Changes in pH Associated with Clotting of Fibrinogen. Kinetic Studies of the pH Shift and Correlation of the pH Change with the Release of Fibrinopeptides and the Ensuing Polymerization†

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ABSTRACT: The effect of the initial pH and the concentrations of thrombin, fibrinogen, and Ca^{2+} upon the rate of pH change associated with clotting of bovine fibrinogen by human thrombin was investigated at pH 6.80, 7.80, and 8.80, 0.3 ionic strength, 25 °C, and 19.5 mg/mL final fibrinogen concentration. At pH 6.80 and 7.80, the reaction was first order, with rate constant k_1. At pH 8.80, a first-order reaction of the release of H^+ (k_1) was followed by a partial rebinding of these in a reaction consecutive to the first one (k_2). At each of the above pH values, k_1 was proportional to thrombin concentration in the 0.05–3.0 min^{-1} range investigated. The k_1 constants were 0.111 ± 0.001, 0.250 ± 0.005, and 0.190 ± 0.002 min^{-1} (NIH thrombin units)$^{-1}$ mL^{-1} at pH 6.80, 7.80, and 8.80, respectively. Plots of log rate vs log thrombin concentration of these data were linear with slopes close to 1 at all three pH values. The rate of the second reaction (k_2) was independent of both the thrombin and the initial fibrinogen concentration. The pH dependence of k_1 exhibited a bell-shaped curve that could be resolved into the effect of one group with a pK of 7.27 that increased the rate and another with a pK of 9.22 that decreased the rate. With constant thrombin concentration but varying fibrinogen concentration, plots of $1/k_1$ vs [fibrinogen] were linear, but the lines did not pass through the origin. From the slope and intercept, k_{cat} and K_M of the Michaelis–Menten equation could be calculated. The same parameters were obtained also from initial velocity vs [fibrinogen] plots. Values of k_{cat} were consistent and accurate; those of K_M were more scattered. K_M was (22.4–34.2) × 10^{-6} M at pH 6.80 and approximately 7 × 10^{-6} M in the pH 7.26–8.80 range. The latter value, pertaining to the release of H^+ ions, is in agreement with values in the literature for K_M of the release of fibinopeptide A by thrombin in the 7.4–8.0 pH range. The value of k_{cat} s^{-1} (unit of thrombin)$^{-1}$ mL^{-1} increases from 1.2 × 10^{-10} s^{-1} unit of thrombin^{-1} mL^{-1} at pH 6.80 to 2.46 × 10^{-10} at pH 7.80 and then decreases to 2.01 × 10^{-10} 10^{-1} (units of thrombin)$^{-1}$ mL^{-1} at pH 8.80. The k_{cat} values are significantly lower than those in the literature for the release of fibrinopeptide A. The difference may be caused by the higher ionic strength (0.3 vs 0.15). The amounts of H^+ liberated were 3.0, 1.9, and 3.0 equiv of H^+ mol of fibrinogen at the respective pH values. The pH shift was correlated with the release of fibrinopeptides and with polymerization, the latter monitored by the increase of turbidity. Release of fibrinopeptide A followed first-order kinetics; release of fibrinopeptide B followed a first-order reaction consecutive to release of fibrinopeptide A. At pH 6.80, the pH shift lagged behind the release of FPA, by a factor of 0.6. Most of the H^+ at this pH originates in the polymerization or changes following this. Therefore, the delay must occur in this phase. At pH 7.80, pH shift and release of FPA are simultaneous. There is no indication of contribution from release of FPB to the pH shift. The stoichiometry of H^+ released also supports this contention. The most likely explanation of this finding is an increase by the polymerization reaction of the pK of the αNH_2 groups formed by release of FPB. At pH 8.80, the pH shift again lags behind release of FPB; however, the reaction curve can be reconstructed accurately by assuming that FPA contributes fully, while FPB to about half the extent, to the pH shift. This places the pK of the groups produced by release of the latter to approximately 8.8. At all three pH values, the turbidity curves coincided or were slightly ahead of the FPA release curves, showing that the latter have a determining role in polymerization and FPB is not directly involved. The pH shift and release of fibrinopeptides was also investigated with bovine thrombin on bovine fibrinogen at the above 3 pH values. Significant differences were found between the human and the bovine enzyme.

Clotting of fibrinogen by thrombin is associated with a complex set of changes in hydrogen ion equilibria, resulting in a shift in pH (Mihalyi, 1954b; Mihalyi & Billick, 1963). A 2-fold approach was used in the studies reported in this paper to elucidate this phenomenon. First, a detailed kinetic analysis of the pH shift was performed. Then the correlation of the pH shift with the cleavage of peptide bonds by the action of thrombin was investigated. The latter for a long time was hindered by lack of an accurate and simple method to estimate the release of the fibrinopeptides. The advent of high-performance liquid chromatography (HPLC)[1] methods and their

† This is paper 5 in the series "Clotting of Fibrinogen". For paper 4, see Mihalyi (1988b).

[1] Abbreviations: HPLC, high-performance liquid chromatography; FPA, fibrinopeptide A; FPB, fibrinopeptide B; ΔpH, pH shift.

application to the estimate of fibrinopeptides (Martinelli & Scheraga, 1979) made the study of this correlation feasible.

The pH change also occurs in a pH range where the αNH_2 groups produced by the cleavage of peptide bonds are nearly completely in their charged form and consequently hydrogen ions are not liberated by the cleavage. Therefore, polymerization and/or conformational changes associated with clotting must also contribute to the pH change. The role of polymerization, or its absence, can be elucidated by comparing the development of turbidity with the kinetics of pH shift, although the former is a monitor of fiber formation, a more advanced stage of the clotting process, that is following the polymerization proper (Hantgan & Hermans, 1979).

Experimental Procedures

Materials

Fibrinogen. Bovine fibrinogen (Pentex, lot 28, 95% clottable) was obtained from Miles Laboratories, Inc., Research Products Division, Naperville, IL. This was freed of material precipitated in the cold at pH 6.5 by the method of Laki (1951). The preparation was dialyzed exhaustively in the cold against 0.3 M NaCl. Later it became apparent that the above commercial preparation could be used directly, only dialyzed against 0.3 M NaCl, with results identical with those with the purified material. The concentration of the fibrinogen solution was estimated from its optical density, using 15.06 for $E_{1\%/cm}$ at 280 nm corrected for turbidity (Mihalyi, 1968). The concentration was adjusted to 20 mg/mL by dilution with the solvent. Clottability of the preparations was 96–97%.

Calcium-free fibrinogen was prepared as described previously (Mihalyi, 1988a).

Thrombin. A highly purified preparation of human thrombin, lot 192, was obtained from Dr. John W. Fenton, II. From the stock solution proper dilutions were made with 0.3 M NaCl containing 0.1 mg/mL bovine serum albumin, the latter added to prevent losses of thrombin by adsorption to surfaces (Horne, 1985). The activity of the solutions was estimated from the first-order rate constant of the pH shift obtained under standard conditions: 19.5 mg/mL fibrinogen in 0.3 M NaCl, pH 7.80, 25 °C. The rate was a linear function of the thrombin concentration. With a standard human thrombin preparation, obtained from the Bureau of Biologics, Food and Drug Administration, Bethesda, MD, a specific rate constant, calculated with natural logarithms, of 0.250 min^{-1} (NIH unit)$^{-1}$ was obtained. This procedure proved more reliable than the generally used methods based on clot formation, mainly because it obviates comparison with standard solutions of fibrinogen and thrombin. Details of this method will be described elsewhere.

