Strydom, D. J. (1973) *Nature (London), New Biol. 243*, 88–89.
Taylor, J., Bidard, J., & Lazdunski, M. (1984) *J. Biol. Chem. 259*, 13957–13967.
von Haux, P., Sowerthal, H., & Habermann, E. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 737–738.
Wernicke, J. F., Vanker, A. D., & Howard, B. D. (1975) *J. Neurochem. 25*, 483–496.

Clotting of Bovine Fibrinogen. Calcium Binding to Fibrin during Clotting and Its Dependence on Release of Fibrinopeptide B†

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ABSTRACT: Polymerization of bovine fibrinogen acted upon by thrombin is accompanied by binding of Ca^{2+} and a concomitant decrease of the free Ca^{2+} concentration. The latter can be recorded by a Ca^{2+}-selective electrode as a shift in the electrode potential. The shift shows marked dependence on the initial free Ca^{2+} concentration, being maximal at about $10^{-4.1}$ M and decreasing sharply on either side of this. Thus, the effect is limited to the 10^{-3}–10^{-5} M free Ca^{2+} concentration range. From the initial and the final value of the electrode potential during a clotting experiment, the amount of Ca^{2+} bound to fibrinogen and fibrin, respectively, can be calculated. The difference between the two, plotted against free Ca^{2+} concentration, gives a bell-shaped curve. This indicates that the reason for the Ca^{2+} binding is a shift of the pK of some groups from a lower to a higher value. The recordings can be used for evaluation of the kinetics of the Ca^{2+} uptake. However, they have to be corrected for the effect of the continuous shift in the free Ca^{2+} concentration during the experiment. The reaction does not follow simple kinetics, showing a lag period. Therefore, rates were estimated from inverse half-reaction times. Half-times of the corrected curves show that the reaction is first order with respect to thrombin. Moreover, the rate of Ca^{2+} uptake is identical with that of the conformational change seen in differential scanning calorimetry [Donovan, J. W., & Mihalyi, E. (1985) *Biochemistry 24*, 3434]. The inverse rate and the final corrected Ca^{2+} uptake increase linearly with the initial fibrinogen concentration. Concomitant estimates of fibrinopeptide A and B release showed that the Ca^{2+} uptake runs parallel to the release of fibrinopeptide B. Fibrinopeptide A was released largely during the lag period of the Ca^{2+} uptake. In agreement with this, clotting with Ancrod, an enzyme that liberates only fibrinopeptide A, was not accompanied by binding of Ca^{2+}. Thus, polymerization is not sufficient for the Ca^{2+} uptake to occur; liberation of fibrinopeptide B seems to be obligatory. Further support for this was obtained with experiments with the polymerization inhibitor Gly-Pro-Arg-Pro. The tetrapeptide inhibits polymerization and also, proportional to this, release of fibrinopeptide B [Hurlet-Jensen, A., Cummins, H. Z., Nossel, H. L., & Liu, C. Y. (1982) *Thromb. Res. 27*, 419; Lewis, S. D., Shields, P. P., & Shafer, J. A. (1985) *J. Biol. Chem. 260*, 10192]. Calcium uptake was also depressed by the tetrapeptide in a way similar to its effect upon firbinopeptide B release.

The fibrinogen molecule undergoes a conformation change during polymerization manifested by an increase of the enthalpy of denaturation of the D domain and a shift of the transition curve of this domain to higher temperature (Donovan & Mihalyi, 1985). These two changes are affected in different ways by the presence of calcium ions: a large increase of the enthalpy of unfolding occurs in the absence of calcium ions, and this increases only moderately further by increasing concentrations of calcium ions. On the other hand, the transition temperature is not affected by polymerization without calcium, while increasing concentrations of the latter elicit an increasing shift that eventually levels off.

Estimates of the shift as a function of free calcium concentration were used to calculate the pK value of the calcium binding sites governing this reaction. This can be compared with the values determined by equilibrium dialysis for the binding constants of calcium to bovine (Marguerie et al., 1977) and to human fibrinogen (Nieuwenhuizen et al., 1981). For human fibrinogen, the pK from the titration curve coincided with that of the high-affinity binding sites, whereas with bovine fibrinogen it was more than 1 unit below this. The data suggest that the sites governing the shift of the transition temperature may be different from the high-affinity calcium binding sites. They may be new sites created by the polymerization, or sites shifted from lower to higher affinity. In either case, it is expected that polymerization will be associated with additional binding of calcium and a corresponding decrease in free calcium concentration. This paper describes studies with the calcium electrode to test this hypothesis.

Experimental Procedures

Materials. Bovine fibrinogen, Pentex (lot 28, 95% clottable), was obtained from Miles Laboratories, Inc., Research Products Division, Naperville, IL. Calcium-free fibrinogen was prepared as described (Donovan & Mihalyi, 1985). An

† This is paper 3 in the series "Clotting of Fibrinogen". For paper 2, see Mihalyi and Donovan (1985).

8–9% solution was obtained in 0.3 M NaCl and 0.025 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)[1] buffer, pH 7.26, with an average calcium content of 0.05 atom per mole of fibrinogen. Protein concentration was estimated spectrophotometrically, using 15.06 for $E^{1\%}_{1cm}$ at 280 nm corrected for turbidity (Mihalyi, 1968). Clottability of the preparations was 96–97%. The high viscosity of the fibrinogen solutions made it necessary that all the dispensing of the material be made by weight.

Human thrombin of high purity was lot 192 obtained from Dr. John W. Fenton, II. Dilutions of this were made with 0.3 M NaCl containing 1 mg/mL bovine serum albumin; the latter addition prevents adsorption of thrombin on surfaces and stabilizes the enzyme (Horne, 1985).

Two commercial preparations of the coagulase from *Agkistrodon rhodostoma* venom were used: "Ancrod", from Abbott Laboratories, North Chicago, IL, lot 33-657-DB, and "Arvin", from Knoll Pharmaceutical Co., Whippany, NJ, lot 3070. The first one contained some unidentified material that caused an instantaneous drop of the free calcium concentration. This contaminant was removed by dialysis. The second one contained 4 mM phosphate buffer. Since in control experiments with thrombin twice the amount of phosphate introduced in the runs with Arvin did not affect the calcium uptake on clotting, the latter was used as supplied.

The polymerization inhibitor tetrapeptide Gly-Pro-Arg-Pro was purchased from Sigma Chemical Co., St. Louis, MO.

Sodium chloride, specially purified, "Aristar" grade from BDH Chemicals, Ltd., Poole, England, was used in all these experiments. Tris base, "Ultra Pure", was obtained from Schwarz/Mann, Cambridge, MA. For both the NaCl and Tris preparations, the manufacturers give a calcium content of 0.1 ppm. With this figure, the calcium content of a 0.3 M NaCl/0.025 M Tris solution, used as solvent in these experiments, can be calculated as 0.11×10^{-6} M. Actual estimates by atomic absorption spectrophotometry of the fifth dialysate of one of the fibrinogen preparations gave 0.7×10^{-6} M. Even if the latter is the correct value, it is still acceptable, since none of our experiments had to be run at lower than 10^{-6} M calcium concentration. HPLC-grade reagents from Fisher Chemical Co., Fair Lawn, NJ, were used for the preparation of the elution buffers for the chromatographic analyses. All other reagents used were of analytical purity.

Methods. Total calcium content was obtained by atomic absorption spectrophotometry of samples hydrolyzed in 6 N HCl to which lanthanum was added.

