

Comparison of Polymerization of Ancrod and Thrombin Fibrin Monomers

By G. G. Spellman, Jr., J. A. Macoviak, and H. R. Gralnick

The polymerization of thrombin and ancrod fibrin monomers was studied with a standardized technique that evaluated turbidity changes and protein incorporation into the clot. Ancrod fibrin monomers were found to polymerize more slowly and form less turbid clots (at identical protein concentrations). Changes in ionic strength and pH influenced ancrod fibrin monomer polymerization to a greater extent than thrombin fibrin monomer polymerization. Benzyltriethylammonium chloride was shown to be a potent inhibitor of fibrin monomer polymerization, with a greater inhibitory effect on ancrod fibrin monomers than on thrombin fibrin monomers. The differences between ancrod and thrombin fibrin may play a role in the infrequent thrombotic complications reported with ancrod therapy.

ANCROD, A PURIFIED FRACTION of the venom obtained from the Malayan pit viper (*Ancistrodon rhodostoma*), directly converts fibrinogen to fibrin by the release of fibrinopeptide A.¹ Despite this thrombinlike activity, when ancrod is given by intravenous infusion in the proper dosage it rapidly produces hypofibrinogenemia with little risk of dangerous intravascular coagulation or thrombotic complications.² To explain this effect, Sharp et al.³ have hypothesized that either the ancrod fibrin monomers form defective polymers or they are defective in their ability to polymerize. Pizzo et al.⁴ have shown that ancrod fibrin clots are more susceptible to plasmin digestion than are thrombin fibrin clots.

The present work, utilizing a standardized procedure of fibrin monomer aggregation, investigates the hypothesis that ancrod fibrin polymerization is defective when compared with thrombin fibrin polymerization. Such defective polymerization would suggest an explanation for the infrequency of acute thrombotic complications.

MATERIALS AND METHODS

Human thrombin (obtained from Dr. David Aronson, Bureau of Biologics, Bethesda, Md.) was diluted in Tris chloride 0.02 M-NaCl 0.15 M, pH 7.4, to a final concentration of 400 NIH U/ml.

The ancrod used was Arvin (batch T.159), prepared by Twyford Laboratories and kindly supplied by Abbott Laboratories, North Chicago, Ill.

Human fibrinogen was prepared as described by Gralnick et al.⁵ Fibrinogen prepared from a pool of three normal plasmas was used in all experiments. The stock fibrinogen solution contained 20 mg/ml and was greater than 92% clottable.

Fibrin monomers were prepared by clotting 32 ml of a fibrinogen solution (1 mg/ml in 0.06 M sodium phosphate buffer, pH 6.8) with thrombin 2 NIH U/mg of fibrinogen or ancrod 2 U/mg of fibrinogen. At 30 min the clots were wound on a glass rod, washed with 34 ml 0.06 M sodium phosphate buffer (pH 6.8), and then dissolved in 14.4 ml 0.02 M acetic acid. After 30 min 54.4 ml 0.06 M sodium phosphate buffer (pH 6.8) was added, raising the pH to 6.6 and a clot reformed.

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Address for reprint requests: Harvey R. Gralnick, M.D., Chief, Hematology Service, Clinical Pathology Department, Clinical Center, NIH, Building 10, Room 5N-236, Bethesda, Md. 20014.

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After 30 min the clot was again collected, rinsed, redissolved, and clotted as above. This process was repeated a total of seven times with the final clot dissolved in 6 ml of 0.02 *M* acetic acid. The final solution was centrifuged for 10 min at 2000 *g* and stored at 4°C. The mean final yield using thrombin was 63.5% ($n = 7$, SD 2.7%), and the mean final yield using ancrod was 55.5% ($n = 3$, SD 3.7%).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), with and without the reducing agent dithioerythritol (5 *mM*), was performed on the final fibrin monomer preparations as described by Shapiro et al.⁶

Fibrin monomer polymerization was performed in a quartz microcuvette with a 1-cm light path at 30°C using a Gilford 2400 constant temperature cuvette chamber recording spectrophotometer (Oberlin, Ohio). The components of the standard reaction mixture were added as follows: 0.4 ml of 0.2 *M* NaCl or inhibitor solution (see below) of identical ionic strength, 0.6 ml of 0.1 *M* sodium phosphate buffer (pH 6.8), and 0.2 ml of fibrin monomer solution. The final pH and ionic strength (*I*) of the standard reaction mixture were 6.6 and 0.16, respectively.