Bovine thrombin (purified) was obtained from Miles Inc., Diagnostic Division, Kankakee, IL, lot 13. The material was dissolved in 0.3 M NaCl, and the activity of the solution was determined by the pH shift method described previously.

The stability of the thrombin solutions in 0.3 M NaCl and 0.1 mg/mL bovine serum albumin was investigated by adjusting the pH of samples to 7.0, 8.0, and 9.0, incubating these, hermetically sealed, at 25 °C for 4 h, and determining the activity of aliquots at time intervals with the pH shift method. After an incubation of 4 h, the activity was unchanged at all three pH values tested. Thus, thrombin is stable under the conditions of the experiments described in this paper.

Methods

Recording of the pH Shift. This was performed with a system consisting of a Beckman Futura pH combination electrode (General Purpose Glass), part 39505, coupled to a Beckman SS Century pH meter (Model 76) operated on the expanded scale. The output from the pH meter was shunted to deliver ca. 1.5 mV/0.1 pH unit, and this was fed into a Kipp & Zonen Model BD 41-mV recorder operated on 5-mV range, with variable sensitivity. The sensitivity was adjusted to give a full-range deflection on the chart for the maximal pH shift expected. The reaction vessel and the upper portion of the electrode containing the reference electrode were surrounded by a jacket through which water of 25 ± 0.1 °C was circulated. The pH meter was standardized with Fisher certified standard buffer of pH 7.00. Then the recorder was calibrated by moving the needle of the pH meter in a negative direction with the buffer control knob in increments of 0.05 pH unit and recording the steps.

The reaction was performed in flat-bottomed 1.2-cm i.d. tubes. A sufficient amount of fibrinogen solution was pipetted into the vessel to cover the electrode to above the porous ring of the junction of the reference electrode. The tube was closed with a rubber stopper through which the glass electrode was inserted and which also accommodated gas inlet and outlet tubes. A continuous gentle stream of argon washed through 40% NaOH, 5% H_2SO_4, and water excluded CO_2 from the system. Additions were made by injection through the stopper with Hamilton microliter syringes. Stirring was provided by a magnetic Teflon-coated microstirring bar. The pH of the solution was adjusted to the desired value with CO_2-free NaOH, and after stabilization, the galvanometer of the pH meter was zeroed with the buffer adjustment knob and the recorder started. The reaction was started by injecting thrombin solution adjusted to the same pH and diluted to give a reasonable reaction time. When the reaction was biphasic, with a fast ascending phase followed by a descending slow one, two recorders were operated simultaneously, one with a fast and the other with a slower chart speed.

The response of the electrode system was checked by adding a suitable amount of 0.1 N HCl to either a buffer or a fibrinogen solution and recording the pH shift. A nearly instantaneous response was observed with 95% of the change within 3 s and stabilization in less than 15 s. This is the response of the glass electrode, but the stability of the system depends mainly on the liquid junction potential. This should stabilize in a few minutes when, for example, the electrode is transferred from a low into a high ionic strength buffer. An absolute requirement for the reliability of these experiments is a stable base line before thrombin addition and a stable asymptotic value after the reaction. As the liquid junction became clogged with precipitated, or clotted, fibrinogen, the equilibration time may have been extended up to 30 min. Once the junction was clogged, various methods suggested for unclogging were mostly unsuccessful. Some electrodes of the type indicated above were usable for up to 50 experiments, but newer models of the Beckman combination electrodes gave much poorer performance and may necessitate the use of the more cumbersome separate reference electrodes with renewable liquid junction.

Release of Fibrinopeptides. Two procedures were employed: (1) Samples of 1 mL of fibrinogen (20 mg/mL) were pipetted into flat-bottomed, open-ended 1.2-cm i.d. tubes thermostated to 25 °C. Contamination by atmospheric CO_2 was prevented as it was described in the preceding section. The pH was adjusted to the desired value with 0.1 N HaOH, and after a stable base line was obtained, the reaction was started by injecting a thrombin solution of the appropriate concentration. The pH shift was recorded as described in the preceding

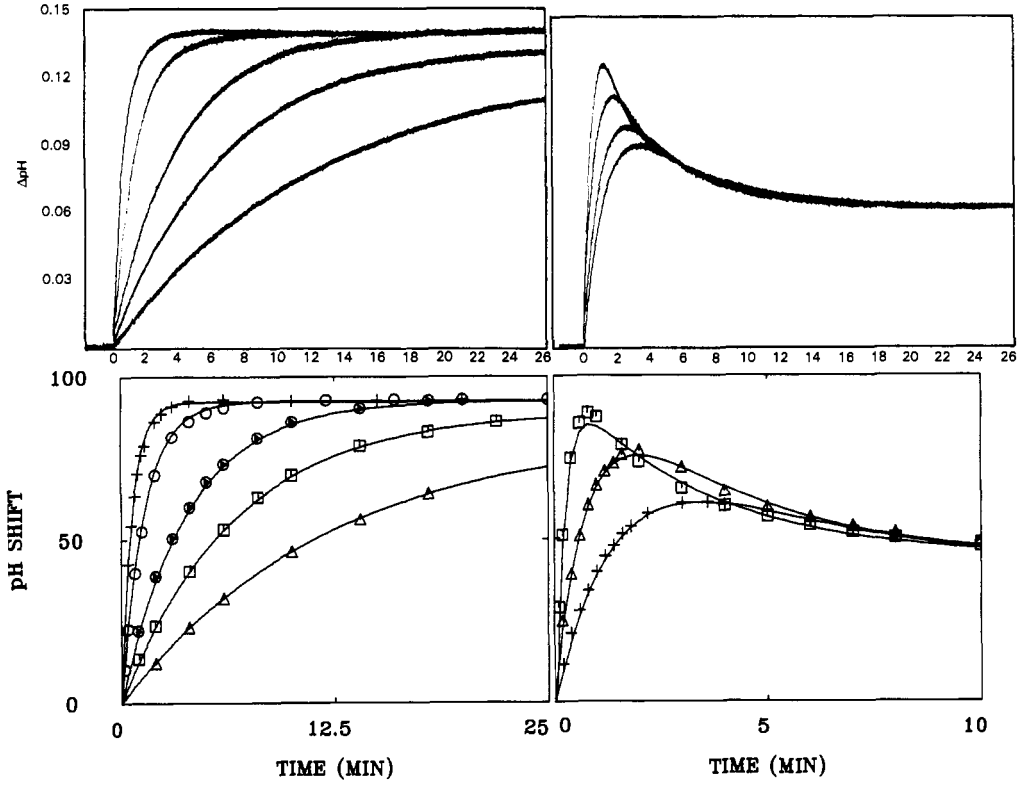

FIGURE 1: Recordings of the pH shift with constant fibrinogen concentration and varying thrombin concentrations at pH 7.80 in the left half of the figure and with constant thrombin concentration and varying fibrinogen concentrations at pH 8.80 in the right half of the figure. Below the recordings are shown curve fittings with eq 1 at pH 7.80 and with eq 2 at pH 8.80. Experimental points, read on the curves, are denoted by various symbols; the calculated curve is depicted with a solid line. Time scale expanded for fitted curves and curve 3 omitted at pH 8.80 to avoid overcrowding. Reaction conditions for pH 7.80: fibrinogen concentration, 19.5 mg/mL; thrombin concentratins, 5.580, 2.790, 0.930, 0.558, and 0.279 NIH units/mL for the successive curves. Reaction conditions for pH 8.80: thrombin concentration, 11.08 NIH units/mL; successive fibrinogen concentrations, 15.95, 39.91, 59.60, and 79.25 mg/mL. For both pH values: 25 °C, 0.30 ionic strength.

