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TRANSFORMATION OF FIBRINOGEN INTO FIBRIN

III. KINETICS OF THE pH CHANGE ASSOCIATED WITH THE CLOTTING OF FIBRINOGEN*

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SUMMARY

The kinetics of the pH change accompanying the transformation of fibrinogen into fibrin has been investigated at different initial pH values. From pH 5.5 to 8 the reaction follows first-order kinetics. Above pH 8, two reactions are apparent: one producing, the other absorbing hydrogen ions. This biphasic curve can be analyzed in terms of two consecutive, first-order reactions. It is concluded that the rate of the single reaction below pH 8 and of the first reaction above this pH is equal to the rate of the proteolytic step, whereas the rate of the second reaction above pH 8 is equal to the rate of the polymerization step.

INTRODUCTION

A comprehensive kinetic study of the conversion of fibrinogen into fibrin, under the action of thrombin (EC 3.4.4.13) is rendered difficult by the multistep nature of this process. In this reaction the sequence of events is briefly the following: an enzymic step of a well-defined chemical nature takes place first, which is then followed by the polymerization of the reaction products, through much less defined secondary forces, into a three-dimensional network. The polymerization very likely occurs in two stages, with an intermediate polymer appearing first***. The appearance of the insoluble fibrin at the end of the reaction makes the kinetic study of the overall reaction very easy, but at the same time imposes great technical difficulties on the isolated study of the first step, the direct action of thrombin upon fibrinogen.

A kinetic study of the enzymic step can be performed by: 1, determination of the peptidic materials released[2]; 2, estimation of the new N-terminal groups liberated by thrombin[3,4]; or 3, determination of the fibrin monomer produced by converting it quantitatively into fibrin-clot, after inhibition of the thrombin action with an

Abbreviations: TAME, tosylarginylmethyl ester; Ac-globulin, accelerator globulin of Seegers; NIH, National Institutes of Health, Bethesda, Md. (U.S.A.).

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*** Our present knowledge on this subject is summarized in SCHERAGA AND LASKOWSKI's recent review[1].

inhibitor which does not interfere with the polymerization of monomers already present[5,6]. The first two obviously would be the methods of choice, since they give a direct estimate of the thrombin action, however, they are very laborious and not very accurate. The third one, beside the numerous correction factors involved, suffers from our present uncertainty with respect to the relationship between thrombin action and polymerization[1]. Thus, it is now well established that thrombin splits off four peptides from the fibrinogen molecule[7,8], but it is not known which of the 24 possible species formed by removal of one, two, three, or four peptides are able to polymerize and which are not. A detailed discussion of the problems involved and of the results of kinetic measurements of the fibrinogen–fibrin transformation has been given by SCHERAGA AND EHRENPREIS[9].

In the present paper a kinetic analysis of the pH changes associated with conversion of fibrinogen into fibrin will be presented. This method gives a direct estimate of the hydrogen ions liberated, or taken up, during the overall reaction. However, the interpretation of the data is complicated by the fact that both the enzymic and the polymerization steps contribute to the pH change. Since our method is unable to distinguish between the two polymerization steps, the entire polymerization process will be treated subsequently as a single step. A detailed analysis of the final changes in hydrogen-ion equilibria, without considering the kinetics of these effects, has been presented already[10]. Together with the titration data pertaining to the first step[11], these experiments made it possible to formulate a reaction mechanism of the clotting process. In this paper it will be shown that on the basis of this mechanism a reasonable interpretation of the kinetics of the pH change is possible.

MATERIALS AND METHODS

Fibrinogen and thrombin

Fibrinogen was purified from Armour's Bovine Fraction I, lot 128-215 and I. 210, according to LAKI'S procedure[12], resulting in preparations which were 94–95% clottable, as estimated by the method of the same author[12]. The fibrinogen solutions were dialyzed exhaustively against 0.3 M KCl in the cold and clarified by high-speed centrifugation. Concentrations were determined by Kjeldahl-nitrogen estimations, on the basis of a nitrogen content of 16.7% (ref. 13) and ranged from 1.21 to 1.66%.