Free calcium was determined with an electrode system consisting of an F2110 calcium ion selectrode from Radiometer, Copenhagen, Denmark, and an Ag/AgCl reference electrode, MI 401, from Microelectrodes, Inc., Londonderry, NH, coupled to a Beckman Century SS pH meter, operated in the millivolt mode on the expanded scale. The solution to be measured was thermostated to 25.0 °C and stirred with a Teflon-coated micro stirring bar. The electrode vessel was of 1.2 cm i.d., sufficient to accommodate the electrodes, and about 0.8 mL was the minimum volume necessary for proper stirring and contact with the electrodes. Although the solution was thermostated, the electrodes themselves, especially the stem of the reference electrode, were not. Therefore, room temperature had to be constant to about 1 °C; otherwise, small drifts in the EMF were observed. Stirring was sufficient, in spite of the high viscosity, to cause complete mixing in 10–20 s, when checked with dye solution injected into the fibrinogen solution. To avoid dead space, the electrodes were lowered only to touch the surface of the solution, and bubble formation was avoided. Equilibration of the EMF of the electrode system was not much longer than the mixing time with both saline and the fibrinogen solutions, if everything was working properly. The main problem seemed to be the reference electrode. This was the sealed-in asbestos fiber type and was chosen because of its small size that enabled use of small volumes. Several of the electrode tubes supplied by the manufacturer had to be tested to find one with fast response time and low noise. Unfortunately, even a good one did not last long. The fiber junction became clogged probably by fibrinogen precipitated in contact with the electrode-filling solution, and this caused increasing sluggishness and noise. With this, the response time may have extended to 8–10 min, that is unacceptable for kinetic studies. Therefore, the response time had to be checked frequently, and if it was longer than 1 min for 90% and 2 min for complete equilibration, the reference electrode tube had to be replaced.

Cleaning of the electrodes presented no problems. With fibrinogen solutions, rinsing with 0.3 M NaCl followed by distilled water was sufficient. When fibrinogen was clotted, especially at higher concentrations, the clot pealed off the electrodes and vessel in one piece, and only gentle wiping with wet tissue paper, followed by rinsing with distilled water, was necessary. However, once the reference electrode clogged up, attempts to unclog it were unsuccessful.

The standard curve of the EMF response was constructed from measurements of a progression of 10-fold dilutions of 1 M $CaCl_2$ solution from 10^{-1} to 10^{-6} M. The EMF of the electrode system is dependent on pH and the presence of other than calcium ions. Therefore, the dilutions of the standard were made with the solvent used in these experiments, 0.3 M NaCl/0.025 M Tris, pH 7.26. EMF plotted against log $CaCl_2$ concentration was linear from 10^{-1} to 10^{-4} M with a slope close to the theoretical value of 29.58 mV and was less by 1–3 mV of the linear plot at 10^{-5} M. Below this concentration, the slope decreased drastically. The electrode is not usable below 10^{-6} M, that was also the limit imposed by the presence of calcium contamination in the solvent, regardless of the electrode response. The measurements reported in this paper fall between 10^{-5} and 10^{-2} M concentration, within the near-linear response of the electrode. While the slope remained constant, the magnitude of the EMF at a given concentration slowly decreased with aging of the electrode. Therefore, standardization of the electrode was performed before and after each series of measurements.

The change in EMF was recorded on a Kipp-Zonen Model BD 41 mV recorder. The recorder was calibrated with respect to the pH meter by short-circuiting the input of the latter, followed by displacing the needle of the pH meter with the standardization knob in several steps within the range desired. With the variable range adjustment of the recorder, the magnitude of the steps on the chart could then be adjusted to fill the chart.

Clotting activity of thrombin and the *Agkistrodon* coagulase preparations was determined with the BBL FibroSystem from Becton, Dickinson and Co., Cockeysville, MD, on human fibrinogen as described by Fenton and Fasco (1974).

Estimation of the fibrinopeptides was performed by HPLC using a Beckman-Altex C_{18} Ultrasphere ODS reversed-phase column (5 μm, 4.6 × 250 mm) connected to Beckman Model 344 CRT-based gradient liquid chromatograph system with Beckman Model 165 variable wavelength UV–vis detector and

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; EMF, electromotive force.

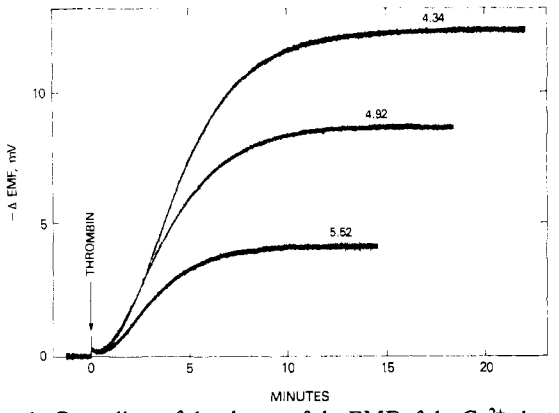

FIGURE 1: Recordings of the change of the EMF of the Ca^{2+} electrode upon clotting of bovine fibrinogen by thrombin at various initial concentrations of free Ca^{2+}. Fibrinogen concentration, 8.56%; thrombin concentration, 5.3 NIH units/mL; −log free $[Ca^{2+}]$ given by the numbers above each curve; 0.3 ionic strength; pH 7.26; 25 °C.

a Shimadzu Chromatopac C-R1B data processor. The peptides were eluted isocratically with a buffer system similar to that of Martinelli and Scheraga (1979): 0.1 M ammonium phosphate buffer of pH 3.12 and acetonitrile, 83:17, at 1 mL/min at room temperature, with detection at 206 nm.

For kinetic experiments, to compare the calcium uptake with release of the fibrinopeptides, individual samples incubated with thrombin were monitored by recording the calcium uptake. At the desired point, the flat-bottomed tube containing the reaction mixture was rapidly transferred into a boiling water bath. Coagulation occurred in 50–55 s. The coagulum was transferred into a centrifuge tube and spun in the Sorvall SS-34 head at 4 °C for 15 min. The clear supernatant was removed, diluted 4-fold with distilled water, and run without filtration in the HPLC apparatus. The enzyme concentration in these experiments was chosen to give reaction times of the order of 100 min. This appeared sufficiently long to minimize the importance of the delay between the interruption of the recording and inactivation of the enzyme by heating. Heating was chosen as a means of stopping the reaction, rather than a precipitating agent or inhibitor, because it did not introduce material which may have shown up in the chromatograms; furthermore, the difficulty of homogenizing the tough gel to ensure rapid diffusion of the reagent was circumvented. The supernatants checked with trichloroacetic acid showed no or only traces of protein which did not interfere with the HPLC runs.

Increase of the optical density upon polymerization was recorded at 450 nm with the Zeiss PMQ II spectrophotometer. Output from the amplifier was fed through a linear-log converter, operated on 0.2 optical density range, into the Kipp-Zonen millivolt recorder. The solution was in a 1-cm square cuvette, with a Star-Head stirring bar (Nalgene) at the bottom. The stirring bar and the top of the solution were masked by black tape that formed a horizontal slit of 3-mm width. Into 1 mL of fibrinogen solution, 0.075 mL of thrombin was injected and the recorder zeroed immediately. Recording was continued until a stable level of optical density was reached.

RESULTS

Changes of the Free Ca^{2+} Concentration during Clotting of Fibrinogen. Effect of the Initial Free Ca^{2+} Concentration. The initial free Ca^{2+} concentration was adjusted to various levels by adding 0.1 M $CaCl_2$ to the fibrinogen solution. Then the EMF of the calcium electrode was read, and after a stable base line, thrombin was added and the EMF change recorded until a constant value was reached. A few of the recordings

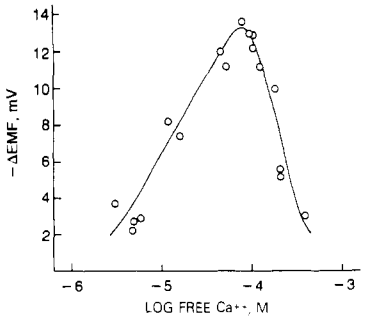

FIGURE 2: Asymptotic values of the change of the EMF of the Ca^{2+} electrode upon clotting of fibrinogen by thrombin plotted against the initial log free Ca^{2+} concentration. Data taken from the recordings in Figure 1 and others not shown there.

