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# Fibrin Formation: Effect of Calcium Ions

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**Using laser fluctuation spectroscopy, a technique that measures particle size change in solution, the kinetics of fibrin clot formation from fibrinogen can be studied. With this technique the effect of calcium on the three distinguishable phases of clot formation, (1) proteolysis of fibrinogen, (2) fibrinogen-fibrin monomer complex formation, and (3) fibrin monomer polymerization, were investigated. Only a small change in the length of the induction period that results from the fibrinogen-fibrin monomer interactions was observed. However, there was a marked increase in the rate of fibrin monomer polymerization in the presence of calcium ions. These data show that calcium decreases the time required for fibrin formation from fibrinogen by markedly accelerating the phase of fibrin monomer polymerization.**

CALCIUM IONS are known to accelerate the formation of a fibrin clot from fibrinogen in the presence of thrombin.[1,2] The conversion of fibrinogen to the fibrin clot is a multistep process, and many studies have attempted to examine the specific step influenced by calcium. These studies traditionally have divided fibrin clot formation into two steps, (1) the conversion of fibrinogen to fibrin monomer by the action of the proteolytic enzyme thrombin and (2) the aggregation of the fibrin monomer to form the visible fibrin clot. Examination of fibrin clot formation from this perspective has led several investigators to conclude that the proteolytic action of thrombin on fibrinogen is not altered by calcium ions and that the accelerated appearance of the fibrin clot results from an increased rate of fibrin monomer polymerization.[3-5]

Using laser fluctuation spectroscopy, we found a third step in the fibrinogen-thrombin system. During the early period of the reaction, the fibrin monomer that is generated forms a reversible complex with fibrinogen.[6] As fibrin monomer begins to accumulate, the next stage in fibrin clot formation, polymerization, proceeds rapidly. This interaction between fibrinogen and fibrin monomer can have dramatic effects on the kinetics of the fibrinogen-thrombin system, as shown by the fibrinogen concentration dependence of the system.[6] Previous studies did not examine the effect of calcium on the fibrinogen-fibrin monomer interaction. Furthermore, many of the systems used to study fibrin monomer polymerization contain fibrinogen. Thus the fibrinogen-fibrin monomer complexing reaction might be influencing the results obtained from these results.[3,4]

To further define the effect of calcium in the fibrinogen-thrombin system, we

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monitored the increase in particle size in the system as a function of time in the presence of calcium at various concentrations. Our results were consistent with the hypothesis that the major action of calcium occurs on fibrin monomer polymerization.

## MATERIALS AND METHODS

Human fibrinogen (fraction $I_4$) was prepared from outdated plasma by the method of Blombäck and Blombäck[7] as previously described[6] and was stored at −80°C. This protein, when subjected to reduction with urea and mercaptoethanol followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), was free of contaminating proteins. Fibrin formed from this fibrinogen in the presence of calcium was soluble in 5 *M* urea, indicating that fibrin stabilizing factor (XIII) activity was not present. Fibrinogen concentrations were determined by the method of Ratnoff and Menzie.[8]

Thrombin was purified from crude bovine thrombin (Parke-Davis, Detroit, Mich.) by the method of Prentice et al.[9] Stock solutions were stored at −25°C.

Fibrinogen solutions and buffers used in experiments were treated with Chelex 100 (Calbiochem, Los Angeles, Calif.) to remove calcium as suggested by Boyer et al.[4] Chelex was either used in the form of a column or was mixed with samples, allowed to equilibrate, and centrifuged out of the solution.

All other chemicals used were of reagent grade.

Particle size was monitored by laser fluctuation spectroscopy.[6] Experiments were initiated at time zero by addition of thrombin and were conducted at 23°C in barbital-saline buffer (pH 7.4, ionic strength 0.15, plus any additional salts specified). The final fibrinogen concentration in all experiments was 1.11 mg/ml; that of thrombin was 0.075 NIH units/ml.

## RESULTS

Figure 1 shows the relationship between particle size and time obtained from the fibrinogen-thrombin system in the absence and presence of 2.0 m*M* $CaCl_2$. Both experiments showed the expected induction period followed by a period of rapid increase in particle size. The time course of the reactions can be conveniently characterized by two variables, (1) the length of the induction period

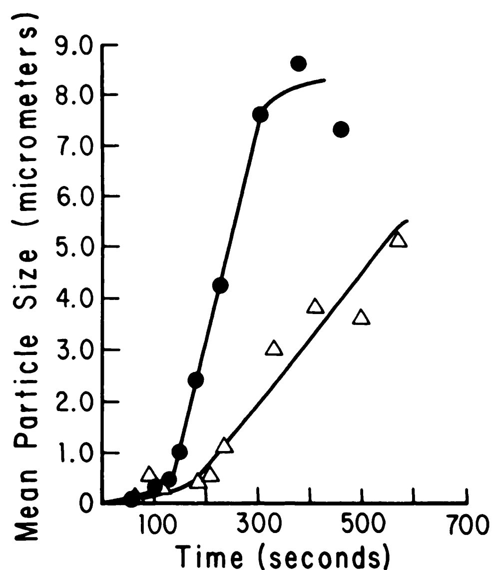


**Fig. 1. Particle size as function of time in absence and presence of calcium. Particle size in fibrinogen-thrombin system was measured in the absence (△) or presence (●) of 2.0 mM calcium chloride.**