Thrombin (EC 3.4.4.13) was obtained from prothrombin purified from bovine plasma according to SEEGERS[14], by either citrate, or brain thromboplastin-Ac-globulin activation. Activity of the former, determined on standard fibrinogen solutions was 314 NIH units/mg, whereas of the latter was 137 NIH units/mg. There was no difference in the behavior of these two different preparations in the experiments described in this paper, therefore, no further distinction will be made between the two. In latter stages of this work the pH-shift reaction was standardized in terms of TAME-units[15], one unit being the amount of thrombin which hydrolyzes 0.1 μmole of TAME in one minute at pH 8.0 and 25° from 0.01 M TAME solution in 0.15 M KCl. All the rate constants were subsequently recalculated on this basis.

pH recording

Since the pH changes observed during the clotting reaction are small, of the order of 0.15 pH units or less, a very sensitive, and at the same time stable, electro-

meter was needed. Most of the experiments were performed with a Cambridge, Model R, pH meter, on which the line-operated power supply was disconnected and batteries substituted. With carefully selected tubes, this apparatus satisfied the above mentioned requirement. However, it proved very sensitive to external disturbances and very careful grounding and shielding of the entire system was essential. In later experiments a Cary, Model 31, vibrating-reed electrometer was used, which proved ideal in every respect. After reading the initial pH, the output of the electrode system was compensated with a dry-cell. When a measurable pH shift occurred, it was recorded as a deviation from this base line on a Leeds and Northrup Speedomax, Type G, Model S recorder, of 30 mV adjustable range and a response time of 1 sec full scale.

The electrode system consisted of a Beckman No. 280 General Purpose glass electrode and a saturated KCl–calomel electrode connected to the solution with a saturated KCl–agar bridge. The latter has given more stability, more reproducibility and less noise than the commercially available reference electrodes. A 30-ml beaker, surrounded with a jacket through which water at 25 ± 0.02° was circulated, served as the electrode vessel. Contamination by atmospheric CO_2 was avoided by blowing a current of air, washed successively through conc. H_2SO_4, 40% NaOH and water, over the solution. The whole electrode system was placed in a Faraday cage and also the components of the water circulating system were grounded.

Typical experiments were performed as follows: 10-ml samples of fibrinogen were pipetted into 25-ml stoppered Erlenmeyer flasks. To these, varying amounts of CO_2-free 0.05 N NaOH, 0.3 M KCl, or 0.05 N HCl, 0.3 M KCl solution were added and the volumes made up to 11 ml with 0.3 M KCl. All the solutions were prepared with CO_2-free glass-distilled water. Acid addition to fibrinogen resulted in floccules which redissolved very slowly. Therefore, acidification was avoided, as much as possible, and instead the pH of the stock fibrinogen solution was kept low, so that most of the samples necessitated alkali addition. The mixture was left in the refrigerator for approximately one hour, then warmed to room temperature and transferred into the reaction vessel. Sufficient time was allowed for temperature equilibration and for obtaining a stable base line. After the pH was read, 0.2 ml of thrombin solution of approx. 66 TAME units/ml was blown in, with a serological pipette, and the solution quickly mixed by shaking the whole reaction vessel. After this, the mixture was left standing and the pH recording continued until a final stable value was attained. In most of the experiments the final fibrinogen concentration was 1.37% and the final thrombin concentration 1.18 TAME units/ml.

It was essential to clean all glassware with extreme care, in order to avoid spontaneous pH drifts. Cleaning with hot chromic acid solution was used, followed by thorough rinsing and then boiling twice in large volumes of distilled water.

The electrode system was standardized with 0.05 M potassium hydrogen phthalate, to which a pH value of 4.00 was assigned at 25°. The standardization was repeated at the end of a series of recordings and no significant drift was ever observed. From time to time, the pH response of the glass electrode over a wider range was also checked with phthalate of pH 4.00, phosphate of pH 6.80 and borate buffer of pH 9.18. In this pH range a perfectly linear response was obtained.