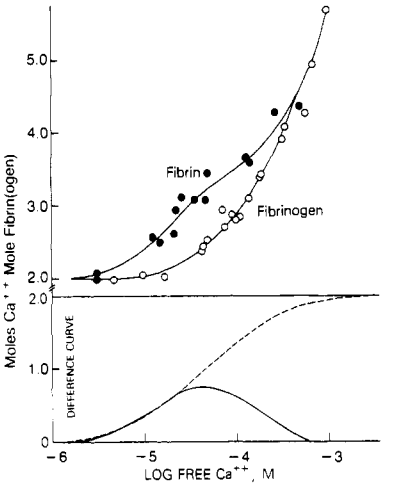

FIGURE 3: (Upper panel) Calcium bound to fibrinogen or fibrin plotted against log free Ca^{2+} concentration. Values calculated from the initial and the final EMF of the Ca^{2+} electrode for fibrinogen clotted by thrombin. (Lower panel) (—) Difference of the fibrinogen and fibrin curves plotted against log free Ca^{2+} concentration. (---) Theoretical titration curve with pK of 4.3, fitted to the ascending part of the difference curve.

are reproduced in Figure 1. All these and subsequent ones display an identical initial jump caused by the dilution of the solution by the addition of thrombin. The jump is of the same magnitude regardless of initial calcium or varying fibrinogen concentrations, because it is determined solely by the log of the dilution factor. After the initial jump, the recordings display a lag period when there is little change, or they may dip even below the base line, then rise to varying heights, and finally level off. The final stable values reached are a sharp function of the initial free Ca^{2+} concentration, as shown in Figure 2. Below 10^{-5} M, the EMF shift is negligible, then increases sharply with increasing free Ca^{2+} concentration, and reaches a maximum at about pCa 4.1, followed by a decrease to low values above 10^{-3} M. Thus, the changes in free Ca^{2+} concentration upon clotting are restricted to a fairly narrow range of the initial concentration, from 10^{-5} to 10^{-3} M. To avoid overcrowding, the examples of recordings shown in Figure 1 were chosen only from the Ca^{2+} concentration range of the ascending limb of Figure 2.

From the total Ca^{2+} added, the free Ca^{2+} concentration, the total volume, and the amount of protein in the sample, the Ca^{2+} bound to fibrinogen or fibrin can be calculated. The initial EMF served for the calculation of Ca^{2+} bound to fibrinogen, the final EMF for that to fibrin. The results of a series of experiments are shown in Figure 3. Bound Ca^{2+} concentration is plotted against log free Ca^{2+} concentration, the latter determined with the Ca^{2+} electrode. The vertical

difference of the two lines drawn through the experimental points is plotted in the lower half of the figure as the difference curve. This curve represents the amount of Ca^{2+} taken up during clotting at a given value of the free Ca^{2+} concentration, if it were possible to run the experiments at a constant free Ca^{2+} concentration. The bell-shaped curve suggests that the uptake is caused by a shift of the binding curve of some sites from the low-affinity group to a higher affinity. The curve should be symmetrical, but the descending side is slightly distorted, probably because of the increasing errors in estimating a small difference with increasingly high numbers. Also, there is no plateau region. For the latter, the shift in pK must be of the order of 4 units, and it is evident that the actual shift is less than 1 pK unit. As a consequence of this, the curve is truncated, and the actual number of sites taking part in this shift is higher than the height of the peak. In our previous paper (Donovan & Mihalyi, 1985), we have estimated the pK of the Ca^{2+} binding groups that govern the conformational change on clotting of fibrinogen as 4.3. The dashed line in the figure is an ideal titration curve with this pK, whose height was adjusted so that its initial segment matched the ascending limb of the difference curve. The height of this curve should give the number of sites actually taking part in this reaction. This estimate is obviously inaccurate, but it suggests that when clotting occurs approximately two calcium ions are bound with a pK of 4.3, intermediate to that of the low- and high-affinity sites of fibrinogen.

Effect of Cross-Linking by Factor XIIIa. Fibrinogen at 10^{-4} M free Ca^{2+} concentration was clotted in the absence and in the presence of 0.01 M iodoacetamide, and the change of free Ca^{2+} concentration was recorded. This reagent had no effect on the clotting time of fibrinogen or the appearance of the clot but prevented cross-linking, as evidenced by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of reduced samples. The two recordings, one with non-cross-linked and the other with cross-linked fibrin gel, were identical, showing that cross-linking had no effect upon the calcium binding during clotting.

Calculation of the Course of the Ca^{2+} Uptake Reaction from ΔEMF Data. The variable recorded in these experiments was the shift of the calcium eletrode potential, ΔEMF. The potential itself is proportional, within the linear response range of the electrode, to the log of Ca^{2+} activity. Thus, if the activity coefficient remains constant, ΔEMF is proportional to the log of the ratio of shifted to initial calcium concentration. The proportionality factor with the divalent calcium ion, at 25 °C, is 29.58 mV. With the ratio and the initial concentration, one can calculate the decrease of the free calcium concentration during the reaction and from this the amount bound to the protein.

The data could be easily handeled if the reaction were run in a calcium-stat, where the calcium concentration could be kept constant by resupplying the amount bound to the protein. This is not possible with a system that forms a gel, that makes rapid equilibration after adding the ligand impossible. Recording the decrease of free Ca^{2+} concentration circumvents this problem; however, the complication arises of running the reaction at constantly changing free Ca^{2+} concentration. The situation is illustrated schematically in Figure 4. The upper curve is the calcium titration of fibrin and the lower one that of fibrinogen, corresponding to the curves shown in Figure 3. The capital letters represent bound calcium values in moles per mole. Vertical difference between the two curves, at a given free Ca^{2+} concentration, is the amount bound during polymerization at the respective free Ca^{2+} concentration.

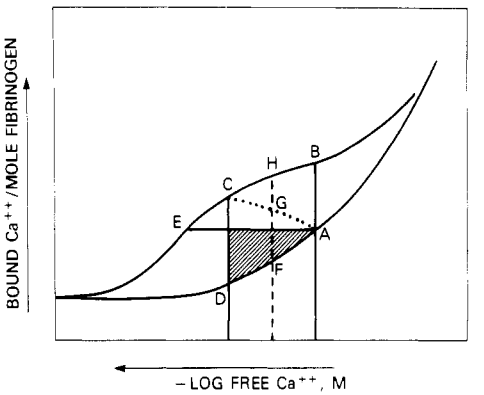

FIGURE 4: Schematic diagram of the shifts in free and bound Ca^{2+} that occur during a clotting experiment. For details, see text.

However, in our case, the free Ca^{2+} concentration shifts continuously as the reaction proceeds, and at each point, the situation is different from the initial one. For one thing, the vertical difference of the two curves changes. This is of relatively minor importance, because as seen in Figure 3 the difference is fairly constant in the range chosen for these experiments. More important is the fact that fibrinogen acts as a calcium buffer and will readjust to the lower level of free calcium by releasing bound calcium. This is shown by the shaded area in the scheme. If the reaction were conducted at constant free Ca^{2+} concentration, the amount of bound Ca^{2+} would move along the vertical line corresponding to this concentration, from e.g., A to B. However, because of the free Ca^{2+} concentration shifts, it will proceed along some oblique path, shown by the dotted line from A to C. Each point along this line corresponds to a certain degree of reaction, so this is a kinetic curve where the time scale has been replaced by the shift in log free Ca^{2+} concentration.