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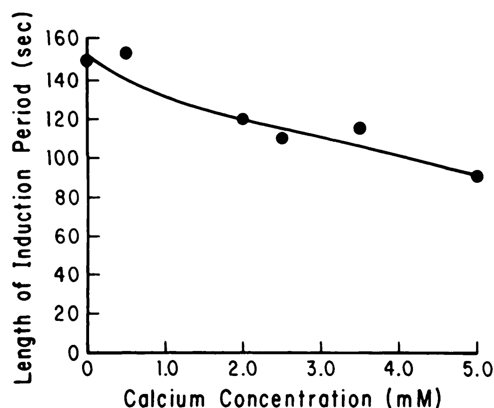


**Fig. 2.** Length of induction period as a function of calcium concentration. Particle size in fibrinogen-thrombin system was measured in the presence of varying concentrations of calcium. Length of induction period was determined from particle size as a function of time plots from each experiment.

and (2) the rate of particle size increase during the period of rapid particle growth (fibrin monomer polymerization).

Figure 2 shows that addition of calcium ions decreased the length of the induction period in a concentration-dependent manner. At physiologic plasma calcium concentrations (2.0 m*M*) the length of the induction period was 80% of that seen in controls. When physiologic calcium concentrations were exceeded, a small additional effect on the induction period was noted. This effect was not due to changes in ionic strength, since the addition of sodium chloride to an ionic strength equal to that with calcium chloride had no effect on the length of the induction period.

In contrast to the minor effect on the length of the induction period, the rate of particle size increase was dramatically accelerated (400%) by addition of calcium (Fig. 3). The calcium concentration dependence of this effect was similar to that seen in Fig. 2, with physiologic calcium concentrations producing a near-maximum acceleration. Again, addition of sodium chloride showed that this increased rate was not a result of increased ionic strength.

The separate measurements of the length of the induction period and the rate of polymerization can be combined to predict the effect on the overall time to form a visible clot. Such a prediction can be made by adding to the length of the induction period the time it would take to reach a given size at the observed

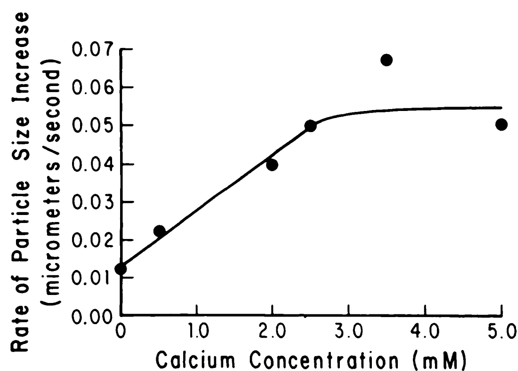


**Fig. 3.** Rate of particle size increase as a function of calcium concentration. Rate of particle size increase following induction period was determined by comparing increase in particle size and time from a series of fibrinogen-thrombin reactions.

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**Table 1. Prediction of Visual Clotting Time Based on Acceleration of Polymerization Reactions**

| $Ca^{2+}$ (mM) | Predicted Visual Clotting Time (Percent of Control) | Actual Visual Clotting Time (Percent of Control) |
|---|---|---|
| 0 | 100 | 100 |
| 0.5 | 70 | 71 |
| 2.0 | 53 | 55 |
| 2.5 | 49 | 53 |
| 3.5 | 45 | 52 |
| 5.0 | 49 | 46 |

The visual clotting time is predicted by adding to the control length of the induction period (150 sec) the time it would take to reach a 4-μm particle size at the observed rate of particle size growth. A 4-μm particle size was chosen because of the onset of gel formation observed previously in this region.[6] This calculated time can then be compared to the visual clotting time using the same reagents.

polymerization rate. Thus this predicted time will compare the portion of the overall clotting time that is spent during polymerization as opposed to that time during which no polymerization occurs, i.e., the induction period. In Table 1 predictions are made for calcium's effect on the visual clotting time based on its observed acceleration of polymerization by laser fluctuation spectroscopy. Table 1 also presents the actual observed effect of calcium on the visual clotting time. It can be seen that the effect of calcium in shortening the visual clotting time can be completely accounted for by its acceleration of polymerization.