RESULTS

The interval in which the pH shift takes place is sufficiently narrow to assure a direct proportionality between the magnitude of the shift and the amount of hydrogen ions liberated. Therefore, the recordings can be used directly for the kinetic analysis of the redistribution of hydrogen ions during the clotting reaction.

It may be mentioned here, that the magnitude of the pH shift, in the region where the buffering effect of water is negligible, is independent of the fibrinogen concentration. This follows from the fact that there is no extraneous buffer in the system and the fibrinogen acts both as a hydrogen ion-producing system and as a buffering system. When the absolute magnitude of the change in hydrogen ions was needed, the pH shift was multiplied by the buffering capacity of fibrinogen in the respective pH range. Values of the buffering capacity, defined as the increment of bound hydrogen ions over the increment of pH, dh/dpH, were calculated at 0.1-pH intervals from the data read on a large scale titration curve of fibrinogen. The titration curve was constructed with the data published in the first paper of this series[11].

The recordings obtained below pH 8 are reproduced on Fig. 1. The curves accurately obey a first-order reaction law over more than 90% of the reaction as demonstrated in the plots shown on Fig. 2. The rate constant, calculated from the slope of the straight lines, shows a steady increase with pH. When its logarithm was plotted against pH a straight line was obtained with a slope of 0.5 as shown on Fig. 3. It is very difficult to ascertain the significance of this fractional slope in a system where the pH effect on the enzyme–substrate complex formation and on the rate of decomposition of the latter are not separated and where both these effects may be linked to ionogenic groups on enzyme and substrate alike.

Above pH 8 the pH-shift recordings have a biphasic character. First, the pH changes in an acidic direction, reaches a maximum, then turns more alkaline and levels off before attaining the initial value. The curves, reproduced on Fig. 4, can be analyzed in terms of two opposing, simultaneous first-order reactions. First the logarithm of the difference of the final height reached and the height at time t was

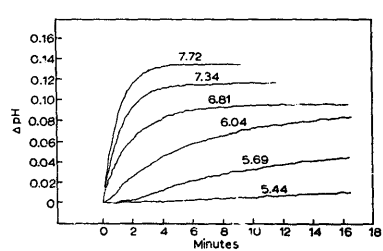

Fig. 1. Recordings of the change in pH of fibrinogen solutions after addition of thrombin. Final fibrinogen concentration 1.37%, final thrombin concentration 1.18 TAME units/ml., I 0.3, temp. 25°. Numbers on each curve indicate the initial pH.

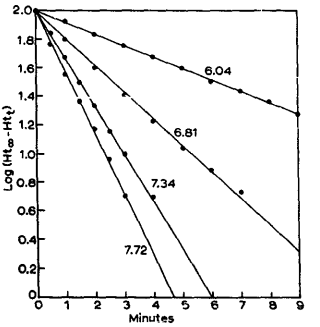

Fig. 2. First-order plots of some of the curves shown on Fig. 1.

plotted against time. This plot corresponds to the second reaction and the points fall on a straight line in the region where there is no interference from the first reaction. With the slope and intercept of the line the second reaction was reconstructed in its entirety and added to the experimental curve. The resulting curve is that of the first reaction. It levels off, as expected, and gives a reasonable first-order plot. Figs. 5a and 5b give an example of the analysis of one curve along the lines suggested above. The experimental curve was then recalculated using the parameters of the two reactions and readjusted when necessary to obtain a better fit. The points on Fig. 4 are calculated in this way and apparently a reasonably good fit was obtained.

