to cause a solution to gel, is a function of the fibrinogen and thrombin concentrations, the pH, ionic strength, etc., and that this quantity may be altered by the addition of materials such as gum acacia, phenol, formaldehyde, urea, or glycerol. A quantitative relationship between even a limited number of pertinent variables is of importance in understanding the way in which clot structure is developed, and will be of eventual use in interpreting clinical and other data.

In previous work (*11*), the interaction of fibrinogen and thrombin at pH 6.85, added ionic strength 0.15 (0.05 phosphate and 0.1 sodium chloride), and 22.7° C was studied over the ranges 0.009–0.45 N.I.H. units per ml thrombin and 0.036–0.35 mg clottable nitrogen (C.N.) per ml. The fibrinogen and thrombin preparations were those of Armour & Co.[2] In the following, Th_0 and ϕ_0 are the total thrombin and initial fibrinogen concentrations in the above units.

A typical reaction curve, which measures the amount of fibrinogen, activated fibrinogen, and noncompactible fibrin remaining in solution after compactible materials have been removed, is shown in Fig. 1, curve 1. In this reaction $\phi_0 = 0.18$, and $Th_0 = 0.045$. $\theta = \phi/\phi_0$ represents the fraction of total clottable nitrogen remaining in solution at time t in min. In plotting $ln\ 1/\theta$ vs. t, a pseudo first-order reaction is assumed. Beyond 5.5 min a linear plot is obtained, the extrapolation of which passes through the origin. The difference between corresponding points on the nonlinear portion and linear extrapolation represents the accumulation of activated fibrinogen and noncompactible fibrin. Linearity is established when sufficient compactible fibrin is present to keep these components at negligible values. Thus, the linear portion and linear extrapolation may be considered to represent the formation of activated fibrinogen whatever its physical state. Fig. 1, curve 2, represents the same reaction run in the presence of 1.54% purified gum acacia (for the effects of which see [*1*]) and shows that the main effect of acacia is to promote compaction and reduce the nonlinear events, a minor effect being an increase in reaction velocity.

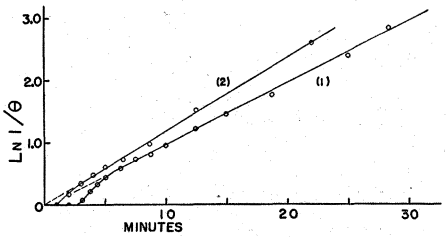

FIG. 1. Typical reaction curves for $\phi_0 = 0.18$ mg C.N./ml and $Th_0 = 0.045$ N.I.H. μ thrombin/ml (curve 1). Curve 2 represents the same reaction run in the presence of 1.54% purified gum acacia.

The linear portions of the reactions examined conform to the equation:

$$ln\frac{\phi_0}{\phi} = \frac{0.482\ Th_0}{0.051 + \phi_0}t. \qquad (1)$$

The most reasonable interpretation of equation (1) is that thrombin combines mainly in an equal, reversible, inactive complex with fibrinogen and fibrin, the life of any assumed active complex being short compared to the diffusion time between fruitful collisions.

Clotting times, using 1.5 ml fibrinogen solution in a tube of 1.0 cm I.D., 0.15 ml thrombin, and taking the first traces of visible fibrin in the gently agitated tube as an end point (as in [*2*]), have been determined for essentially the same ranges of Th_0 and ϕ_0 as for the kinetic studies. To give comparable data, acacia was not present. The fibrin and potential fibrin, $\phi' = \phi_0 - \phi$, present at a given clotting time, T_c, may be calculated by equation (1). When ϕ' is plotted vs. ϕ_0, Fig. 2, a series of lines is obtained having a common intercept at $\phi_0 = -0.11$ and slopes given by

[1] Clotted material which can be separated easily after compaction will be termed fibrin. The sum of activated fibrinogen and noncompactible fibrin (i.e., fibrin particles not yet incorporated into the compactible structure) will be termed potential fibrin.

[2] We are greatly indebted to the Chemical Research and Development Department of Armour & Co., Chicago, Ill., for supplying these materials and for generous financial support.

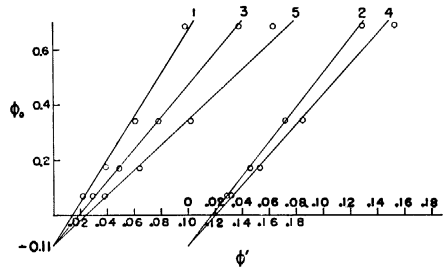

FIG. 2. Potential fibrin, ϕ', plotted as a function of the initial fibrinogen concentration, ϕ_o, both in mg C.N./ml. The curves and corresponding thrombin concentrations, in N.I.H. units/ml, are (1), 0.045; (2), 0.091; (3), 0.136; (4), 0.227; and (5), 0.454. Lower values on the abscissa refer to curves (1), (3), and (5).