The degree of reaction in this scheme, at an intermediate stage represented by the dashed line, is given by $(G - F)/(H - F)$. The measured quantities are A and G, which are the amounts of Ca^{2+} bound initially and at the intermediate stage, in moles per mole. The difference $(G - A)$, derived from these, is equal to $\Delta[Ca^{2+}]_{obsd}$, the change in the amount of Ca^{2+} bound observed in the experiment. As seen in Figure 4, the actual amount bound on polymerization is larger than $\Delta[Ca^{2+}]_{obsd}$ by the amount liberated from fibrinogen at this point, given by $A - F$. F and H may be read on the titration curves shown in Figure 3, F being the amount of Ca^{2+} bound to fibrinogen and H to fibrin at the free Ca^{2+} level considered. The degree of reaction as defined above is a dimensionless normalized quantity and has to be multiplied by the total Ca^{2+} bound on polymerization at the initial free Ca^{2+} concentration, i.e., $B - A$. The result is the corrected Ca^{2+} uptake at the given point along the reaction path, as if this had occurred at a constant free Ca^{2+} concentration, equal to the initial one. The complete equation for the correction is

$$\Delta[Ca^{2+}]_{cor} = \{\Delta[Ca^{2+}]_{obsd} + (A - F)\}\frac{B - A}{H - F}$$

The corrected curves give the true course of the reaction; however, the presence of the lag period indicates that they do not follow simple kinetics. Instead of attempting to resolve the complex kinetics, for the purpose of the present paper it seemed sufficient to evaluate the half-reaction times. Reciprocals of these are a measure of the rates, if the kinetic relationships are not perturbed by changes of the various variables investigated. Thus, these will be used with all the curves presented in this paper, and a more sophisticated analysis will be given in the following paper (Mihalyi, 1988).

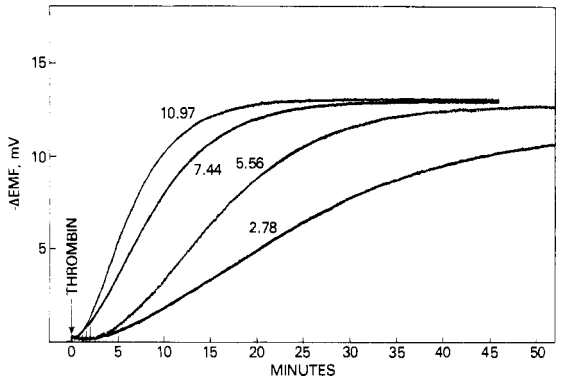

FIGURE 5: Effect of thrombin concentration on changes of the EMF of the Ca^{2+} electrode during clotting of fibrinogen. Fibrinogen concentration, 7.76%; thrombin concentration given by numbers next to each curve in NIH units per milliliter; log free $[Ca^{2+}]$, −4.00; 0.3 ionic strength; pH 7.26; 25 °C.

The rate of Ca^{2+} uptake, as judged by the $1/t_{1/2}$ values, appeared to be little affected by the increase of the free Ca^{2+} concentration in the 10^{-5}–10^{-3} M range, decreasing slightly with the increase of the latter. A double-logarithmic plot of $1/t_{1/2}$ vs free Ca^{2+} concentration gave a straight line with a slope of −0.1, showing that probably some nonspecific ion effect was involved in the change. These findings justify the use of the corrected curves for kinetic analysis. If the effect of the free Ca^{2+} concentration on the rate were more pronounced, this would not be the case, because under these circumstances the shifting free Ca^{2+} concentration during the reaction would affect the rate continuously. Another correction would become necessary to eliminate this effect. Since the shifts and their effect on the rate are small, this was neglected.

In summary, the ΔEMF data are converted in two steps, first into $\Delta[Ca^{2+}]_{obsd}$ and then into $\Delta[Ca^{2+}]_{cor}$, resulting in two modified reaction curves. The shape of all three normalized curves is very similar, but that of $\Delta[Ca^{2+}]_{obsd}$ is shifted slightly to the left, while that of $\Delta[Ca^{2+}]_{cor}$ is shifted to the right of the ΔEMF curve. While these shifts and those of the reaction half-times ensuing from these are relatively small, the magnitude of the conversion of $\Delta[Ca^{2+}]_{obsd}$ into $\Delta[Ca^{2+}]_{cor}$ may be substantial. This correction depends on the magnitude of the shift in free Ca^{2+} concentration, since as it is apparent in Figure 4, the relative importance of the amount of Ca^{2+} released from fibrinogen increases with the shift. For example, with the curve obtained with the high fibrinogen concentration in Figure 7, it amounts to 2.9-fold for the asymptotic value.

With an increase of the initial fibrinogen concentration, clotting brings about an increase in the asymptotic value of $-\Delta$ log free $[Ca^{2+}]$. This, however, cannot continue indefinitely, because when it reaches the intersection of the horizontal line drawn from *A* with the titration curve of fibrin at the point designated in Figure 4 as *E*, there will be no further decrease in free Ca^{2+} concentration. At this point, the amount of Ca^{2+} bound to fibrin will be exactly balanced by the amount released from fibrinogen.

Effect of Thrombin Concentration. Recordings of the change in EMF of the Ca^{2+} electrode on clotting with increasing thrombin concentrations are shown in Figure 5. Initial free Ca^{2+} concentration was chosen in all cases to have a value of 10^{-4} M, where ΔEMF is maximal. The character of the reaction curves is the same as described previously, but with all the curves leveling off at the same height. The lag times are noticeable and increase as the rate decreases with decreasing thrombin concentration.

The ΔEMF data were manipulated as described in detail in the preceding section, and from the $\Delta[Ca^{2+}]_{cor}$ vs time

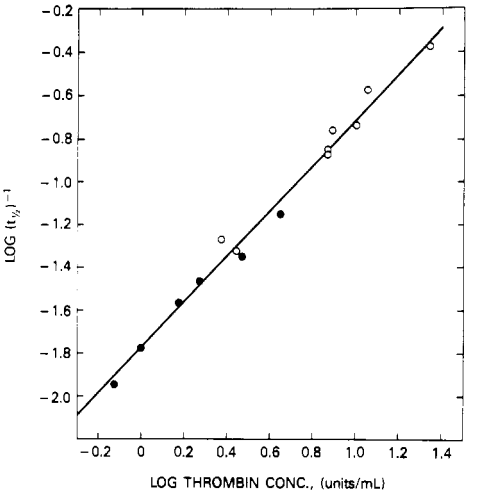

FIGURE 6: Logarithm of the reciprocal half-time of Ca^{2+} uptake calculated from the recordings shown in Figure 5, and others not shown there, plotted against log of thrombin concentration, denoted by open symbols. Similar data points obtained with differential scanning calorimetry taken from Figure 7 of Donovan and Mihalyi (1985) shown with closed symbols.

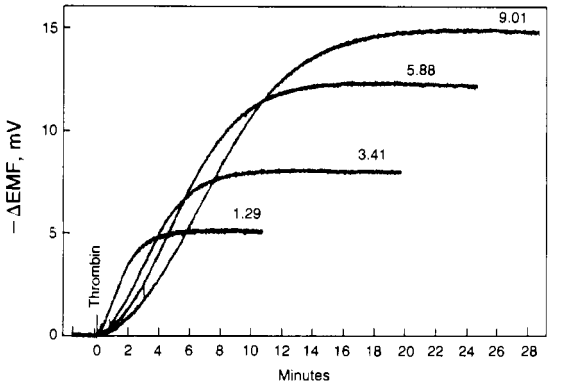

FIGURE 7: Recordings of ΔEMF during clotting with various fibrinogen concentrations. Fibrinogen concentration given in percent above each curve; thrombin concentration, 2.84 NIH units/mL; log free Ca^{2+} concentration, −4.00; 0.3 ioinic strength; pH 7.26; 25 °C.

curves, the $t_{1/2}$ values were determined. A plot of log $(1/t_{1/2})$ vs log thrombin concentration is shown in Figure 6. The experimental points fall reasonably well on a straight line with a slope of 1. This indicates that the rate-limiting reaction is first order with respect to thrombin. It may be recalled that the same result was obtained with the rate of conformational changes associated with clotting, detected by differential scanning calorimetry (Donovan & Mihalyi, 1985). Moreover, the points of both experiments fall on the same line as shown in the figure, and this is confirmed also by the near identity of the least-squares parameters of the lines defined by the two sets of data. Thus, within the time resolution of these methods and their different systematic errors, and uncertainties of the corrections, the two phenomena, conformational change and uptake of Ca^{2+}, are simultaneous. The main concern in this respect is the stability of the thrombin solutions. The two sets of experiments were performed at different times, with different dilutions, although from the same thrombin stock solution, and the activity of the latter was the same before and after the experiments. Therefore, we are reasonably certain that as far as the thrombin activities go the two sets of data are consistent.