The usual pitfalls of fitting reaction curves with exponentials are even more pronounced in our case of two opposing reactions than with exponentials of the same sign. However, as long as the rate constants are sufficiently different, the calculations seem to yield unique answers, but obviously the answers become more ambiguous as the overlap of the two curves becomes more extensive. This situation

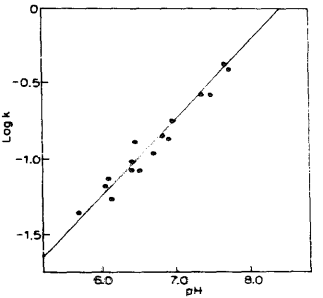

Fig. 3. Logarithm of the rate constants, calculated from the plots shown on Fig. 2 and other similar ones, plotted against pH.

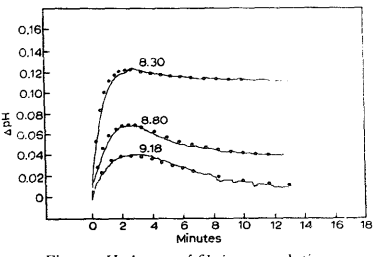

Fig. 4. pH change of fibrinogen solutions upon thrombin addition at initial pH's more alkaline than 8. The circles are values calculated as indicated in the text.

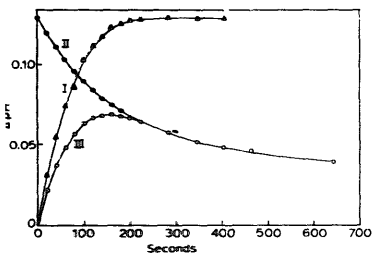

Fig. 5a. Analysis of the reaction curve obtained at pH 8.80. The experimental curve (○) is resolved into the two component curves of the first (▲) and the second (●) reaction.

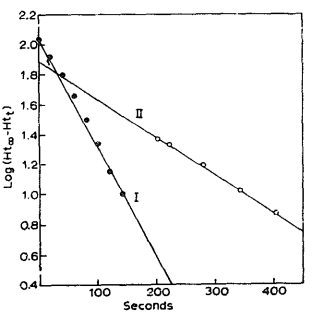

Fig. 5b. First order plot of the isolated first (●) and second (○) reactions

limits the usefulness of our data to the region below pH 9.2, which is rather unfortunate, because data at higher pH values would make the estimate of the groups involved, with pK values in this pH range, much more accurate. Perhaps more accurate recordings and the use of computers could remedy this situation to some extent.

The rate constants calculated for the two reactions are plotted against pH on Fig. 6. It is reassuring that the rates of the first reaction calculated from the composite curve fit smoothly with the points of the single reaction on the acidic side. The curve of the first reaction is a symmetrical, bell-shaped curve typical of the pH dependence of a large number of enzymic reactions. It is nearly identical in shape and location of its maximum at pH 8.2 with the pH dependence of the rate of splitting of TAME by thrombin[15], of the rate of formation of fibrin monomer[9] and of the extent of inhibition of thrombin by diisopropylfluorophosphate[16].

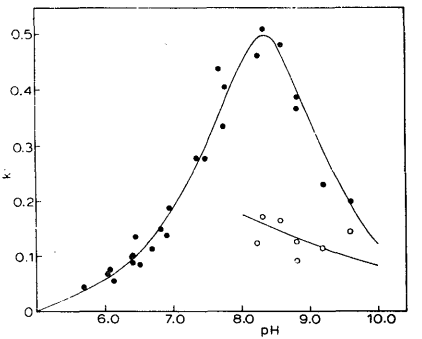

Fig. 6. Rate constants of the first (●) and of the second (○) plotted against pH. Rate constants calculated with decimal logarithms and time in min/1 TAME unit/1 ml in the reaction mixture. Final fibrinogen concentration in the four experiments collected in this figure varied between 1.02 and 1.44 %. I 0.30, temp. 25°.

The rate of the second reaction is much slower than that of the first and it decreases gently with pH. It appears that at approx. pH 10 the rate of the two reactions becomes nearly equal. This, and the steeply increasing buffering capacity of fibrinogen in this pH range, decreases the magnitude of the pH change to a very great extent and also results in almost complete loss of the biphasic character of the curves.