## DISCUSSION

The ability to divide the conversion of fibrinogen to the fibrin clot into individual reactions or groups of reactions has provided details of the mechanism by which clot formation is regulated or perturbed. The formation of the fibrin clot from fibrinogen and thrombin includes the proteolytic action of thrombin and the subsequent polymerization of the generated fibrin monomer. In addition, the importance of an intermediate series of steps, the formation of a reversible complex between fibrinogen and fibrin monomer, was recently shown.[6] Two dysfibrinogens (Cleveland I[10] and Detroit[11]) would appear to be examples of abnormal complex formation rather than abnormal polymerization, as initially suggested by studies that did not include methods to evaluate this step of the reaction.

In earlier work we monitored the increase in particle size, a direct measure of the growth of the fibrin clot, as a function of time in the fibrinogen-fibrin system. Such monitoring permits the kinetic effects of the fibrinogen-fibrin monomer interaction to be evaluated and visualized in the form of an induction period prior to the onset of rapid polymerization. The kinetics of the fibrinogen-thrombin system predict that the length of the induction period is sensitive to changes in the proteolytic action of thrombin and to changes in the fibrinogen-fibrin monomer interaction. In contrast, the rate of size increase after the induction period is a measure of the polymerization reaction.

The present study shows that with the addition of calcium ions the induction period is decreased only slightly, while there is a dramatic increase in the rate of particle size increase. This increase in the rate of polymerization is similar to

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that observed by previous investigators and by ourselves studying polymerization of purified fibrin monomer in the absence and presence of calcium as monitored by optical density changes (data not shown). Since it was shown previously that calcium ions do not alter the rate of thrombin's proteolytic attack of fibrinogen,[3,4] the small change in the length of the induction period eliminates the possibility of a major effect of calcium on the fibrinogen-fibrin monomer complex. Furthermore, a small decrease in the length of the induction period can be expected when the polymerization reactions are enhanced. The increased rate of polymerization will remove fibrin monomer, favoring dissociation of the fibrinogen-fibrin monomer complex by mass action. The major change observed in the rate of polymerization with a minor change in the length of the induction period accurately predicts the behavior of the fibrinogen-thrombin system as measured by visual clotting time. Thus an increase in polymerization rate of the magnitude observed is sufficient to quantitatively account for the acceleration by calcium during fibrin formation.

Kanaide and Shainoff[12] reported that the reversible fibrinogen-fibrin monomer complex can be converted to an irreversible complex by the action of fibrin stabilizing factor (FSF, factor XIII). The time for this irreversible complex formation is long compared to our studies. Since the action of FSF on the complex irreversibly removes fibrin monomer from the system, the induction period would be lengthened or the rate of polymerization retarded. Neither of these effects are consistent with calcium's effect of accelerating fibrin polymerization.

Thus the experiments presented here provide strong support for the conclusion that the major effect of calcium in the fibrinogen-fibrin system is to increase the rate of fibrin monomer polymerization, thereby decreasing the time required to form a visible clot. Further studies are required to delineate the molecular mechanism by which calcium interacts with fibrin monomer during polymerization.

## REFERENCES


1. Rosenfeld, G. Janszky B: The accelerating effect of calcium on the fibrinogen-fibrin transformation. Science 116:36, 1952

2. Ratnoff OD, Potts AM: The accelerating effect of calcium and other cations on the conversion of fibrinogen to fibrin. J Clin Invest 33:206, 1954

3. Godal HC: Delayed fibrin polymerization due to removal of calcium ions. Scand J Clin Lab Invest 24:29, 1969

4. Boyer MH, Shainoff JR, Ratnoff OD: Acceleration of fibrin polymerization by calcium ions. Blood 39:382, 1972

5. Endres GF, Scherago HA: Equilibria in the fibrinogen-fibrin conversion. IX. Effects of calcium ions on the reversible polymerization of fibrin monomer. Arch Biochem Biophys 153: 266, 1972

6. Brass EP, Forman WB, Edwards RV, Lindan O: Fibrin formation: The role of the fibrinogen-fibrin monomer complex. Thromb Haemostasis 36:37, 1976

7. Blombäck B, Blombäck M: Purification of human and bovine fibrinogen. Ark Kemi 10: 415, 1957

8. Ratnoff OD, Menzie C: New method for determination of fibrinogen in small samples of plasma. J Lab Clin Med 37:316, 1951

9. Prentice CR, Ratnoff OD, Breckenridge RT: Experiments on the nature of the prothrombin-converting principle: Alteration of proaccelerin by thrombin. Br J Haematol 13: 898, 1967

10. Forman WB, Brass EP, Edwards RV, Lindan O: Fibrinogen Cleveland. I. Abnormal fibrinogen-fibrin complex formation. (Manuscript in preparation)

11. Kudrky B, Blombäck B, and Blombäck M: Fibrinogen Detroit. An abnormal fibrinogen with non-functional $NH_2$-terminal polymerization domain. Thromb Res 9:25, 1976

12. Kanaide H, Shainoff JR: Cross-linking of fibrinogen and fibrin by fibrin-stabilizing factor (factor XIIIa). J Lab Clin Med 85:574, 1975

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