paragraph. After a given time interval, the pH electrode was removed, and the reaction vessel was rapidly transferred into a boiling water bath for 7 min. Coagulation was apparent after about 45 s. The coagulae were transferred into microfuge tubes and centrifuged at high speed in the Eppendorf microfuge for 10 min. The supernatants contained no protein detectable by trichloroacetic acid precipitation and were used directly, without filtering or dilution, for the HPLC[1] runs. The whole procedure was repeated for each point along the reaction path. The pH shift recordings were all superimposable and the points provided by the end points of the recordings fell on the same curve. (2) The above procedure was simplified to conserve time and material. The pH of a larger volume of fibrinogen solution was adjusted to the desired pH, and aliquots of 0.5 mL were pipetted directly into the microfuge tubes. These were capped immediately and placed in a 25 °C water bath. Thrombin solution was injected, and the tubes were incubated at 25 °C for timed intervals after which they were rapidly transferred into the boiling water bath. From here on, they were treated as above. The pH shift for the whole reaction course was recorded in a separate experiment.

Estimation of the fibrinopeptides by HPLC was performed with the equipment and procedure described previously (Mihalyi, 1988b). Recovery of the fibrinopeptides from heat-coagulated fibrinogen was checked by adding various amounts of the peptides to fibrinogen and heat-coagulating the samples. Recoveries in the supernatants of 97–100.5% showed that these are not adsorbed by the coagulated protein.

Optical Density Recordings. These were performed as described previously (Mihalyi, 1988a) with a Zeiss PMQ II spectrophotometer whose output was fed into a linear-log converter and from there into the Kipp-Zonen recorder.

Mathematical analysis of the pH shift recordings, fibrinopeptide release, and optical density increase curves was performed by curve fitting to various mathematical models by the MLAB interactive computer program developed at NIH (Knott, 1979).

Results

Kinetic Analysis of the pH Shift

Effect of pH. The experiments reported in this paper were performed at pH 6.80, 7.80, and 8.80. Data at pH 7.26 were borrowed from a previous paper (Mihalyi, 1988b). Two types of pH shift curves were described in previous publications (Mihalyi, 1954b; Mihalyi & Billick, 1963): one that is observed below approximately pH 8 and the other above this pH. These are illustrated in a series of recordings obtained at pH 7.80 and 8.80 in Figure 1, top two quadrants. The curves of the first type can be described by the equation of a first-order process and those of the second type by an equation that is the expression for the difference of a first-order reaction and of a second first-order reaction consecutive to the first one. These equations are

$$\Delta\text{pH} = A[1 - e^{-k_1(t+C)}] \quad (1)$$

$$\Delta\text{pH} = A[1 - e^{-k_1(t+C)}] - B\left[1 - \frac{k_2}{k_2 - k_1}e^{-k_1(t+C)} + \frac{k_1}{k_2 - k_1}e^{-k_2(t+C)}\right] \quad (2)$$

A and B in the second equation correspond to the final pH shifts connected to the first and to the second reaction if these could be measured independently. The quantity actually measured is their difference, and as with all differences, es-

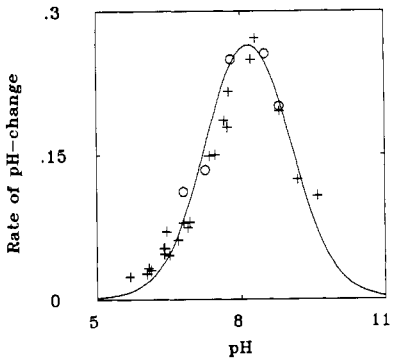

FIGURE 2: Rate of pH change per unit of thrombin plotted against pH. (+) Data taken from Mihalyi and Billick (1963); (O) data obtained in the present studies. Solid curve calculated by curve fitting with eq 4. Reaction conditions: 19.5 mg/mL fibrinogen, 25 °C, 0.30 ionic strength.

timates of the magnitude of A and B are uncertain to some extent.

The correction factor C, that corrects for the uncertainty of the starting point of the recordings, was close to zero at all three pH values, because electrode response and mixing times are negligible compared to the time course of the reaction. In order to compare the course of the various reactions, they were all normalized to 100 with the aid of the respective asymptotic values.

Fitting the recordings with the corresponding equations is shown in the lower two quadrants of this figure. The fit was good in all cases, and it was worse if other more complicated reaction mechanisms than those expressed by the above equations were tested. The average standard error of the rate constants obtained with all the curves with eq 1 was ±5.0% at pH 6.80 and ±2.3% at pH 7.80. Those of the two opposing reactions at pH 8.80 were slightly larger, ±6.8% and ±8.4%, respectively, except at the lower extremity of the range of rates, where $k_2 - k_1$ approaches zero and consequently the expression for B becomes indeterminate. Since the experimentally determined quantity at pH 8.80 is the difference $A - B$, curve fitting permits some leeway in the magnitude of these terms, as long as their differences is kept constant. However, in the series of experiments where the rates were varied by changes in either thrombin or fibrinogen concentration, the average standard deviation was ±4.4% for A and slightly larger, ±8.4%, for B.

Equation 2 can be rearranged to give eq 3 (Mihalyi & Billick, 1963). This is the expression of two simultaneous first-order reactions with the rate constants identical with those

$$\mathrm{pH} = A - B - \left(A - B\frac{k_2}{k_2 - k_1}\right)e^{-k_1(t+C)} - B\frac{k_1}{k_2 - k_1}e^{-k_2(t+C)} \quad (3)$$

in eq 1 and 2, but with different coefficients for the exponential terms. Consequently, all the reaction curves above pH 8, fitted with eq 2, could be fitted just as well with eq 3; the rate constants were identical in both cases, and the A and B values were only slightly different, with their difference being the same with both equations. Thus, mathematically it is not possible to differentiate between the consecutive and the simultaneous model.

The rate constant k_1, calculated with eq 1 or eq 2 depending on the type of the curve, is plotted against pH in Figure 2. Data taken from an early publication [Figure 6 in Mihalyi and Billick (1963)], converted to the present units and corrected for differences in fibrinogen concentration, are also included in this figure. Inspite of the fact that human thrombin

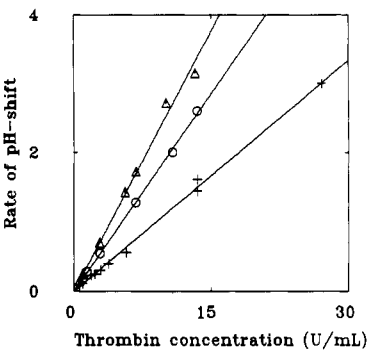

FIGURE 3: Rate of pH shift plotted against thrombin concentration. Successive curves from top to bottom at pH 7.80, 8.80 (k_1), and 6.80. Other reaction conditions as in Figure 2.