DISCUSSION

In the first two papers of this series[10,11] it was shown that in the overall clotting reaction below pH 8 hydrogen ions are released, whereas above pH 8 are both produced and absorbed. It was also possible to assign the various components of this complex process, either to the first enzymic step, or to the second polymerization step: In the first step hydrogen ions are liberated in increasing amounts as the pH runs from 6 to 10 by the splitting of the peptide bonds. In the second step, when the fibrin monomers polymerize, the pK's of two groups are shifted by hydrogen bond

formation, or some other mechanism. The first group (A), with the lower pK becomes more acidic, whereas the second, (B) with the higher pK less acidic, and as a result hydrogen ions are liberated when the pH is in the titration range of the first and absorbed when the pH is in that of the second group. The apparent pK's of these two groups were determined as 7.0 and 8.2. A more detailed theory of the hydrogen bonding mechanism and calculation of the true pK's was given by STURTEVANT *et al* [17]. The α-amino group produced in the first step has an apparent pK of 7.5.

The amount of hydrogen ions liberated, or taken up by either of these groups approaches approximately the same maximal value, about one equiv/10^5 g of fibrinogen. However, at a given pH these quantities will vary from one group to the other because of the different pK values.

A schematic representation of the hydrogen-ion shifts is given in Fig. 7. The ordinate of the heavy line (I) at any particular pH gives the amount of hydrogen ions released in the first step at that pH, that of the thin lines the amounts released

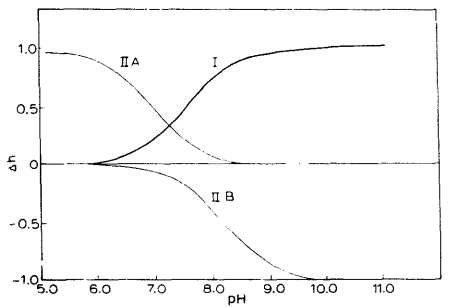

Fig. 7. Schematic representation of shifts in hydrogen-ion equilibria brought about by the clotting of fibrinogen. Curve I represents hydrogen ions liberated by the first, proteolytic step, curve IIA by the second polymerization step and curve IIB shows hydrogen ions reabsorbed during the polymerization step.

(IIA), or absorbed (IIB) in the second step. The algebraic sum of all the ordinates gives the change in hydrogen ions during the overall reaction.

Thus, the reaction at the two extreme pH's of the clotting reaction can be written as follows:

	pH 6	pH 10
Step one (I)	$n\ F \longrightarrow n\ f$	$n\ F \longrightarrow n\ f + n\ H^+$
Step two (II)	$n\ f \longrightarrow f_n + n\ H^+$	$n\ f + n\ H^+ \longrightarrow f_n$
Overall	$n\ F \longrightarrow f_n + n\ H^+$	$n\ F \longrightarrow f_n$

In this scheme F denotes fibrinogen, f fibrin monomer, f_n fibrin polymer and n the number of units of 10^5 gm taking part in the reaction.

It is apparent that at pH 6 hydrogen ions are produced only in the second step, whereas at pH 10 they are produced in the first and absorbed again in the second step. Because the titration ranges of these groups overlap to a considerable degree at an intermediate pH hydrogen ions are liberated by both the first and the second step.

These conclusions were reached by an analysis of the pH differences between the initial and the final stages of the first and of the overall reaction. The kinetic curves presented in this paper fully support the above reaction mechanism. At low pH, there is only one reaction, producing hydrogen ions; at high pH, however, one can see that hydrogen ions are first liberated and then absorbed again, as required by the above mechanism. That the two reactions at high pH are resolved is, of course, due to the fact that the second one is much slower than the first one. Only one reaction would be apparent, if the first step were the rate limiting one.