Effect of the Initial Fibrinogen Concentration. Figure 7 shows a series of recordings performed at the same initial free Ca^{2+} concentration of 10^{-4} M and thrombin concentration of

2.84 units/mL, but with increasing fibrinogen concentrations. These experiments were performed primarily for practical reasons, to delineate the usable concentration range of fibrinogen; however, at the end, they yielded valuable insights for the explanation of the experimental data. As expected, ΔEMF increased with the fibrinogen concentration. The increase, however, was not linear but progressively declined, and the asymptotes seemed to reach a limiting value at high fibrinogen concentrations. This behavior is expected from the considerations presented in the previous section. In the concentration range employed, the data were reliable as it is shown in this and also in the next paper (Mihalyi, 1988). The signal to noise ratio of the recordings naturally decreases with the fibrinogen concentration, but even at 5 mg/mL, with recordings not shown here, the data were still usable. Increasing the fibrinogen concentration above the highest in these experiments has no advantage, because there is little further increase in the final heights of the ΔEMF curves and also there are experimental difficulties with the highly viscous solutions.

The corrected amount of Ca^{2+} taken up during polymerization increased with the fibrinogen concentration, but, even with the highest concentration of 2.65×10^{-4} M, was only about 65% of the Ca^{2+} originally present. Since fibrinogen was in 2.65-fold molar excess over the calcium initially present, the latter should have been exhausted at 55% of the reaction, even with the less than stoichiometric amount bound of 0.67 mol/mol read on the titration curves of Figure 3. This did not happen because of the shifts in equilibria already discussed. As mentioned previously, the maximal shift of the calcium concentration can be calculated from the geometry of the scheme of Figure 4, being point *E* in this figure. With 10^{-4} M initial Ca^{2+} concentration, this turns out to be at $10^{-4.60}$ M. The shift with the highest concentration of fibrinogen in these experiments reached $10^{-4.57}$, close to the above limit.

Whereas the total amount of calcium bound upon polymerization increases with increasing initial fibrinogen concentration, the amount bound per mole of fibrinogen changes in the opposite direction, dropping from 0.97 to 0.24 as the concentration increases from 0.183×10^{-4} to 2.65×10^{-4} M. The reason for this is again the shift in free Ca^{2+} concentration and the resulting changes in equilibria as shown in Figure 4. An extrapolation to zero fibrinogen concentration, where the shift would also be zero, should give the amount of bound Ca^{2+} as if the experiment were performed at constant calcium concentration. The extrapolated value is 1.15 mol/mol while the value read on the difference curve of Figure 3 is 0.67 mol/mol. The discrepancy is possibly more than the experimental error and uncertainty of extrapolation, and the reason for this might be that the Ca^{2+} binding to both fibrinogen and fibrin seems to depend on protein concentration below 10–15 mg/mL, the dependence being more pronounced for fibrinogen than for fibrin. This results in a larger difference at the lower concentrations.

The half-reaction times read on the corrected curves increase linearly with the fibrinogen concentration, but the line passes slightly above the origin. Thus, the rates are approximately inversely proportional to this concentration.

Correlation of Calcium Binding during Polymerization with the Release of Fibrinopeptides. The change in EMF of the calcium electrode was recorded with individual samples and then after various time intervals, the reaction was stopped by heat coagulation. Fibrinopeptides were analyzed in the supernatants of the coagula by HPLC. The possibility of the fibrinopeptides being adsorbed to the coagula was examined in control experiments. Various amounts of the fibrinopeptides were added to fibrinogen, and the mixture was heat-coagulated. The recovery of the fibrinopeptides in the supernatants of the coagula was 99.1 ± 2.2%, proving the feasibility of the method.

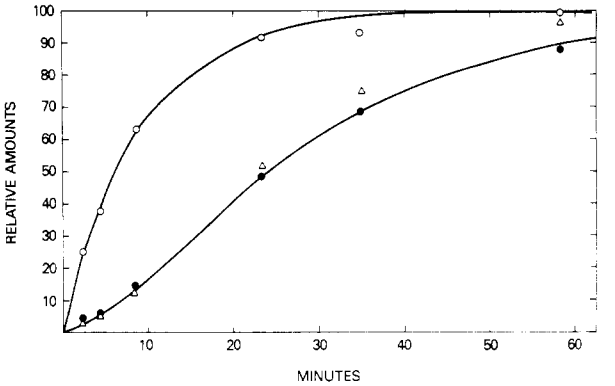

FIGURE 8: Correlation of Ca^{2+} uptake with release of fibrinopeptides. Normalized Ca^{2+} uptake (△), fibrinopeptide A (○), and fibrinopeptide B (●) releases plotted against reaction time. Fibrinogen concentration, 1.24%; thrombin concentration, 0.20 NIH unit/mL; log free Ca^{2+} concentration, −4.00; 0.3 ionic strength; pH 7.26; 25 °C.

Figure 8 shows the relative proportion of fibrinopeptides released at each time point together with ths similarly normalized values derived from the corrected calcium uptakes. The areas of the peaks were obtained from the integrator. These, for either fibrinopeptide A or fibrinopeptide B, reached a maximum and then declined slowly and slightly as the reaction proceeded. The reason for this decline was probably the presence of some enzyme contaminant that slowly degraded the fibrinopeptides. The data were normalized with respect to the maximal values. The experiment depicted in the figure was performed at relatively low fibrinogen and thrombin concentrations, 0.364×10^{-4} M (12.37 mg/mL) and 0.2 unit/mL, respectively. It is apparent that the rate of release of fibrinopeptide A, judged by the half-reaction times, is about 4 times faster than that of fibrinopeptide B. In the initial phase, the ratio of the rates is even larger, indicating an induction period for the release of fibrinopeptide B. The important fact emerging from this experiment, clearly shown in the figure, is the coincidence of the release of fibrinopeptide B with the Ca^{2+} uptake associated with clotting. Since it was demonstrated in a previous section that the conformational change associated with clotting and calcium binding are simultaneous within the time resolution of these experiments, the conclusion seems warranted that all three phenomena are interlinked.

An experiment performed at approximately 6-fold higher fibrinogen and thrombin concentrations, 2.387×10^{-4} M (81.18 mg/mL) and 1.36 units/mL, respectively, yielded similar results. The reaction curves were nearly superimposable with the ones obtained at the lower fibrinogen concentration, a fact expected from what has been said with respect to the effect of fibrinogen and thrombin concentrations on the reaction rates; namely, the rate is increasing proportionally with the increase of thrombin concentration and decreases in the same way by increase of fibrinogen concentration. Thus, the same 6-fold increase in both these variables canceled out their effect. The coincidence of the normalized, corrected Ca^{2+} uptake curves with similarly expressed fibrinopeptide B release with both experiments also increased our confidence in the correction procedure, since this was of considerably different magnitude with the two runs. For the low fibrinogen concentration run, it was negligible because the free Ca^{2+} concentration shift was also small, whereas it was

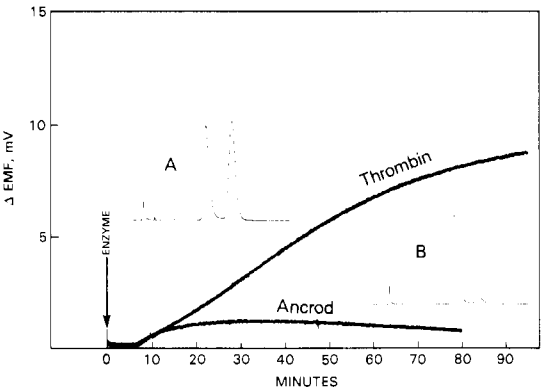

FIGURE 9: Recordings of the EMF change of the Ca^{2+} electrode with clotting of fibrinogen; upper curve with thrombin, lower curve with Ancrod. Fibrinogen concentration, 8.66%; thrombin concentration, 0.61 NIH unit/mL; Ancrod concentration, 1.32 NIH units/mL; log free Ca^{2+} concentration, −3.91; 0.3 ionic strength; pH 7.26; 25 °C. Inserts: HPLC patterns of peptides liberated (frame A) by thrombin and (frame B) by Ancrod.