The addition of the fibrin monomer solution was considered time 0. The reaction mixture was mixed at time 0 with a glass rod. Four tests were run simultaneously, recording the turbidity by measuring the absorbance at 350 nm (A_{350}) and the cuvette chamber temperature every 17 sec. At the end of 15 min the clot was removed with a glass rod and the supernatant fluid was dialyzed against 0.02 *M* acetic acid to dissociate any intermediate polymers not incorporated into the clot. The amount of protein that had been incorporated into the clot was calculated by subtracting the concentration of protein not incorporated into the clot from the total starting protein concentration.

Experiments were done using final monomer concentrations of 0.125 mg/ml, 0.225 mg/ml, 0.325 mg/ml, and a fourth concentration varying between 0.390 and 0.522 mg/ml (using diluted stock solution). The pH of the reaction mixture was changed by altering the sodium phosphate buffer so that the *I* of the final reaction mixture remained 0.16. Alterations in the *I* were made by adding sodium chloride to the buffer and making slight corrections in the buffer with NaOH so that the final pH of the reaction mixture remained 6.6 and the *I* of the reaction mixture was 0.4, 0.7, or 1.0. The solutions used for ancrod and thrombin fibrin monomer tests were identical and the pH values were measured for each experiment.

Inhibitors of fibrin monomer polymerization were added to the reaction mixture in place of the 0.4 ml of 0.2 *M* NaCl. Stock solutions of benzyltriethylammonium chloride and tetraethylammonium chloride were 0.2 *M* in deionized water and the stock solution of hexamethylene glycol was 0.2 *M* in 0.2 *M* NaCl. These solutions were diluted in 0.2 *M* NaCl to the concentration used.

Protein concentrations of the fibrinogen solution and fibrin monomer solution were computed from the absorbance at 280 nm assuming $A_{1\text{cm}}^{1\%}$ to be 15^7 in a concentration range where deviations from Beer's laws were minimal.

Statistical analysis was done with the help of Robert Baird, Division of Computer Research and Technology, NIH, using standard computer programs for regression analysis and analysis of covariance to study the relationships between the following: the lag phase (time from start of reaction to intersection of baseline of polymerization curve with tangent of the maximum slope of polymerization curve) and the initial protein concentration; A_{350} at 15 min and the initial protein concentration; the protein incorporated into the clot and the initial protein concentration; and the A_{350} at 15 min and the protein in the clot. The significance levels given are from the analysis of covariance using all results obtained at the four experimental monomer concentrations.

RESULTS

A comparison of thrombin and ancrod fibrin monomers at pH 6.6 and *I* 0.16 is shown in Fig. 1. Representative examples of the polymerization curves show that the ancrod fibrin monomers had longer lag phases ($p < 0.025$) and developed less turbidity ($p < 0.001$) than the thrombin fibrin monomers. Increasing the initial monomer concentration accentuated these differences. The analysis of covariance graph shows that as the amount of protein in the clot

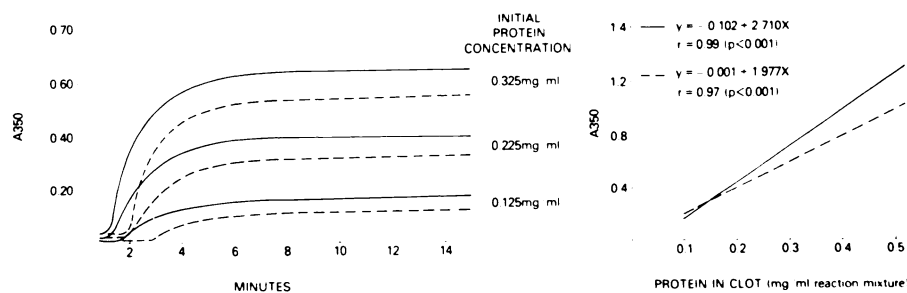


Fig. 1. Comparison of thrombin (—) and ancrod (----) fibrin monomer polymerization at pH 6.6 and *I* 0.16. The difference between the slope of ancrod and thrombin monomer regression lines is significant ($p < 0.001$).

increased the turbidity of the thrombin fibrin clot increased more than the ancrod fibrin clot ($p < 0.001$). The greater increase in turbidity for a given increase in protein in the clot with thrombin monomers compared to ancrod monomers existed under all experimental conditions of pH, *I*, and initial fibrin concentrations.