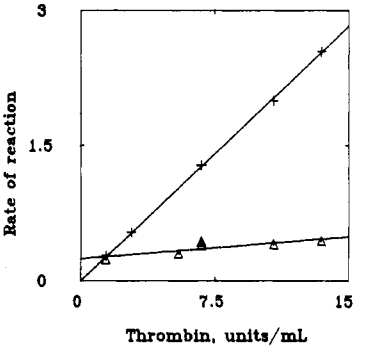

FIGURE 4: Rate constants k_1 and k_2 at pH 8.80 plotted against thrombin concentration. Reaction conditions as for Figure 2.

of high purity was used in the present studies and a much less pure bovine thrombin was used in the old experiments, the two sets of data agree very well. The data were fitted to eq 4 which

$$k_1 = N\frac{10^{pK_1-\mathrm{pH}}}{1 + 10^{pK_1-\mathrm{pH}}} - N\frac{10^{pK_2-\mathrm{pH}}}{1 + 10^{pK_2-\mathrm{pH}}} \quad (4)$$

was derived on the assumption of two ionizing groups governing the reaction, one with a lower pK increasing the rate and another with a higher pK decreasing the rate. The curve has the familiar bell shape characteristic for all serine proteinases, and the pK values found, pK = 7.27, pK = 9.22, are also in the same range as those of the other enzymes of this group.

Effect of Thrombin Concentration. At pH 6.80 and 7.80, a first-order reaction course was observed over the whole range of thrombin concentrations tested. The rate constants ranged from about 0.05 to 3 min^{-1}, the latter being close to the higher limit still manageable with the equipment used. These are plotted against thrombin concentration in Figure 3. The rate appears to be a linear function of the thrombin concentration at each of the two pH values, and the lines pass through the origin.

At pH 8.80, the curves obtained at various thrombin concentrations should have been superimposable if both k_1 and k_2 were proportional to the thrombin concentration and the time scale were expanded proportionally with the latter. Inspection of the recordings shown in the right panels of Figure 1 suggests that this is not the case. The results of curve fittings with either eq 2 or eq 3 confirmed this assumption. The rate constants obtained are plotted against thrombin concentration in Figure 4. It is apparent that k_1 pertaining to the ascending limb of the curves is proportional to the thrombin concentration whereas k_2 connected with the descending limb is practically independent of this. Consequently, the shape of the curves depends on the relative rates of the two reactions. The biphasic

Table I: K_M and k_{cat} at Various pH Values Calculated from the Dependence of the Rate of the pH Shift on Fibrinogen Concentration

	direct plot		reciprocal plot	
pH	$K_M \times 10^6$ (M)	$k_{cat} \times 10^{10}$ (s^{-1})	$K_M \times 10^6$ (M)	$k_{cat} \times 10^{10}$ (s^{-1})
6.80	34.2 ± 5.2	1.30 ± 0.05	22.4 ± 3.9	1.21 ± 0.01
7.26			4.7 ± 6.2	1.36 ± 0.05
7.80	15.3 ± 6.3	2.61 ± 0.13	7.5 ± 5.5	2.46 ± 0.08
8.80	8.3 ± 0.7	2.06 ± 0.03	7.2 ± 7.1	2.01 ± 0.09

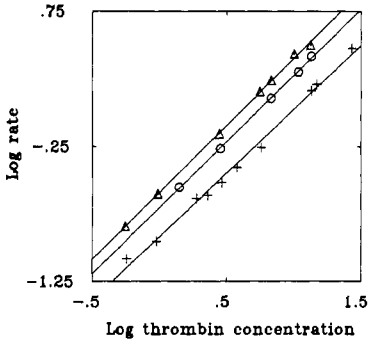

FIGURE 5: log rate vs log thrombin concentration plots of data shown in Figure 3. Successive lines at pH 7.80, 8.80, and 6.80.

character (a) is very sharp when the first reaction is much faster than the second one, (b) becomes less pronounced as the two rates approach each other, and (c) disappears entirely when the two rates are equal, leaving a straight first-order course. Further decrease of the rate of the first process, below that of the second one, results in progressively flatter curves, without regaining the biphasic character. Since only the first reaction, shifting the pH in an acidic direction, is connected to the action of thrombin, only k_1 of the pH 8.80 runs is plotted in Figure 3.

The slope of the lines obtained at all three pH values by least-squares fitting is given in the abstract. Similar data given in Table I are averages of three estimates each, encompassing a much narrower range.

A plot of log rate vs log thrombin concentration, with the same data, is shown in Figure 5. The slope of the linear plots is close to 1 at all three pH values, demonstrating that the process liberating the hydrogen ions, or the rate-limiting step in a more complex process, is first order with respect to thrombin.

Effect of Fibrinogen Concentration. Increasing the fibrinogen concentration with constant thrombin concentrations decrease the rate of pH shift in a nearly hyperbolic fashion at pH 6.80 and 7.80. The course of the reaction remains first order throughout the range of fibrinogen concentrations. Also, at a given pH the asymptotic value of the shift is the same regardless of the fibrinogen concentration. At any pH, however, the curves level off at a different height, which is determined by the buffering capacity of fibrinogen at this point and the amount of hydrogen ions liberated. At pH 8.80, the shape of the pH shift curves goes through the same metamorphosis with a decrease of the fibrinogen concentration as was seen with the increase of thrombin with constant fibrinogen concentration. Thus, the shape changes are the same, regardless of the cause that increases the rate of the ascending component of the reaction. The analysis of the curves shows that k_1 decreases hyperbolically with the fibrinogen concentration while k_2 remains constant. A plot of $1/k_1$ vs fibrinogen concentration at all three pH values is linear; however, the lines do not pass through the origin, as seen in Figure 6. From the slope and intercept of these lines, the Michaelis–Menten

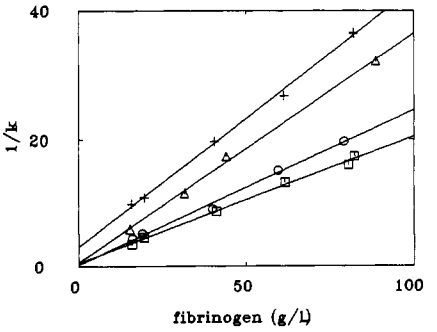

FIGURE 6: Reciprocal rate plotted against fibrinogen concentration. Rates were converted to 1 NHI unit/mL thrombin concentration. Lines from top to bottom at pH 6.80, 7.26, 8.80, and 7.80.

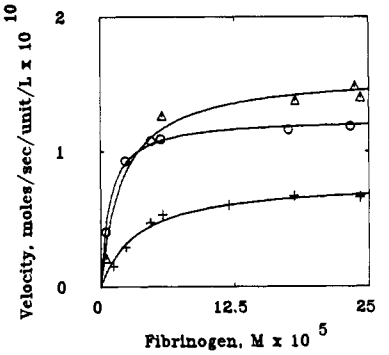

FIGURE 7: Initial velocity, given by the product of the first-order rate constant per units of thrombin per milliliter and initial fibrinogen concentration, plotted against initial fibrinogen concentration. Curves from top to bottom for pH 7.80, 8.80, and 6.80.

parameters, K_M and k_{cat} can be calculated with eq 5 [see Mihalyi (1988b)]. The results, given in Table I, show that

$$\frac{1}{k_{app}} = \frac{K_M}{k_{cat}} + \frac{1}{k_{cat}} S_0 \qquad (5)$$

K_M is high at pH 6.80 and decreases sharply and varies little between pH 7.26 and 8.80. The value of k_{cat} increases about 2-fold from pH 6.80 to 7.80 and then decreases somewhat at pH 8.80. The efficiency of thrombin, given by k_{cat}/K_M, is low at pH 6.80, because of the high K_M, and then increases about 6-fold at pH 7.26 and 7.80 and decreases again at pH 8.80.