As it was shown in the previous section, the experimental curves above pH 8 can be resolved into two simultaneous and opposing first order reactions. Denoting by a_0 the quantity taking part in the first reaction and by b_0 that taking part in the second reaction, the equation of two opposing simultaneous first order reactions, with rate constants k_1 and k_2 is:

$$\Delta h = (a_0 - b_0) - a_0 e^{-k_1 t} + b_0 e^{-k_2 t} \tag{1}$$

The experimental curves are accurately described by an equation of this form, however, the reaction scheme adopted calls for consecutive and not simultaneous reactions. This apparent discrepancy was resolved by deriving the equation of opposing consecutive reactions and showing that it is formally identical with Eqn. 1. Thus, a curve amenable to a mathematical description in terms of two opposing simultaneous reactions, may in fact represent consecutive reactions, but in the latter case, the parameters a_0 and b_0 are only apparent, related in a more complex way to the actual quantities taking part in the reaction.

Let us denote the momentary concentration of fibrinogen, fibrin monomer and fibrin polymer by n_1, n_2 and n_3, all expressed in the same weight unit, *e.g.* 10^5 g, per volume. The amount of hydrogen ions released will be proportional to the amount of fibrin monomer produced whether present as such, or polymerized, *i.e.* to the sum of fibrin monomer and fibrin polymer, and the amount of hydrogen ions absorbed will be proportional to the amount of polymerized fibrin. If α and β are proportionality factors themselves dependent on pH, then the amount of hydrogen ions released, Δh_1, absorbed, Δh_2, and the overall change, Δh, at a given time and a certain pH will be:

$$\Delta h_1 = \alpha(n_2 + n_3) \tag{2}$$

$$\Delta h_2 = -\beta n_3 \tag{3}$$

$$\Delta h = \Delta h_1 + \Delta h_2 = \alpha n_2 + (\alpha - \beta) n_3 \tag{4}$$

The proportionality factors are not equal, even though the number of groups taking part in each step are approximately equal because the pK's of the groups are different.

The rate equations of two consecutive first order reactions can be integrated to give:

$$n_2 = n_0 \frac{k_1}{k_1 - k_2} \left[e^{-k_2 t} - e^{-k_1 t} \right] \tag{5}$$

$$n_3 = n_0 \left[1 + \frac{k_2}{k_1 - k_2} e^{-k_1 t} - \frac{k_1}{k_1 - k_2} e^{-k_2 t} \right] \tag{6}$$

where $n_0 = n_1 + n_2 + n_3$, *i.e.* the original fibrinogen concentration. Combination of these two equations with Eqn. 4 results in:

$$\Delta h = (\alpha - \beta)n_0 - \left[\alpha n_0 + \beta n_0 \frac{k_2}{k_1 - k_2}\right] e^{-k_1 t} + \beta n_0 \frac{k_1}{k_1 - k_2} e^{-k_2 t} \qquad (7)$$

Eqns. 7 and 1 differ only in the coefficients of the exponential terms. Therefore, curves of either simultaneous, or consecutive reactions can be fitted by the sum of two exponential curves. Moreover, the exponents in the two expressions are identical, *i.e.* the rate constants calculated by such curve fitting will be the true ones, whether the reactions are simultaneous, or consecutive.

When the reactions are consecutive a_0 and b_0 are only apparent and the true values of the quantities taking part in the reaction can be determined by comparing coefficients in Eqns. 1 and 7. This gives:

$$\alpha n_0 = a_0 - b_0 \frac{k_2}{k_1} \qquad (8)$$

$$\beta n_0 = b_0 \frac{k_1 - k_2}{k_1} \qquad (9)$$

The a_0 and b_0 values, obtained from each experimental curve with the procedure already described, were used to calculate the corresponding αn_0 and βn_0 values with the help of Eqns. 8 and 9 and the latter were then plotted against pH on Fig. 8 (αn_0 by open circles, βn_0 by full triangles). Below pH 8, where only one reaction was present, the amount of hydrogen ions liberated was calculated from the final value of the pH shift. These points are represented by full circles on Fig. 8. The upper solid curve of this figure represents the amount of hydrogen ions released and the lower one that of the hydrogen ions absorbed, irrespective of their origin, *i.e.*, whether they come from the first, or the second reaction.