The effects of pH, *I*, and fibrin polymerization inhibitors on ancrod and thrombin fibrin monomer polymerization are shown in Figs. 2-4. Altering the pH from 6.6 (Fig. 2) reduced ancrod fibrin monomer polymerization more than thrombin monomer polymerization ($p < 0.001$). Increasing the ionic strength (Fig. 3) impaired ancrod fibrin monomer polymerization more than thrombin fibrin monomer polymerization ($p < 0.001$). The effects of two previously unreported fibrin monomer polymerization inhibitors, tetraethylammonium chloride and benzyltriethylammonium chloride, as well as hexamethylene glycol are shown in Fig. 4. Benzyltriethylammonium chloride completely blocked ancrod fibrin monomer polymerization but not thrombin fibrin monomer polymerization.

In SDS-polyacrylamide gel electrophoresis, greater than 98% of the protein on the gel of the thrombin fibrin solution was in the α , β , and γ chain areas. The SDS-polyacrylamide gel electrophoresis patterns of the reduced ancrod fibrin monomers revealed α -chain degradation and a small amount of cleavage products (Fig. 5).

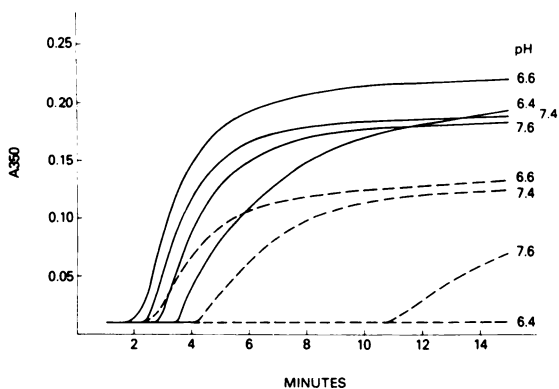


Fig. 2. Comparison of effect of pH on ancrod (----) and thrombin (—) fibrin monomer polymerization at *I* 0.16 and initial fibrin monomer concentration of 0.125 mg/ml.

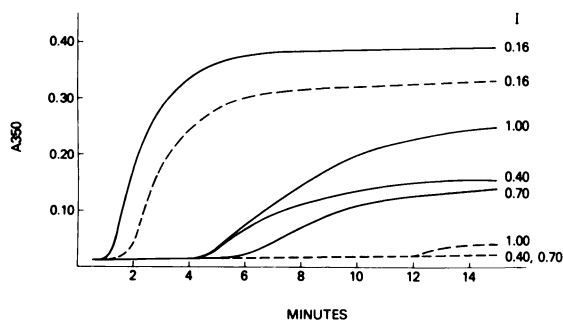


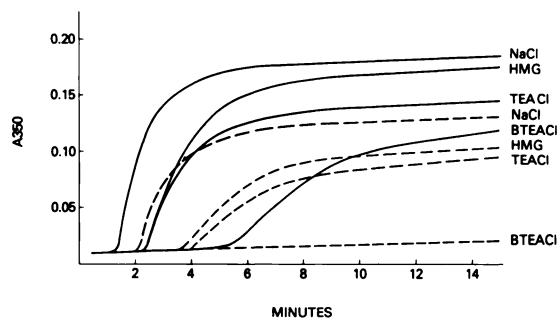
Fig. 3. Comparison of the effect of I on ancred (----) and thrombin (—) fibrin monomer polymerization at pH 6.6 and initial fibrin monomer concentration of 0.225 mg/ml.

DISCUSSION

The simple reproducible method used in this study for evaluating fibrin monomer polymerization facilitated the systematic evaluation of the effect of monomer protein concentration, pH, and I on the lag phase, the amplitude of the polymerization curves, and the amount of protein in the clot. This evaluation demonstrated significant differences between ancred and thrombin fibrin monomer polymerization. It also pointed to the necessity of coupling examinations of light-scattering polymerization curves with quantitation of protein in the clot since the turbidity that develops for a given amount of protein in the clot not only varies with the type of monomer but also with the pH and I .