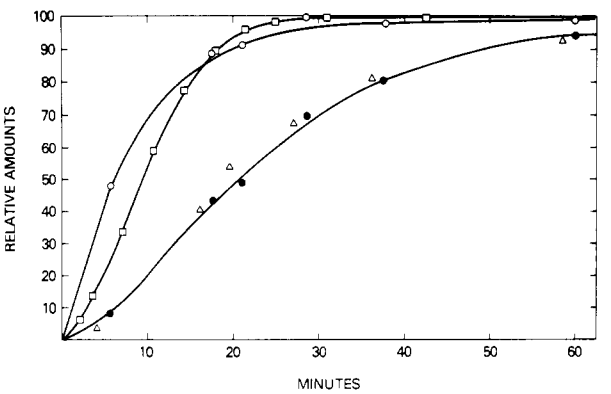

FIGURE 10: Correlation of turbidity increase with Ca^{2+} uptake and fibrinopeptide release. Relative amounts of increase of optical density at 450 nm (□), of Ca^{2+} uptake (△), and of fibrinopeptides A (○) and B (●) released during clotting plotted against reaction time. Fibrinogen concentration, 8.11%; thrombin concentration, 1.36 NIH units/mL; log free Ca^{2+} concentration, −4.10; 0.3 ionic strength; pH 7.26; 25 °C.

appreciable with the large shifts associated with the high fibrinogen concentration.

Clotting times in these experiments were measured in an approximate way by the time at which the stirring bar in the reaction mixture stopped. This represents roughly the time necessary for the rigidity of the sample to reach a certain level determined by the torque of the spinning bar. The data are not sufficient for a full analysis but only give a starting point for further experiments. Clotting time as defined above was 3.5 min with the high fibrinogen concentration and 22.5 min with the lower one. Thus, regardless of the fibrinogen concentration, the times were inversely proportional to the thrombin concentration. However, with high fibrinogen concentration, clotting occurred when about 30% of fibrinopeptide A, but only 3% of fibrinopeptide B, was liberated, while with low fibrinogen concentration this happened with liberation of over 90% of the first and 50% of the second fibrinopeptide.

Gel point occurs when the concentration of the activated fibrinogen molecules reaches a certain level. This critical concentration is achieved at a lower degree of reaction with the higher than with the lower fibrinogen concentration. The situation, though, seems to be more complicated than this. At the gel point, the degree of reaction, given by the relative amount of fibrinopeptide A released, is only 3-fold larger at the lower than at the higher fibrinogen concentration, although the concentration difference between the two runs if 6-fold. Of course, with the degree of fibrinopeptide A release being already over 90%, it could not have increased much more than this, so other factors must also be operative. The release of fibrinopeptide B may have an adjuvant effect, or the slower rate gives sufficient time for a more effective organization of the activated molecules, or the dynamics of the measurements may be different at different rates, among other things. Certainly, with an ample supply of des-A-fibrinogen, the removal of the B peptide does not seem to be necessary for gel formation.

Calcium binding and release of fibrinopeptides were investigated further in experiments with the coagulase of the *Agkistrodon rhodostoma* venom, that cleaves off only the A peptide (Ewart et al., 1977), but this is sufficient for clot formation. Identical results were obtained with the two preparations used: Ancrod and Arvin. With the venom coagulase, calcium binding during clotting was almost entirely absent, as shown in Figure 9. The enzyme is not absolutely specific for fibrinopeptide A, according to the HPLC pattern of the peptides in the right lower corner of the figure, obtained with the sample at the end of the recording. A small amount of fibrinopeptide B was also liberated, and the Ca^{2+} uptake recorded is commensurate with this. The figure shows for comparison also the Ca^{2+} uptake curve and the peptide pattern at the end of the recording obtained with thrombin. Both enzyme concentrations were adjusted so that the clotting time was approximately the same with both, about 7 min.

Correlation between Release of Fibrinopeptides, Polymerization, and Binding of Calcium. The courses of the release of fibrinopeptides and of Ca^{2+} binding were obtained from the experiment with high fibrinogen concentration described in the preceding section. Polymerization was followed by recording the turbidity increase in a separate experiment. Formation of the gel occurs in two steps. In the first, protofibrils are formed by end-to-end polymerization, and in the second one, these associate laterally into thick fibers. Because light scattering by the protofibrils is insignificant compared to that of fibers, with the less sensitive turbidity measurements only the formation of the thick fibrin fibers is recorded. This experiment, therefore, will enable one to place fiber formation in context with liberation of peptides and calcium uptake. Figure 10 shows the normalized turbidity curve, together with the normalized peptide release and Ca^{2+} uptake curves. The turbidity curve, i.e., fiber formation, clearly shows a lag period that can be assigned to the time necessary for the formation of a sufficient number of protofibrils which then start building up the fibers. Fiber formation appears to lag behind release of fibrinopeptide A also in the later phases of the polymerization reaction, but certainly is much ahead of fibrinopeptide B release. Similar observations were made already by Blombäck et al. (1978). Fiber formation at high ionic strength is slow and is even slower from activated fibrinogen molecules from which only fibrinopeptide A has been removed (Hantgan & Hermans, 1979). This may be the explanation for the delay of the fiber formation with respect to release of fibrinopeptide A through practically the whole course of the reaction. Release of fibrinopeptide B accelerates fiber formation, but this occurs too late according to the above experiment to be of much use. Figure 10 also shows that release of fibrinopeptide B and binding of Ca^{2+} follow a parallel course, reproducing exactly the pattern seen in Figure 8 with low firbinogen concentration. It is certain from these experiments that release of fibrinopeptide A and fiber formation precede the release of fibrinopeptide B and the associated Ca^{2+} uptake, but if the first has any effect upon the second, one could not ascertain whether this is connected to the release of fibrinopeptide A or the

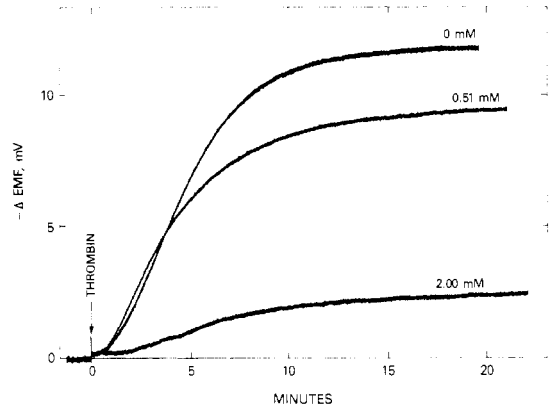

FIGURE 11: Effect of Gly-Pro-Arg-Pro on the EMF shift of the Ca^{2+} electrode during clotting. Fibrinogen concentration 4.66%; thrombin concentration 3.9 NIH units/mL; numbers above each curve give final concentrations of the tetrapeptide; log free Ca^{2+} concentration, −3.94; 0.3 ionic strength; pH 7.26; 25 °C.

ensuing association of the activated molecules into fibers, or both of these.

Effect of the Polymerization Inhibitor Gly-Pro-Arg-Pro. The effect of the tetrapeptide on Ca^{2+} binding during clotting is shown in Figure 11. The reaction was run at approximately 10^{-4} M free Ca^{2+} and 1.37×10^{-4} M fibrinogen concentration, with increasing concentrations of the inhibitor. The recordings show a drastic reduction of the Ca^{2+} uptake, with little apparent change in the rate.