According to Fig. 7, above pH 8 the hydrogen ions are liberated exclusively by the first reaction, therefore, the points of the upper curve in this region should

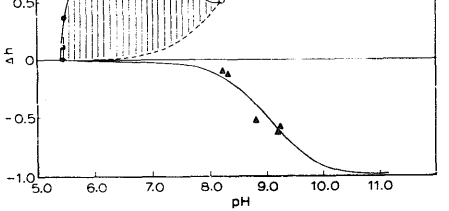

Fig. 3. The upper curve shows the combined release of hydrogen ions by the first and the second reactions expressed as equiv. of H^+ per 10^5 g of fibrinogen, plotted against the initial pH. The lower curve is a similar plot for the hydrogen ions reabsorbed during the polymerisation step. For explanations see text.

represent the titration of the α-amino groups liberated by thrombin. As a first approximation, a classical titration curve was fitted through these points. The extension of the titration curve below pH 8, shown by the broken line, separates thus the domain of hydrogen ions liberated in the first from that of hydrogen ions liberated in the second reaction. To emphasize the complex origin of each point on this curve, the domain corresponding to the liberation of hydrogen ions by the polymerization reaction is shaded in Fig. 8. It is perhaps worthwhile to point out that the curves of Fig. 8 are not differential curves and the amount of hydrogen ions liberated is not given by the areas delineated on the graph. These are titration curves, more exactly, the difference of two titration curves, where the total amount of hydrogen ions liberated during the first and the second step, at a certain pH is given by the ordinate at that pH and the portion of this passing through the clear area represents hydrogen ions liberated during the first, that passing through the shaded area those liberated during the second step.

Returning now to the question of whether the pH shift is made up of simultaneous or consecutive reactions, it is evident from the equations give that this question can be settled only by an independent knowledge of the quantities involved. From the differences of titration curves[11] and estimates of the new N-terminal groups formed[7,8] there are approx. 1.1 α-amino groups produced per 100000 g of fibrinogen. This is in fair agreement with the quantity derived from Fig. 8, thus suggesting consecutive reactions. Analysis in terms of simultaneous reactions yields quantities 30–50 % higher, but in view of the low accuracy of the data at the high pH end, a difference of this magnitude does not constitute a definite proof.

The titration curve of the α-amino groups, as shown in Fig. 8, is slightly shifted from that found in the difference of titrations in the presence of urea[11]. The cause of this might be simply the errors involved in these fairly complicated measurements, or alternatively, the insufficient correction of the effect of 5 M urea on the fibrinogen-titration curves. Also, it should be remembered that the correction for the urea effect was based on the shift caused by urea of the titration curve of native fibrinogen. However, there might be more here than a pure solvent effect. Indeed, the position of the new α-amino groups might be quite different in the fibrin gel than in the urea solution where the gel structure is disrupted. Consequently, larger shifts than those solely due to the presence of urea might be expected. The pK of 8.0 arrived at in the present experiments is closer to that expected for an N-terminal group[18] than the 7.5 value found previously. The apparent pK values of the groups taking part in the polymerization process are also affected by the relocation of the curve of the first step. Their new values are 7.4 and 8.9.

Inspection of Fig. 8 reveals that up to pH 7 the hydrogen ions are liberated largely by the polymerization reaction, between pH 7 and 8 both reactions contribute to their release and finally above pH 8 only the proteolytic step. The different rates of release of the peptides may complicate this picture further[19,20]. Surprisingly, none of this complex behavior is reflected in the kinetic curves. As seen on Fig. 2 the reaction curves below pH 8 strictly obey a first-order law. Between pH 7 and 8 one would expect a more complex curve, revealing the two consecutive reactions and conforming to an equation analogous to Eqn. 7 with the sign of the β-coefficient changed. Moreover, there is no indication of a transition in the magnitude of the rate constants, neither on an ordinary, nor on a logarithmic plot of the rate against pH (see Figs. 6 and 3).