$(3.8 - Ln\ \mathrm{Th}_o)$. The lines of Fig. 2 were drawn according to the final equation, which is:

$$\phi' = \frac{\phi_o - 0.11}{3.8 - Ln\ \mathrm{Th}_o}. \qquad (2)$$

On combination, equations (1) and (2) yield:

$$T_c = \frac{0.051 + \phi_o}{0.482\ \mathrm{Th}_o}\, Ln \frac{\phi_o\,(3.8 - Ln\ \mathrm{Th}_o)}{\phi_o\,(2.8 - Ln\ \mathrm{Th}_o) - 0.11}. \qquad (3)$$

Equation (3) should describe the clotting times obtained when Th_o and ϕ_o are varied. The lines shown in Figs. 3 and 4 were calculated by equation (3), the experimental points, averages of several determinations, being shown. In view of the subjective nature of clotting time measurements, the agreement is satisfactory.

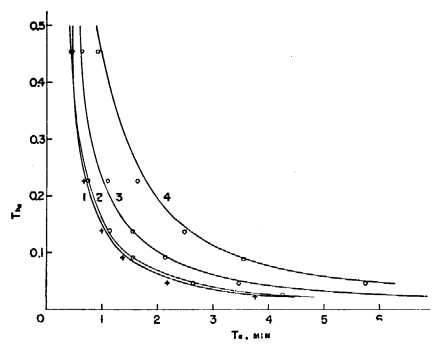

FIG. 3. Clotting time in min as a function of thrombin concentration in N.I.H. units/ml of final solution. The curves and initial fibrinogen concentrations in mg C.N./ml are: (1), 0.07; (2), 0.17; (3), 0.345; and (4), 0.68.

Equation (3) predicts, as has been found (*4, 6, 10, 12*), that clotting times will decrease with decreasing ϕ_o until a critical value is reached, after which they will increase. $T_c = \infty$ is obtained when $\phi_o = \infty$ and when $\phi_o\ (2.8 - Ln\ \mathrm{Th}_o) = 0.11$, in theory for the latter case when the establishment of clotting time requires as potential fibrin all the fibrinogen in solution. These values for several values of Th_o are recorded in column 2 of Table 1. Columns 3 and 4 record the minima in T_c and corresponding values of ϕ_o, obtained in the usual way by differentiating equation (3). The ϕ_o value for min. T_c, corresponding to about 0.1% fibrinogen at

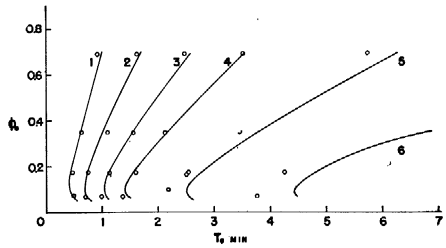

FIG. 4. Clotting time in min as a function of the initial fibrinogen concentration in mg C.N./ml. The curves and corresponding thrombin concentrations in N.I.H. units/ml are: (1), 0.454; (2), 0.227; (3), 0.136; (4), 0.09; (5), 0.045; and (6), 0.023.

$\mathrm{Th}_o = 0.5$, is in accord with similar values reported in the literature (*4, 6, 10, 12*).

The first traces of retractable fibrin are established differently for ϕ_o values above and below the T_c minimum in the $\phi_o - T_c$ curves. Thus the end points are qualitatively different. For ϕ_o values above the minimum, visible fibrin strand formation appears to be

TABLE 1

Th_o	Calculated	Calculated minima	
	ϕ_o at $T_c = \infty$	T_c	ϕ_o
0.022	0.016	4.6	0.092
.045	.019	2.5	.095
.091	.021	1.4	.097
.136	.023	1.0	.10
.227	.026	0.69	.102
0.454	0.031	0.42	0.107

an average property of the solution, a slight turbidity being observed throughout just before strand formation, which also takes place throughout the solution. At ϕ_o values below the minimum, different types of behavior have been observed, two of which seem to depend on the recruitment of fibrin from the entire solution, and a third on the appearance of fine flocculates instead of strands. At $\mathrm{Th}_o = 0.14$ and ϕ_o values between 0.07 and 0.04, a few strands are formed suddenly at the end point; between $\phi_o = 0.03$ and 0.01, a fine flocculation appears; and below $\phi_o = 0.01$, the solution remains clear until, on disturbance, a minute amount of compactible fibrin is observed. Thus, using the end point described, "clotting times" may be observed at $\phi_o = 0.001$ or less, depending in part on the volume of solution in which the test is conducted. T_c assumes a relatively constant value, approximately equal to the minimum for T_c in the calculated $\phi_o - T_c$ curves, for ϕ_o values between the minimum and that at which an infinite clotting time would be calculated, after which T_c increases rapidly. Likewise at values of ϕ_o below the calculated minimum, sol-gel end points, of a subjective nature, are equally difficult or impossible to establish. This fact has led to the acceptance of different end points for different regions of the $\phi_o - T_g$ curves (*6*).