Discussion

Calcium-selective electrodes could satisfy ideally the requirements for measuring binding of Ca^{2+} to proteins and would allow kinetic analysis of changes that are manifested in shifts of the binding equilibria. They would be equivalent in this respect to the glass electrodes used in H^+ binding studies. Although they were used extensively for measuring free Ca^{2+} concentrations in various biological fluids, their use for the above-mentioined purposes appears to have been limited. The reasons for this were the drawbacks of the early electrodes available. These were slow in equilibration or did not equilibrate at all, and interference from other ions was appreciable. Newer electrodes seem to have overcome to a large extent all these shortcomings (Simon et al., 1978). Gallagher and Lauffer (1983) reported careful studies of Ca^{2+} binding to tobacco mosaic virus protein and found the results in agreement with those obtained with other methods. The response of the electrode was nearly ideal also when Ca^{2+} titration of EDTA was investigated. In the latter case, this was almost instantaneous; however, in the presence of protein, it was sluggish, prolonged sometimes to 30 min. Our studies showed that the response time of the Radiometer electrode used, even at high protein concentration, was practically the same as the mixing time, provided the reference electrode was functioning properly.

The effect of Ca^{2+} on the various factors involved in the activation of prothrombin is of no concern for the present subject. However, Ca^{2+} effects on the conversion of fibrinogen into fibrin and on the quality of fibrin clot are also numerous and were the subject of numerous papers. It is beyond the scope of this discussion to review these. They were investigated as a rule at millimolar concentrations of Ca^{2+} and compared with the same properties of the usual preparations of fibrinogen that contained one to two Ca^{2+} bound to the high-affinity sites. Our attention was focused on Ca^{2+} bound upon polymerization in the range from 10^{-5} to 10^{-3} M free Ca^{2+} concentration. The results of these studies are summarized in Figure 3, in particular in the difference curve of calcium binding of fibrin vs fibrinogen. The latter suggests that binding associated with polymerization is the result of the shift of the affinity of some weaker Ca^{2+} binding sites to a higher value. The difference curve cannot be generated mathematically because the parameters of the Ca^{2+} binding curves are uncertain. However, by nonlinear least-square curve fitting of the difference curve, the number of sites could be varied from one to five with little change in the accuracy of the fit. In the same time, the gap between the original and the shifted pK narrowed from 3.7–4.9 to 4.2–4.6. The best fit, although only marginally so, appeared to be for two sites with pK shift from 4.0 to 4.6. This could be a slightly better estimate than the one obtained visually from the difference curve. Moreover, the shifted pK is close to the value of 4.2, found for the groups governing the conformational change associated with clotting (Donovan & Mihalyi, 1985).

Kinetics of the Ca^{2+} uptake associated with clotting do not follow a simple course. A lag period is observed, common also with other aspects of the coagulation phenomena, as, for example, fiber formation and the associated increase of turbidity, or release of fibrinopeptide B. This behavior betrays a multistep process. A detailed kinetic analysis of the reactions observed in the present studies will be given in the following paper (Mihalyi, 1988). Here this is limited to the estimation of half-reaction times, whose reciprocals are a relative measure of the rates involved in these phenomena, as long as the rate and equilibrium relationships of the various processes are not affected by the changing conditions. The reciprocal half-time was proportional to thrombin concentration. This suggests that the rate-limiting step in these reactions is the action of thrombin. This is borne out also by the fact that reaction curves obtained at various thrombin concentrations are nearly perfectly superimposable if the time scale is expanded in proportion to the enzyme concentration. Intermediate steps connected to the various aspects of the polymerization certainly occur, but under the conditions of these experiments, they are much faster than the action of thrombin critical for the Ca^{2+} uptake. Thrombin dependence of the rate of calcium uptake was furthermore identical with that of the conformational change observed during clotting by differential scanning calorimetry (Donovan & Mihalyi, 1985). Thus, for both the Ca^{2+} uptake and the conformational change, the same action is the rate-determining factor. Since we have failed to detect release of any other peptide but the classical fibrinopeptides A and B, within the time frame of the clotting reaction, the above-mentioned changes should be related also to the release of fibrinopeptide A, or B, or both. A choice from these possibilities can be made on the basis of the different kinetics of the release of the two fibrinopeptides. The experiments depicted in Figures 8 and 10 produced convincing evidence that release of fibrinopeptide B is the critical event. Release of fibrinopeptide A occurs before the Ca^{2+} uptake reaction, largely during the lag period of the latter, as shown in these two figures. Calcium uptake and release of fibrinopeptide B, on the other hand, follow strictly the same reaction course.

Binding of Ca^{2+}, although parallel to release of fibrinopeptide B, still follows release of firbinopeptide A and polymerization, as shown in Figure 10. Therefore, one cannot conclude from these experiments that these two events do not contribute to the Ca^{2+} binding associated with clotting. Experiments with the *Agkistrodon rhodostoma* venom coagulase, an enzyme that removes specifically fibrinopeptide A (Ewart et al., 1970), confirmed the necessity for removal of fibrinopeptide B for the reactions studied here. The action of the enzyme is followed by polymerization into a fibrin gel similar

to if not identical with that produced by thrombin (Hantgan et al., 1980). This, however, was not associated either by increase in Ca^{2+} binding, as shown in this paper (Figure 9), or by coformational change, as described in a previous paper (Mihalyi & Donovan, 1985). Thus, removal of fibrinopeptide A and polymerization are not involved directly in the above two reactions, although they may provide some necessary change before these are triggered by removal of fibrinopeptide B.

The tetrapeptide Gly-Pro-Arg-Pro is a powerful inhibitor of the polymerization of fibrin monomers. The inhibition is caused presumably by binding selectively to the fibrin binding sites situated on the D domain of fibrinogen (Laudano & Doolittle, 1980). Parallel with the inhibition of polymerization, the release of fibrinopeptide B is depressed while that of fibrinopeptide A remains unaffected (Hurlet-Jensen et al., 1982; Lewis et al., 1985). Lewis et al. completed their studies with a detailed mathematical analysis of the equilibria and rates involved. Both studies concluded that fibrinopeptide B release is greatly enhanced by polymerization, but occurs at an appreciable rate also from nonpolymerized fibrin monomers. These experiments were performed at very low fibrinogen concentrations of 0.56 and 0.10 mg/mL, respectively, with the tetrapeptide in a large molar excess. In our experiments, the tetrapeptide concentration was in the same range, but because of the high fibrinogen concentration, it was only in a 4–20-fold molar excess. Nevertheless, their curves of release of fibrinopeptide B show a striking resemblance to the EMF shift curves obtained in our experiments. In the latter, the range of effective peptide concentrations is slightly higher, that is explicable by the no longer negligible decrease of free peptide concentration by binding to fibrinogen. It is also noticeable that the EMF curves show little effect of the tetrapeptide on apparent rate, but a definite tendency of the Ca^{2+} uptake to level off at progressively lower values.

Because the Ca^{2+} uptake is a secondary event and the binding data are obtained by a rather complicated conversion of the experimentally determined ΔEMF values, we did not embark on extensive studies to make a detailed mathematical analysis feasible. The qualitative information obtained suggests the role assigned to fibrinopeptide B release in the Ca^{2+} binding, but is equally unable to clarify if either release of fibrinopeptide A or polymerization has any involvement in these processes beyond their effect of promoting release of fibrinopeptide B. Future work with the copperhead snake venom coagulase that removes under certain conditions fibrinopeptide B selectively (Shainoff & Dardik, 1979) may answer this question.

The data presented here and other data borrowed from the literature provide a sufficient basis to reconstruct the sequence of the complex events of fibrin formation. The primary event is obviously the removal of the two firbinopeptides, but each one of these triggers a different set of events. Release of fibrinopeptide A, at moderate enzyme concentrations, is rate limiting for the polymerization of desA-fibrinogen molecules. Removal of the peptide and the ensuing polymerization concurrently increase greatly the susceptibility of the peptide bond cleaved for release of fibrinopeptide B. The latter then is followed by Ca^{2+} uptake and conformational changes within the polymer. Within the time resolution of these experiments, it is not possible to say which is the first one of the latter two. Some conformational change, betrayed by a nearly 50% increase of ΔH of unfolding, occurs without, but an increase of the transition temperature by about 12 °C is apparently governed by binding of Ca^{2+} in the reaction described in this paper. Still, one cannot decide whether there is a Ca^{2+}-dependent conformational change or a conformation-dependent binding of Ca^{2+}. At any rate, under physiological conditions, there is an ample supply of Ca^{2+} for all these reactions to occur.