According to Eqn. 7, there will be only one apparent reaction when k_2 is very much larger than k_1, *i.e.* when the proteolytic step is the rate-limiting one. Our data, presented on Fig. 6, show that the rate of polymerization above pH 8, at our particular fibrinogen concentration of approx. 1.2 %, is lower than the rate of the proteolytic step. However, the rate increases with decreasing pH and may very well take an upward turn below pH 8, increasing substantially over the rate of the first reaction. Unfortunately, our experiments are not able to furnish data below pH 8 and there are very few data in the literature on the rate of the polymerization step. The only available data on its pH dependence are those of SCHERAGA AND EHRENPREIS[9], obtained under substantially different conditions, and they show the expected steep increase of the rate with lowering of the pH. Thus, it seems reasonable to assume, that the first step is rate limiting below pH 7 and that the rate of the pH shift is indeed a measure of the rate of the proteolytic step, even though most of the hydrogen ions released originate in the polymerization step. In the intermediate region the two rates are very similar, therefore, the two reactions cannot be distinguished in the overall curve, whereas at pH values higher than 8, when the polymerization only absorbs hydrogen ions, the rate of the hydrogen-ion production, by necessity, must be equal to the rate of the first step. Thus, the bell-shaped curve, presented on Fig. 6, is actually the rate curve of the first, proteolytic step in the fibrinogen–fibrin transformation.

TABLE I

COMPARISON OF THE RATE CONSTANTS OF THE FIRST REACTION OBTAINED BY SCHERAGA AND EHRENPREIS[9], WITH THOSE FOUND IN THIS WORK

pH	k_1/min/unit	
	Scheraga and Ehrenpreis	*This paper*
6.0	0.183	0.06
7.0	1.49	0.19
8.0	2.50	0.46
9.0	0.93	0.34

The first-order character of the overall clotting reaction was first recognized by LAKI[21]. SCHERAGA and his collaborators, on the other hand, have demonstrated that both the first (proteolytic) step[5,9] and the polymerization step[17,9] obey first-order kinetics. The data presented in this paper are in complete accord with these findings. A quantitative comparison of our rate constants for the first step with those found by SCHERAGA AND EHRENPREIS may be instructive, even though the latter were obtained at somewhat different experimental conditions: 0.2 % fibrinogen concentration at I 0.15, compared with 1.37 % fibrinogen concentration at I 0.30 in our studies. The rates were read on Fig. 6 of ref. 9 and on Fig. 6 of this paper and are given in Table I. It is apparent that our rates are some 5 times lower than those of SCHERAGA AND EHRENPREIS. This difference very probably is not caused by the 6-fold difference in fibrinogen concentration, because the above authors found no difference in rate when the fibrinogen concentration was increased 10-fold from 0.02 to 0.2 % (ref. 6). More likely, it is caused by the difference in ionic strength, since WAUGH AND PATCH[22] found in their experiments on the effect of ionic strength on the rate of

clotting a similar 5-fold decrease in rate when the ionic strength was increased from 0.15 to 0.30. Hence, the agreement in the absolute values of the rate constants in the two sets of experiments seems to be satisfactory.

Careful inspection of the recordings obtained at pH 5.44 and 5.69 revealed the presence of a lag period. After this initial disturbance the curve continues as a first-order reaction. Similar observations were made by WAUGH AND LIVINGSTONE[23] and BLOMBÄCK AND LAURENT[24] in their kinetic studies of the clotting reaction, although at a higher pH, where our curves do not appear to have such a lag period. It can be explained by lack of polymerization at this low pH, until a certain concentration of fibrin monomers is reached. After some nuclei of polymerization are formed, apparently all the available fibrin monomers are incorporated and the reaction proceeds with the rate of formation of the fibrin monomers.

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