Ancred fibrin monomers polymerized more slowly, formed less turbid clots, and were inhibited more by changes in pH, I , and polymerization inhibitors than thrombin fibrin monomers. The effects of pH and I on thrombin fibrin monomer polymerization confirm the conclusions of Ferry and Morrison⁸ and Shulman and Ferry⁹ based on studies of the clotting of fibrinogen with thrombin. They reasoned that pH and I influence primarily fibrin polymerization rather than the thrombin-fibrinogen reaction and that at higher I and pH values fine clots rather than coarse clots form because the attractive forces between fibrin molecules are lower. These studies plus those of Haschemeyer¹⁰ using the venom of *Bothrops jararaca* (which, like ancred, does not release fibrinopeptide B) explain the findings in the present study that ancred fibrin monomers form finer (less turbid) clots at a slower rate than thrombin fibrin monomers. Because ancred does not release fibrinopeptide B,¹¹ ancred fibrin

Fig. 4. Effect of monomer polymerization inhibitors 0.05 M hexamethylene glycol (HMG), 0.05 M tetraethylammonium chloride (TEA Cl), and 0.05 M benzyltriethylammonium chloride (BTEA Cl) on ancred (----) and thrombin (—) fibrin monomers at pH 6.6, I 0.16, and initial fibrin monomer concentration of 0.125 mg/ml.



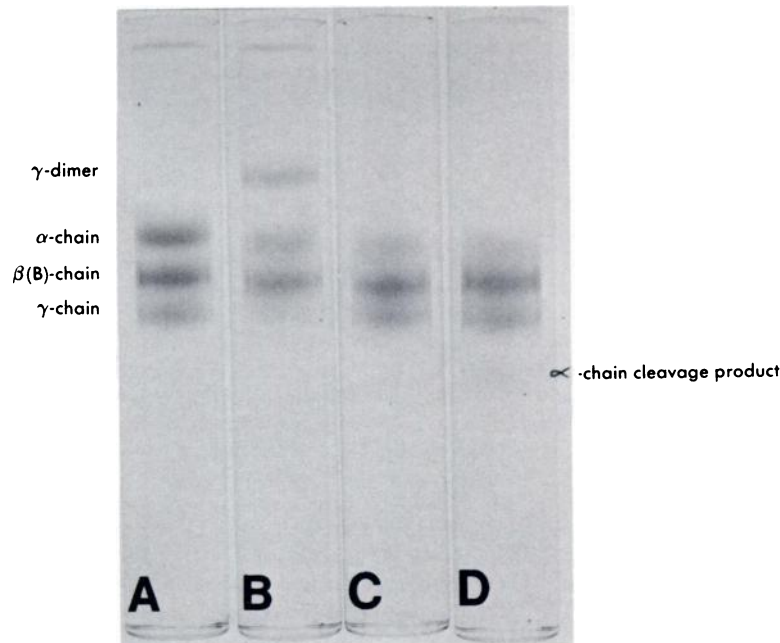


Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reduced fibrin(ogen) preparations. (A) Initial fibrinogen solution. (B) Soluble fibrin; purified fibrinogen was clotted with thrombin and CaCl_2 . The fibrin clot was placed in 5 M urea and the soluble fibrin (γ -chain dimers) was recovered. (C) Thrombin fibrin monomers. (D) Ancrod fibrin monomer with α -chain degradation product.

monomers react only end to end and thus polymerize more slowly, forming less turbid clots.

Another possible explanation for the defective polymerization of ancrod fibrin monomers may be that ancrod's proteolytic degradation of the α chain of fibrin⁴ adversely affects the α -chain interactions in polymerization. It is also possible that the α -chain cleavage products contribute to the difference.

Although the mechanism is not clear, the hypothesis that ancrod fibrin monomer polymerization is not optimal could be demonstrated in vitro. Ancrod monomers formed clots more slowly and the clots were less turbid. Polymerization of ancrod fibrin monomers was more easily affected by pH and *I* changes and by inhibitors of polymerization. It is possible that these significant in vitro physicochemical differences between ancrod and thrombin fibrin monomer polymerization also occur in vivo and result in the delayed formation of defective ancrod fibrin. This difference may be a partial explanation for the infrequency of acute intravascular thrombi with ancrod therapy.

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