The values of K_M and k_{cat} could be obtained also by plotting initial velocities of the reaction, given by the product of the first-order rate constant and the initial fibrinogen concentration, against fibrinogen concentration, as shown in Figure 7. The curves level off at saturation of the enzyme with the substrate. Curve fitting to the Michaelis–Menten equation gave the values of the K_M and k_{cat}. These are also listed in Table I. The errors are large with both fitting procedures, and in view of these, the agreement of the two sets of data is reasonable.

Effect of Calcium Concentration. Fibrinogen of 65.6 mg/mL concentration in 0.3 M NaCl, pH 7.80, was clotted with 8.2 units/mL thrombin in the presence of various concentrations of $CaCl_2$, and the pH shift was recorded. Free calcium concentration, determined with the Radiometer Ca^{2+} selectrode as described (Mihalyi, 1988a), varied from $<10^{-6}$ to 0.4×10^{-3} M. An increase of the Ca^{2+} concentration increased the asymptotic value of the pH shift approximately 2-fold, indicating that the slope of the hydrogen ion titration curve of fibrinogen becomes smaller, or the pK of the groups liberating hydrogen ions become lower. The pH shift curves were first order except at the highest calcium concentration used where a biphasic curve appeared. The latter suggests that the group (or groups) responsible for the second reaction,

Table II: Rate of FPA and FPB Release and of pH Shift with Bovine and Human Thrombin at Various pH Values

	human thrombin				bovine thrombin			
pH	range of [thrombin] (NIH units/mL)	k_{FPA}/unit	k_{FPB}/unit	$k_{\Delta pH}$/unit	range of [thrombin] (NIH units/mL)	k_{FPA}/unit	k_{FPB}/unit	$k_{\Delta pH}$/unit
6.80	0.95–3.82	0.200 ± 0.013	0.065 ± 0.002	0.120 ± 0.007	0.39–0.78	0.187 ± 0.007	0.024 ± 0.001	0.112 ± 0.006
7.80	0.38–0.79	0.255 ± 0.003	0.155 ± 0.010	0.254 ± 0.012	0.19–1.79	0.243 ± 0.001	0.079 ± 0.007	0.245 ± 0.015
8.80	1.70–6.83	0.268 ± 0.023	0.137 ± 0.011	0.197 ± 0.001	0.19–2.35	0.242 ± 0.025	0.103 ± 0.015	0.171 ± 0.012

binding protons at pH values higher than 8, also is affected by the Ca^{2+} ions. From the Ca^{2+} concentration range where these two effects appear (not shown), it is likely that the first one is connected to binding of Ca^{2+} to the high-affinity sites whereas the second one to binding to the low-affinity sites. The rate of the pH shift, on the other hand, was unaffected by the presence of Ca^{2+}. The clots at free Ca^{2+} concentration below 10^{-4} M were not cross-linked, whereas above this increasing cross-linking occurred by factor XIIIa. Since the rate remained unchanged, one may conclude that cross-linking does not affect the pH shift.

Release of Fibrinopeptides and Their Correlation with the pH Shift and Increase in Optical Density

The experiments were performed at pH 6.80, 7.80, and 8.80. The kinetic curve for the release of fibrinopeptide A was fitted to a first-order reaction course analogous to eq 1:

$$\mathrm{FPA} = A[1 - e^{-k_1(t+C)}] \quad (6)$$

That of fibrinopeptide B was fitted to a first-order reaction consecutive to the first one (Higgins et al., 1983; Mihalyi, 1988a):

$$\mathrm{FPB} = B\left[1 - \frac{k_2}{k_2 - k_1}e^{-k_1(t+C)} + \frac{k_1}{k_2 - k_1}e^{-k_2(t+C)}\right] \quad (7)$$

In these equations, A and B are the asymptotic values connected with release of FPA and FPB, respectively. The other parameters were defined previously in eq 1 and 2. The parameter C is now the time elapsed between sample collection and arrest of the reaction by heat coagulation. It is of the order of 0.45 min, no longer negligible. From a large number of experiments, the relationship $B = 1.506A$ was determined, which is the relationship between the molar absorptivities of the two fibrinopeptides at 206 nm, the wavelength of the detection. Because FPB is liberated more slowly than FPA, the reaction had to be continued beyond the complete liberation of FPA. Unambiguous results were obtained only if over 70–80% of FPB was liberated. Still acceptable data could be obtained with less complete reactions by constraining B to 1.506 A. However, A obtained with the constraint must be equal to that obtained with the FPA data fitted alone to eq 1.

At pH 6.80 and 7.80, the pH shift data were fitted as described under Kinetic Analysis of the pH Shift to eq 1 and at pH 8.80 to eq 2 or 3. The first-order relationship with respect to thrombin was obeyed with the data obtained at the first two pH values and with those of the first component of the biphasic curves.

The average values of the data obtained at pH 6.80, 7.80, or 8.80, with various concentrations of either human or bovine thrombin, are assembled in Table II. The rates of release of FPA and FPB are low at pH 6.80 and increase at the higher pH values to a nearly constant value, while the rate of the pH shift shows a clear maximum at pH 7.80. The rate of release of FPA is larger than that of FPB. Fibrinopeptide B is removed more slowly by bovine thrombin than by human

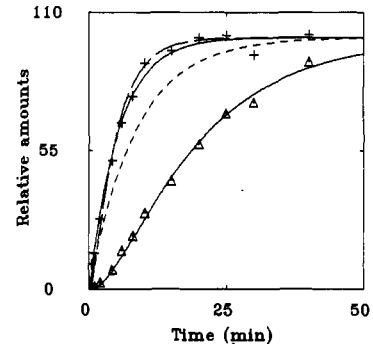

FIGURE 8: Clotting of bovine fibrinogen by human thrombin at pH 6.80. Release of fibrinopeptide A (+) and of fibrinopeptide B (Δ), the pH shift (---), and the increase in turbidity (--) plotted against reaction time. Reaction conditions: 19.5 mg/mL fibrinogen concentration; 0.943 NIH unit/mL thrombin concentration; 0.3 ionic strength; 25 °C. Solid lines, curves fitted to the experimental points with eq 6 for FPA and with eq 7 for FPB.

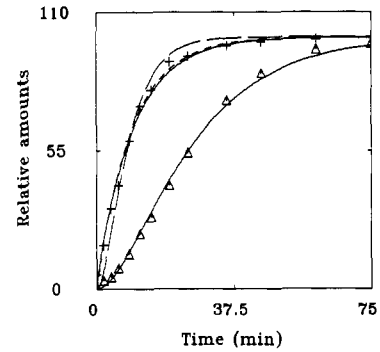

FIGURE 9: Clotting of bovine fibrinogen by human thrombin at pH 7.80. Thrombin concentration, 0.478 NIH unit/mL. All other reaction conditions and symbols are the same as in Figure 8.

thrombin from bovine fibrinogen. The difference between the two enzymes increases as the pH is lowered, as revealed by comparison of the ratios of the rates of release of the two fibrinopeptides. The rates of release of the fibrinopeptides and of the pH shift were proportional to the thrombin concentration.