Since clotting time is established when the first

traces of compactible fibrin are visible, a correlation should be observed between T_c and the time when the corresponding reaction curve leaves the $ln\ 1/\theta$ axis (Fig. 1), at which time the first traces of compactible fibrin are determined experimentally. Deviations from the abscissa, calculated clotting times, and observed clotting times are recorded for a number of reactions in columns 3, 4, and 5 of Table 2, the first two columns defining ϕ_o and Th_o. Close agreement is observed.

The last column of Table 2 records values of ϕ' calculated by equation (2). The initial velocity of the fibrinogen-thrombin reaction is given by the differential form of equation (1), using $\phi = \phi_o$, or

$$-\frac{d\phi_o}{dt} = \frac{0.482\ Th_o}{0.051 + \phi_o}\phi_o. \qquad (4)$$

The rate of formation of activated fibrinogen increases with increasing Th_o and ϕ_o. In all cases, ϕ' increases with increasing rate (equation [2]). This is not unexpected, for, as the reaction velocity increases, more potential fibrin will appear before the retractable clot structure is developed. At constant ϕ_o, clotting times

TABLE 2

ϕ_o	Th_o	First deviation from $ln\ \frac{1}{\theta} = 0$ (min)	Calculated T_c (min)	Observed T_c (min)	Calculated ϕ'
0.185	0.009	9.5	10.7	.....	0.035
"	.023	4.5	4.89	4.26*	.039
"	.045	2.5	2.69	2.5*	.043
"	.068	1.6	1.83	1.6*	.046
"	.091	1.3	1.54	1.24*	.048
"	.227	0.50	0.74	0.75*	.056
"	0.454	0.25	0.47	0.47*	0.064
0.035	0.045	1.3	1.8	.....	0.021
.075	"	1.3	2.0	2.18	.028
.11	"	1.8	2.2	.....	.032
.185	"	2.5	2.6	2.69	.043
0.35	"	.....	3.5	3.46	0.067

* For $\phi_o = 0.172$.

decrease with increasing reaction velocity (increasing Th_o), suggesting that the less effective utilization of potential fibrin at the higher rates is more than balanced by the rate of appearance of activated fibrinogen. At constant Th_o, and increasing ϕ_o, the opposite appears to be the case; the decreased utilization of potential fibrin overbalances the increased rate of activated fibrinogen appearance, and the clotting time increases with increasing reaction velocity. As a partial explanation for the latter phenomenon, it is suggested that the cross-linking of fibrin strands, or incorporation of potential fibrin into the compactible structure, is suppressed by increasing concentrations of residual fibrinogen. A relationship between rate and ϕ' may be obtained from equations (2) and (4). It is probable that the change in end point observed when ϕ_o decreases below the minimum value, and the

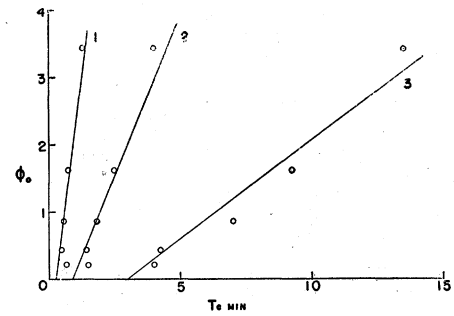

FIG. 5. Sol-gel transformation times in min as a function of fibrinogen concentration (mg C.N./ml) obtained from the data of Ferry and Morrison (4). The curves and corresponding thrombin concentrations are: (1), 4.0; (2), 1.0; and (3), 0.2.

fact that T_c remains relatively constant over a large range in ϕ_o, may likewise be associated with the above effect of fibrinogen.

Clotting times are all considerably smaller than those required to establish linearity. Thus, for the reaction of Fig. 1, curve 1, linearity occurs at 5.5 min, whereas the clotting time occurs at 2.7 min. A comparison of corresponding points on the reaction curve and the extrapolated linear portion at 2.7 min shows that the major portion of ϕ' is not in a compactible form. This is to be expected. On a relative basis, to keep the concentration of activated fibrinogen and fibrin particles negligible, the retractable clot structure must be extensive, the distances between fibrin strands small, and the aggregating area (surface area) of the fibrils large. As a first approximation, the compactible clot may be visualized as strands running along the edges of closely packed tetrahedra. Were the strands to maintain a constant axial ratio, the quantity of fibrin would be independent of the coarseness or fineness of the clot and dependent only on the axial ratio. The variations in ϕ' with ϕ_o and Th_o expressed by equation (2) and illustrated in Fig. 2 and Table 2, in addition to the presence of potential fibrin, may be due to a progressive change in axial ratio (length of tetrahedron edge over fiber diameter) which, if it were operating alone, would of necessity increase as the length of the fiber increased; i.e., the axial ratio in a fine clot would be smaller than that in a coarse clot. Regions of the reaction curves near the point where deviations from the abscissa occur will be examined in detail to determine the relative contributions from nonutilization of potential fibrin and average change in axial ratio (or some similar property of the compactible clot).