Polymerization is also a two-step process as first proposed by Ferry (1954) and later elaborated by Hermans and collaborators (Hantgan & Hermans, 1979) and others. First, by end-to-end polymerization of desA-fibrinogen, two-stranded protofibrils are formed. These associate laterally into thick fibers in the second step. The two steps occur with desA-fibrinogen, as seen, for example, with clotting with Ancrod, but the lateral association is slow. This is accelerated greatly by removal of fibrinopeptide B from the building blocks of the protofibrils (Hantgan & Hermans, 1979). In our experiments, as shown in Figure 10, fiber formation follows with some delay removal of fibrinopeptide A, but in the final stages of the reaction seems to close ranks with this. The initial lag period certainly corresponds to the time necessary to build up sufficient numbers of the protofibrils which then start forming the fibers. Further delay is caused probably by the sluggishness of the lateral association of the protofibrils which are built mostly of monomers with only fibrinopeptide A removed. This situation is corrected later on as removal of fibrinopeptide B from monomers both free and in the protofibrils becomes more and more prevalent and consequently the rate of lateral association is increased.

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REFERENCES

Blombäck, B., Hessel, B., Hogg, D., & Therkildsen, L. (1978) *Nature (London) 257*, 501–505.

Donovan, J. W., & Mihalyi, E. (1985) *Biochemistry 24*, 3434–3443.

Ewart, M. R., Hatton, M. W. C., Basford, J. M., & Dodgson, K. S. (1970) *Biochem. J. 118*, 603–609.

Fenton, J. W., II, & Fasco, M. J. (1974) *Thromb. Res. 4*, 809–817.

Ferry, J. D., Katz, S., & Tinoco, I., Jr. (1954) *J. Polym. Sci. 12*, 509–516.

Gallagher, W. H., & Lauffer, M. A. (1983) *J. Mol. Biol. 170*, 905–919.

Hantgan, R. R., & Hermans, J. (1979) *J. Biol. Chem. 254*, 11272–11281.

Hantgan, R., Fowler, W., Erickson, H., & Hermans, J. (1980) *Thromb. Haemostasis 44*, 119–124.

Horne, M. K., III (1985) *Thromb. Res. 37*, 201–212.

Hurlet-Jensen, A., Cummins, H. Z., Nossel, H. L., & Liu, C. Y. (1982) *Thromb. Res. 27*, 419–427.

Laudano, A. P., & Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3085–3089.

Lewis, S. D., Shields, P. P., & Shafer, J. A. (1985) *J. Biol. Chem. 260*, 10192–10199.

Marguerie, G., Chagniel, G., & Suscillon, M. (1977) *Biochim. Biophys. Acta 490*, 94–103.

Martinelli, R. A., & Scheraga, H. A. (1979) *Anal. Biochem. 96*, 246–249.

Mihalyi, E. (1968) *Biochemistry 7*, 208–223.

Mihalyi, E. (1988) *Biochemistry* (following paper in this issue).
Mihalyi, E., & Donovan, J. W. (1985) *Biochemistry 24*, 3443-3448.
Nieuwenhuizen, W., van Ruijven-Vermeer, I. A. M., Nooijen, W. J., Vermond, A., Haverkate, F., & Hermans, J. (1981) *Thromb. Res. 22*, 653-657.
Shainoff, J. R., & Dardik, B. N. (1979) *Science (Washington, D.C.) 204*, 200-202.
Simon, W., Ammann, D., Oehme, M., & Morf, W. E. (1978) *Ann. N.Y. Acad. Sci. 307*, 52-70.

Clotting of Bovine Fibrinogen. Kinetic Analysis of the Release of Fibrinopeptides by Thrombin and of the Calcium Uptake upon Clotting at High Fibrinogen Concentrations†

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ABSTRACT: Kinetic data on the release of fibrinopeptides A and B from bovine fibrinogen by human thrombin were obtained at high fibrinogen concentrations, within the 0.8–8.8% range (0.227×10^{-4} to 2.60×10^{-4} M), at 25 °C, pH 7.26, 0.30 ionic strength, and 10^{-4} M free Ca^{2+} concentration. Release of fibrinopeptide A followed strictly first-order kinetics at all concentrations, in spite of the fact that the highest concentration was 26 times larger than the value of K_M found in the literature. This behavior can be explained by inhibition of thrombin by the reaction products, with $K_I = K_M$. The equation describing the course of the reaction under these conditions can be rearranged into a linear relationship between $1/k_{obsd}$ and substrate concentration. The slope of the line is equal to $1/k_{cat}$ and the intercept to K_M/k_{cat}. The data points fell accurately on a straight line, and with the parameters of the latter, k_{cat} and K_M were calculated as $(6.3 \pm 0.11) \times 10^{-10}$ M s^{-1} (unit of thrombin)$^{-1}$ L^{-1} and $(11.0 \pm 3.0) \times 10^{-6}$ M, respectively. These values agree well with those found in the literature. Release of fibrinopeptide B follows complex kinetics. Higgins et al. [Higgins, D. L., Lewis, S. D., & Shafer, J. A. (1983) *J. Biol. Chem. 258*, 9276-9282] suggested that it can be described as the result of two consecutive reactions, the first one being the release of fibrinopeptide A and the second one of fibrinopeptide B from those molecules that have already lost fibrinopeptide A in the previous step. An alternate model was also considered which allowed removal of fibrinopeptide B directly from the native molecule, running parallel to the consecutive reactions. These and several other mathematical models were tested by nonlinear least-squares fitting to our experimental data. Best fit was obtained with the model of two consecutive first-order reactions. Release of fibrinopeptide B from the native molecules, i.e., without previous removal of fibrinopeptide A, if at all possible, was nearly an order of magnitude slower. The rate constants for the release of fibrinopeptide B with the two consecutive reactions model were used as with fibrinopeptide A to obtain k_{cat} equal to $(3.5 \pm 0.2) \times 10^{-10}$ M s^{-1} (unit of thrombin)$^{-1}$ L^{-1} and K_M equal to $(6.0 \pm 8.5) \times 10^{-6}$ M. Data points for fibrinopeptide B were more scattered than those for fibrinopeptide A and hence the uncertainty of K_M. Uptake of calcium associated with the clotting reaction followed the same mechanism as the release of fibrinopeptide B, and the rates of the two processes were also similar.

Determination of the time course of the release of fibrinopeptides A and B was presented in the preceding paper (Mihalyi, 1988) to correlate the conformational change and calcium uptake during clotting of fibrinogen by thrombin with release of fibrinopeptide B. Similar data, however, may contribute also to the resolution of the controversy regarding the kinetics of release of the two fibrinopeptides. Therefore, the experiments were expanded and are reported in the present paper.

Detailed kinetic analysis of this process has been undertaken by several authors. The initial fibrinogen concentration was an order of magnitude below the K_M of the thrombin-catalyzed removal of the fibrinopeptides from fibrinogen in some of these studies (Higgins et al., 1983; Lewis et al., 1985) and at about the value of K_M in others (Martinelli & Scheraga, 1980; Hanna et al., 1984). Moreover, in the latter case, data were restricted to the initial phase of the reaction, before clot formation. Our data were obtained under conditions in which the substrate was partially or nearly completely saturating the enzyme, the initial fibrinogen concentration in some runs being up to 26 times higher than K_M, and the whole course of the reaction was followed. From these data, some conclusions can be drawn about whether thrombin releases fibrinopeptide B from native fibrinogen, as suggested by the studies of Martinelli and Scheraga (1980), or only after prior removal of fibrinopeptide A, or after the polymerization ensuring the release of fibrinopeptide A, as suggested by Blombäck et al. (1978) and Shafer and collaborators (Higgins et al., 1983; Lewis et al., 1985). A puzzling observation was the strict adherence in all our kinetic curves to first-order kinetics, in spite of the high fibrinogen concentration that would have mandated zero-order kinetics. A possible explanation for this is also offered in this paper.

† This is paper 4 in the series "Clotting of Fibrinogen". For paper 3, see Mihalyi (1988).