The position of the pH shift curves with respect to that of the release of fibrinopeptides is different at pH 7.80 from that at pH 6.80 and 8.80. At pH 7.80, it coincides within experimental error with the curve of release of FPA, whereas at the other two pH values it is situated in between the curves of the release of fibrinopeptides. This is shown in Figures 8–10. At pH 8.8, the pH shift was resolved into its two components, and the ascending one, corresponding to release of hydrogen ions, was plotted with the release curves of FPA and FPB in Figure 10. At pH 8.80, the relative position of the pH shift and FPA release curves is the same whether human or bovine thrombin is used. Further, the ratio of the rates of these two phenomena is the same, approximately 0.6, regardless of the thrombin concentration.

The relationship of the turbidity increase during clotting to fibrinopeptide release and pH shift is shown also in Figures

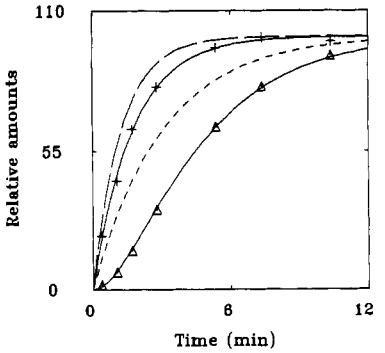

FIGURE 10: Clotting of bovine fibrinogen by human thrombin at pH 8.80. Thrombin concentration, 1.692 NIH units/mL. All other reaction conditions and symbols are the same as in Figure 8. pH shift and optical density increase curves were resolved by curve fitting with eq 2, and the first, fast, component of each curve is plotted in the figure (for fitting of the optical density, curve B should be positive).

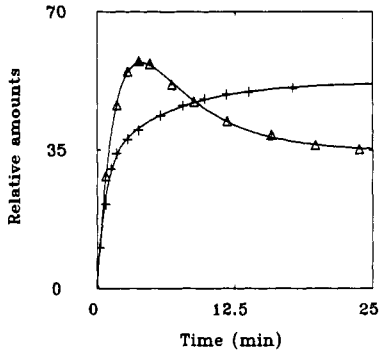

FIGURE 11: Complete reaction curves of the pH shift (Δ) and optical density increase (+) at pH 8.80 plotted against reaction time. Reaction conditions are the same as with Figure 10. Solid curves were obtained by curve fitting with eq 2 to the experimental points.

8 and 9 at pH 6.8 and 7.8. It is apparent that the turbidity curves have a short induction period but, except for this, they are closely situated to the curve of release of FPA. An accurate description of the curves was obtained by using eq 2, with the slower reaction having a rate close to that of FPA release. At pH 6.8 and 7.8, the turbidity curves level off slightly before that of the release of FPA. At pH 8.8, however, the turbidity curve is definitely rising beyond this level. This is described by an equation analogous to eq 2, but with the sign of the second term also positive. The coefficients A and B correspond to the optical density increases associated with the two phases of the reaction. The first, faster, reaction corresponds to the FPA release and the second, slower, reaction to the negative phase of the pH shift curve. This relationship is shown in Figure 11, where the complete pH shift curve is fitted with eq 2 and the complete turbidity curve with the same equation but the sign of the second term changed from negative to positive.

The optical density increase at pH 6.80 was recorded at 675 nm and reached a final value of 0.855; at pH 7.80, recording was at 400 nm with an asymptotic value of 0.145; and at pH 8.80, it was at 390 nm with a final value of 0.193.

Discussion

In the first report of a change of pH during clotting of fibrinogen by thrombin, a hypothesis was also presented to explain the experimental observations (Mihalyi, 1954b). It was recognized in this that both the cleavage of peptide bonds by thrombin and the subsequent polymerization of the resulting fibrin monomers contribute to the change in bound or released hydrogen ions. The two effects were separated by subtracting from the overall change that attributed to the cleavage of peptide bonds, estimated by Mihalyi (1954a) as the difference of the titration of fibrinogen and fibrin. The result showed some groups liberating hydrogen ions with a pK of about 7.2 and other ones binding them with a pK of about 8.2. At pH 7.8, on the other hand, this analysis predicted that there should not be any change of the pH caused by the polymerization. These conclusions were fully justified by the experiments of Endres et al. (1966) of the pH shift connected with the polymerization of isolated fibrin monomers. An acidic pH shift was observed in the lower pH ranged investigated and an opposite one in the higher range, with no pH change at pH 7.6. These studies were performed in 1 M NaBr, whereas those of Mihalyi were in 0.3 M NaCl, and the different solvents are expected to cause moderate shifts in pK values and more important ones in rates.

The amount of hydrogen ions found to be liberated during clotting at lower pH was approximately equal to that observed at the higher pH range; therefore, it was an acceptable assumption that a mutual interaction of the groups involved caused this effect. A hydrogen bond was suggested, and the acceptor group was assumed to be uncharged imidazole, whereas the donor group an unionized amino or phenolic hydroxyl group (Mihalyi, 1954b). Various indirect evidence supported this assumption. Subsequently, the heat of polymerization of fibrin monomers in 1 M NaBr was determined by Sturtevant et al. (1955), and a detailed mathematical model was provided for both the calorimetric and the ionization changes based on hydrogen bond formation.

The three pH values of the experiments described in this paper were chosen on the basis of the interpretation of the pH shifts presented above. At pH 6.8, the amount of protons liberated during clotting is much larger than that expected from the cleavage of peptide bonds and the appearance of an αNH_2 group with a pK of 7.5. Therefore, most of the hydrogen ions should originate with the polymerization or secondary changes connected with the latter. At pH 7.8, polymerization does not produce shifts in hydrogen ion equilibria. Therefore, the change in pH must be caused entirely by the cleavage of peptide bonds by thrombin. Finally, at pH 8.8, the production of hydrogen ions is again ascribable solely to the enzymatic process, with the resulting absorption of protons to the ensuing polymerization reaction.

The pH shift reaction, from the lowest pH where it still can be observed to approximately pH 7.8, follows a first-order course. The rate under these conditions is a linear function of the thrombin concentration. At pH values higher than this, the reaction becomes biphasic: first, the pH shifts in an acidic direction, reaches a maximum, then reverts toward the original value, and levels off before reaching this (Figure 1). Kinetics of the biphasic reaction showed that the rate of the first phase is proportional to thrombin concentration whereas that of the second phase is independent of this (Figure 4). Thus, it appears that the single rate constant of the pH shift in the lower pH range and that of the first component of the shift above pH 7.8 are of first order with respect to thrombin concentration (Figure 3). The first-order relationship was confirmed by the log rate vs log thombin concentration plots shown in Figure 5.

The analysis of the descending limb of the pH shift curve at pH 8.80 shows that the rate of the reaction responsible for this is independent not only of thrombin concentration (Figure 4) but also of fibrinogen concentration (not shown). The first finding is understandable, since thrombin is not involved in the reactions following the enzymatic step. However, the fact that the reaction is independent also of fibrinogen concen-

tration is unexpected, because the rate of protofibril and of the subsequent fiber formation both increase linearly with the fibrin monomer concentration (Hantgan & Hermans, 1979). The lack of effect of the initial fibrinogen concentration indicates that this reaction is not connected to polymerization but presumably to a slow conformational change, or a rearrangement of the monomers in the already formed fibrin network, resulting in shifts in hydrogen ion equilibria. The fact that this reaction coincides with a further increase in turbidity, after the sharp rise corresponding to the initial gel formation, as shown in Figure 11, indicates that the alteration of the fibrin network is the more likely explanation. Also, this change is different from the one detected by differential scanning calorimetry (Donovan & Mihalyi, 1985), because the latter is dependent on the release of FPB, whereas this one is independent of the action of thrombin.