Sol-gel transformation times in the above experiments are somewhat longer than clotting times, but lie also on the nonlinear portions of the reaction curves, and will conform to an equation similar to (3). That such is the case in another system may be observed from Fig. 5, which has been calculated from the data of Ferry and Morrison (4) obtained with human fibrinogen and thrombin at pH's near 6.8 and ionic

strength 0.3. The points represented conform to the empirical equation:

$$T_g = \frac{\phi_0 + 0.9}{0.76\ \mathrm{Th}_0 + 0.16}. \quad (5)$$

Similarly, the curves of Fig. 4 may be expressed by the empirical equation:

$$T_c = \frac{\phi_0 + 0.3}{2.66\ \mathrm{Th}_0 + 0.05}. \quad (6)$$

Both equations are useful only at ϕ_0 concentrations above the T minimum in the $\phi_0 - T$ curves. Equations (5) and (6) indicate a similar basis of measurement in the two systems.

The presence of 1.53% acacia (Fig. 1) increases the reaction velocity by about 20%. On the basis of clotting time measurements the "reaction" has been accelerated by a factor of about 1.8, since acacia has decreased the clotting time from 2.5 to 1.4 min. The major portion of the effect, as indicated above, is due to a shift in the distribution of fibrin and potential fibrin. Thus, accelerators or inhibitors might not affect the rate of a reaction appreciably, but might cause large changes in clotting time by altering the distribution between fibrin and potential fibrin, and by changing the absolute value of the former. For this reason, the use of clotting times alone to compare reaction velocities at different pH's and ionic strengths or in the presence of accelerators or inhibitors is open to question.

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Radiocarbon Age Measurements and Fossil Man in Mexico

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The final release of radiocarbon dates obtained by Arnold and Libby (*1*) includes a number of age determinations of plant materials from prehistoric sites in the basin of Mexico that I collected for that purpose. Considering the geographic location of these localities, intermediate between North and South American prehistoric stations, a brief commentary is warranted, with special reference to the geologic antiquity of man in that region. Basic for this problem are the two age measurements (*1*, p. 13, samples No. 204 and No. 205) for two samples, wood and peat, from the Becerra formation, which represents the Upper Pleistocene in that area. The time range indicated is 10,603–20,000 years ago, the latter date referring to results previously communicated to me. It happens that the peat sample was collected by my Mexican collaborator, A. R. V. Arellano, from a geological horizon and level that corresponds in topographic elevation and stratigraphic position with the swamp deposit at Tepexpan where I had found the partly fossilized remains of "Tepexpan Man." At both localities fossil bones of elephant and horse occur, a tooth of the latter having been found in a later excavation 5 yards distant from the original position of the human bones. As the new excavation trench substantiated the impression of an undisturbed occurrence of human remains with fossil elephant, the date for the Becerra peat being 11,-300 ± 500 years ago, my original estimate of 11,000–12,000 years for the human bones appears to be supported by the radiocarbon count.

A special effort was made last year to secure plant samples from the Tepexpan Man site itself, a task which involved the removal of some 4 tons of clay, from which delicate plant roots were extracted. Although it was by no means certain that these roots and stems had not grown from a younger lake floor into deeper and older sediments, such an effort nevertheless promised information on their mode of origin and age. A botanical examination of the roots was made by J. Beal, head of the Botany Department, University of Chicago, who believes that they belonged to an unidentifiable water plant, uncarbonized and in a fresh state of preservation. At the site, single roots were seen to extend 10–12 in. into laminated sandy clay of buff coloring, the roots standing upright and fading out toward the overlying caliche soil (4 in.). The latter showed faint traces of dark root canals that must have extended from the overlying marsh deposit, a sandy marl 15 in. thick, from which fresh-water shells and roots had previously been reported (*2*, p. 38). Considering the nature of the sediment below the caliche, a laminated deposit requiring a slow rate of sedimentation, it is most unlikely that roots could have grown at the same rate as deposition of sediment proceeded. The fresh preservation is obviously due to the sealing effect of the overlying caliche, which prevented bacterial action at a depth of 30–40 in. below the marl. Some modern water plants in the relic Lake Texcoco, in the basin of Mexico, showed roots and stems 10–20 in. long, a length that might well have been exceeded at Tepexpan in view of the water-logged condition of the older swamp deposit below the caliche.

In the light of these considerations, the age measure-