Assuming that the titration curve of fibrinogen is linear in the narrow interval of the pH shift, that is, 0.15 pH unit at the most, the stoichiometry of the release of hydrogen ions is given by the product of the pH shift and the slope of the titration curve at the midpoint of the shift. The latter was estimated from the titration curve of fibrinogen (Mihalyi, 1970), at 0.1 pH unit intervals. At pH 6.80, the buffer capacity of fibrinogen, dh/dpH, is 24.8, with dh expressed as equivalents of hydrogen ions bound per mole of fibrinogen. The average of the pH shift at this pH is 0.121; that with the above buffering capacity gives 3.0 equiv of hydrogen ions liberated per mole of fibrinogen. The newly formed αNH_2 groups of the two α-chains of the fibrin monomer, with a pK of 7.5, release 0.33 equiv, that is, some 11% of the total amount of hydrogen ions released (the contribution of the β-chains is negligible; see below). The remainder should originate with the polymerization process.

The pH shift followed a first-order course at pH 6.8 in spite of the fact that according to the above scheme it should have been composed of two consecutive reactions, each one of them delivering protons. Equation 2, with the sign of the second term changed, describes such a process, or its rearranged form, eq 3. The observed first-order course can be reconciled with this scheme if $k_2 \gg k_1$, i.e., if the cleavage of peptide bonds is the rate-limiting factor. This is a reasonable proposition, because both the polymerization of fibrin monomers and the following fiber formation are fast reactions (Hantgan & Hermans, 1979; Spellman et al., 1977; Lewis et al., 1985).

The pH shift at pH 7.8 has a straightforward mechanism. The small contributions, both positive and negative, of the steps following thrombin action cancel out [see Figure 4 in Mihalyi (1954b) and Figure 7 in Mihalyi and Billick (1963)], and only the direct release of protons by the enzymatic reaction is observed. Consequently, the reaction is first order, and the rate is a linear function of the thrombin concentration (Figure 3). Moreover, the log k_1 vs log thombin concentration plot is also linear, with a slope close to unity, showing that the reaction is first order with respect to thrombin. As shown in Figure 9, at this pH the release of FPA, the evolution of turbidity, and the release of protons follow closely parallel courses. Release of FPB produces no protons, and this may be explained by a shift to higher values of the pK of the αNH_2 group produced by cleavage of the peptide bond liberating FPB, by formation of hydrogen bonds, or by strong electrostatic effects. This contention is supported also by the stoichiometry of the hydrogen ions released. The slope, dh/dpH, at pH 7.8 is 13.6, and this with a ΔpH of 0.140 unit results in 1.90 equiv of protons released per mole of fibrinogen. If the pK of the αNH_2 group of the β-chain of fibrin is 8.8, as indicated below, the contribution of the release of FPB to this figure should be about 0.18 equiv. The difference should be further corrected, because the pK of the αNH_2 groups formed by thrombin was estimated as 7.5 (Mihalyi, 1954a). Correcting for this results in about 2.57 bonds cleaved per mole of fibrinogen. This is larger than corresponding to the two fibrinopeptides A liberated per mole, but the errors in the pK, ΔpH, and dh/dpH are all considerable and may account for the difference.

At pH 8.80, the contributions of the enzymatic and the postenzymatic steps are well separated, being of opposite signs. The ascending part of the pH shift curve is situated between the curves of the liberation of the fibrinopeptides (Figure 10). However, the stoichiometry again indicates less than four protons released per mole. With a dh/dpH of 20.7 and a ΔpH of 0.147, derived from the A values of eq 2 of computer fittings of the pH shift curves, the released protons amount to 3.0 per mole. Since the pH is 1.3 units higher than the assumed pK of the αNH_2 groups produced by the liberation of fibrinopeptide A, this value suggests that the former are fully titrated, wheres those originating from release of fibrinopeptide B are only about halfway; thus, the pK of the latter should be around 8.8.

With increasing fibrinogen concentration, at a constant thrombin concentration, the first-order rate constant decreased in approximately hyperbolic fashion. Reciprocal rates plotted against fibrinogen concentration were linear; however, the lines did not pass through the origin (Figure 6). From the slope and intercept of these lines, the Michaelis–Menten parameters, K_M and k_{cat}, were calculated.

The Michaelis–Menten parameters given in Table I show that the error of the K_M values is large and there is considerable difference between the data obtained with the two different plots. This is obviously the result of the small value of the intercept in the reciprocal plot with associated large relative errors, and of relatively few experimental points in the low fibrinogen concentration range critical for the estimates with the direct plot. It is clear, however, that K_M is large at pH 6.80 and then decreases sharply and remains nearly constant between 7.26 and 8.80. In contrast, the values of k_{cat} are accurate and identical with the two plots, as expected from the reliance of these on the better defined high fibrinogen concentration points. The value of k_{cat} increases 2-fold from pH 6.80 to 7.80, parallel to the changes of the rate shown in Figure 2.

All the data in Table I are for the process causing the change in pH. The pH shift is determined directly or indirectly by the release of fibrinopeptides. Thus, a comparison of these data with the Michaelis–Menten parameters determined for the release of fibrinopeptides by thrombin may be instructive. The more recent data in the literature (Martinelli & Scheraga, 1980; Higgins et al., 1983; Hanna et al., 1984) were obtained under different conditions. However, neither a change in temperature (25 or 37 °C) nor a change in pH (8.0 or 7.4), nor use of human or bovine materials, had an appreciable effect on these parameters; therefore, any difference between these and the experiments at pH 7.26 and 7.80 would not invalidate a comparison. However, there are no data concerning the effect of ionic strength. Thus, the latter may be the cause of significantly lower k_{cat} values obtained in these studies at 0.3 ionic strength than those at 0.15 ionic strength in all the above: 2.4×10^{-10} s^{-1} at pH 7.80 compared to $(7.3–8.3) \times 10^{-10}$ s^{-1} at pH 8.0 and 7.4, respectively. In our previous studies (Mihalyi, 1988b), K_M and k_{cat} were estimated for release of FPA at pH 7.26, and values of 11.0×10^{-6} M

and 6.3×10^{-10} s^{-1}, respectively, were reported. These data are at variance with the present results suggesting that the activity of the thrombin used might had been grossly underestimated. Recalculating K_M and k_{cat} with the corrected value results in 16.5×10^{-6} M and 2.4×10^{-10} s^{-1} for FPA and 3.2×10^{-6} M and 0.8×10^{-10} s^{-1} for FPB release, respectively. The parameters listed in Table I at pH 7.26 are for the pH shift recorded simultaneously with the peptide release reaction. The agreement between the two sets of data is poor, reflecting the large errors of these estimates. From the data in Table I, it would appear that K_M and k_{cat} are nearly constant in the 7.26–8.80 pH range. Taking this into account, the data for FPA release are more reasonable, being of the same magnitude as those for the pH shift. Unfortunately, the above data are the only ones we have for release of FPA under the conditions of the pH shift experiments. Also, with all the points at pH 7.26, the fibrinogen concentration was above that saturating the enzyme, precluding the use of the direct plot to estimate K_M and k_{cat}. It is, however, certain that the parameters for FPB release are significantly lower, indicating an absence of involvment of this process in the pH shift reaction at pH 7.80.

Finally, two characteristics of the pH shift reaction should be emphasized: (1) The reactions are always first order, or at pH values higher than 8 composed of two reactions of first order, inspite of the fact that the highest fibrinogen concentration used is more than 100 times higher than the K_M determined for the release of FPA. This peculiarity was discussed in a previous paper (Mihalyi, 1988b) and was explained by inhibition by the reaction products with the same association constant as K_M. (2) The asymptotic values of the pH shift varies with the initial pH, but at a constant value of pH, the shift is always of the same magnitude, regardless of the fibrinogen concentration. This is because the system does not contain an external buffer. The same fibrinogen molecule serves as the source of the hydrogen ions and as buffer. Since the ratio of the two is the same at any concentration of fibrinogen, the pH shift should be also the same, as long as the initial pH value is not extreme, or the concentration of fibrinogen too low, so that the ionization of water can no longer be neglected. Also, the presence of an external buffer will increase the total buffering capacity of the system and consequently decrease the magnitude of the pH shift. However, the kinetics of the reaction will not be affected, and the derived rate constants will be the same, unless there is a specific interaction between the buffer ions and fibrinogen or thrombin.

At pH 6.80, the pH shift lags behind the release of FPA, as shown by the data in Table II and Figure 8. Both obey first-order kinetics and a linear dependence of the rate constant on thrombin concentration. The latter for the pH shift was confirmed over a wider range of thrombin concentrations as shown in Figure 3.

The rate difference between FPA release and pH shift is not large in the realm of kinetic experiments, especially when the different events cannot be recorded in a single simultaneous experiment and also the technique used for each one of them is different. The fact that the ratio of the two rates is constant with different thrombin concentrations indicates that a systematic error in one against the other method of monitoring the reaction is not likely. Also, the absence of a lag period in the pH shift curves argues against the consecutive reaction scheme. Thus, the pH shift is not simultaneous with polymerization, but the linear dependence of thrombin concentration indicates that the shift is connected directly to the action of thrombin. One explanation of the slower rate could be that release both of FPA and of FPB in a secondary way, either through polymerization or through a conformational change following the release of the peptides, contributes to the pH shift. With equal contributions, this would place the pH shift curve in a midposition between the curves of release of the two fibrinopeptides. This is appreciably below that of the experimental pH shift curve, but decreasing the contribution of FPB release could bring the two curves together. Curve fitting with a single-exponential term would still be possible with not much larger error than with the composite function, and no lag period would appear. Release of FPB is followed by a structural realignment of the gel and a change in Ca^{2+} binding (Donovan & Mihalyi, 1985; Mihalyi, 1988a), and this process may result also in changes in hydrogen ion equilibria. This scheme is contradicted by a comparison of the reaction rates with human thrombin against those with bovine thrombin. According to the data given in Table II, the rate of release of FPB is 3 times slower with bovine than with the human enzyme. Nevertheless, the pH shift curves with both enzymes coincide. Also, the FPB release curve, especially with bovine thrombin, extends beyond the point where the pH shift curve levels off. Therefore, hydrogen ion release at this pH may be connected with some other slower changes following polymerization. Proving any of these is made difficult because of the mathematical uncertainties of fitting an experimental curve with a combination of exponential functions. The sum of two exponentials cannot be resolved with confidence uneless the rate constants differ by at least 5-fold, and with more complicated systems, the uncertainties are even larger.

The relationship of the pH shift to peptide release is much simpler at pH 7.8. As stated earlier, hydrogen ions are released at this pH exclusively by the cleavage of peptide bonds by thrombin. However, the release of protons coincides with that of FPA, and the release of FPB seems to have no effect, as shown in Figure 9. Also, formation of the fibrin fibers, reflected by the turbidity curves, falls together with the two previous ones. Thus, release of FPA at this pH is the rate-limiting factor of polymerization and the determining factor of the pH shift. The arguments invoked with respect to the contribution of FPB release to these changes at pH 6.8 can be applied in this case too: the pH shift and turbidity curves level off at or before the termination of FPA release, and the rate of pH shift is the same with human or bovine thrombin (see Table II), in spite of the fact that FPB is liberated more slowly by the latter.

The most reasonable explanation of the absence of release of protons by FPB release is an increase by the polymerization process of the pK of the resulting αNH_2 groups. The pK values of the NH_2 groups produced by thrombin on either the α- or the β-chains of fibrin are the same according to the difference of the titration curves of fibrinogen and fibrin (Mihalyi, 1954a). The titration, performed in 3.33 M urea which dissolved fibrin, actually pertains to the difference between fibrinogen and fibrin monomer. If the assumed mechanism is correct, the pK of the αNH_2 groups of the β-chain of the fibrin monomer is shifted to higher values by the polymerization, or by conformational changes associated with polymerization. This may be by either strong electrostatic interactions or hydrogen bonding. In either case, it will contribute to the stability of the polymerized system.

At pH 8.8, the pH shift curves become biphasic. These curves can be resolved unequivocally by eq 2, with the requirement that A is positive and B negative. The positive component is situated between the curves of release of FPA and FPB, a situation similar to that at pH 6.80. However, at pH 8.80, this may be a reflection of a combined effect of

both the release of FPA and the release of FPB. The pH shift curve can be reconstructed fairly accurately by assuming a stoichiometric release of hydrogen ions for each FPA released, and half of this for each FPB. Thus, the pK of the αNH_2 groups of the β-chain in fibrin should be at approximately 8.8. Some evidence for this assumption was given also by the stoichiometry derived from the pH shift experiments.

The descending limb of the pH shift curves above pH 8 was assigned in the original scheme to polymerization (Mihalyi, 1954b), and this was apparently confirmed also by the pH shifts observed in this pH range during polymerization of fibrin monomer (Endres et al., 1966). The present experiments cast doubt upon this assumption. It appears that the descending portion of the pH shift is the result of a conformational change, or a rearrangement of the fibrin gel, that occurs after the polymerization. This is evident in the turbidity recording shown in Figure 11. The turbidity development also follows a biphasic course, although the two compenents have the same sign. The first steeper part coincides with the ascending part of the pH shift curve, and this according to Figure 10 coincides with the release of fibrinopeptide A and also with polymerization. The second slower part, on the other hand, is a mirror image of the descending limb of the pH shift curve, and this should be a reflection of the conformational change, or reorganization of the fibrin network.

In conclusion, the opacity increase during clotting of fibrinogen by thrombin, at all three pH values investigated, seems to be closely related to release of FPA. Since this is a reflection of the formation of fibrin fibers, it appears that release of FPB is not involved in this process. In contrast, release of hydrogen ions has a different mechanism at each of these pH values. At pH 6.80, it follows with a slight delay release of FPA and fibrin formation. Given the pK of the newly formed αNH_2 groups, release of FPA is directly involved in this effect only to a limited extent. Most of the protons should originate in the polymerization and other reactions following this. At pH 7.80, there is a clear-cut correlation between the releases of FPA and the pH shift. At pH 8.80, on the other hand, the release of both FPA and FPB appears to contribute hydrogen ions. The second, negative component of the pH shift reaction at this pH is concurrent with a reorganization of the fibrin gel manifested by a slow increase of the turbidity following the fast rise connected with the polymerization.

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Registry No. Ca, 7440-70-2; H^+, 12408-02-5; thrombin, 9002-04-4.

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