Equilibria in the Fibrinogen–Fibrin Conversion. VI. Ionization Changes in the Reversible Polymerization of Fibrin Monomer*

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ABSTRACT: In order to provide an experimental basis for a more rigorous examination of possible polymerization mechanisms, precise potentiometric studies were made of the pH changes accompanying the reversible polymerization and clotting of fibrin monomer in 1.0 M NaBr. It was found that protons were released during polymerization when the initial pH was below 7.65 at 25.0°, and absorbed at higher initial pH values. The net release or absorption of protons during polymerization was measured quantitatively over about the lower half of the pH range in which polymerization occurs, indicating a maximum release of 1.10 ± 0.03 equiv/10^5 g of protein at initial pH 6.39 ± 0.05. The results at high pH were complicated by a simultaneous slow release of protons from fibrin, probably due to conformational changes. Kinetic studies indicated that the ionization change due to polymerization is essentially a first-order process, with a maximum rate constant at pH 7.5–8, and an apparent activation energy of 22 ± 2 kcal/mole at initial pH 6.92.

Among the important experimental features of reversible fibrin polymerization are its exothermicity (Sturtevant *et al.*, 1955) and (by inference from studies of the over-all fibrinogen–fibrin conversion) significant changes in the degree of ionization of the protein (Mihalyi, 1954). In a previous paper in this series (Sturtevant *et al.*, 1955) it was shown that both of these observations could be accounted for quantitatively by intermolecular hydrogen bond formation between ionizable groups, suggesting that such bonding is largely responsible for the polymerization. It was assumed in this treatment that the heat of formation of an individual hydrogen bond can be taken as −6 kcal/mole. However, evidence has since accumulated that this value is too large, and that −1.5 kcal/mole would be a much more reasonable estimate (Schellman, 1955; Némethy *et al.*, 1963). Use of the latter value leads to a large discrepancy between the experimental and theoretical heats of reaction, based on the hydrogen bonding model. Since this could have been due to the different media employed in the ionization change (0.3 M KCl) and calorimetric (1.0 M NaBr) measurements, or to interference by the other substances present in the ionization change experiments, it was considered necessary to measure directly the pH changes accompanying the isolated polymerization of fibrin monomer in 1.0 M NaBr. The results of this investigation are reported in the present paper, and their significance with regard to the polymerization mechanism will be analyzed in the following paper of this series.

Experimental Section

Materials. Bovine fibrinogen was prepared from plasma fraction I (Armour, Lot No. U4704) by the procedure of Laki (1951), as modified by Sturtevant *et al.* (1955). Solutions of fibrin monomer in 1.0 M NaBr at pH 5.3 were prepared from this purified fibrinogen essentially by the procedure of Donnelly *et al.* (1955), except that the fibrin was usually reclotted and redissolved only once. (Identical pH changes during polymerization were observed with fibrin prepared in this manner, and with fibrin which was reclotted three times). The final solution was dialyzed at 4° *vs.* unbuffered 1.0 M NaBr, pH 5.3. Dialysis was continued for 48 hr with gentle rocking of the vessel, with three changes of NaBr solution. After clarification by centrifugation and determination of protein concentration, the preparation was diluted to the desired concentration with unbuffered 1.0 M NaBr, pH 5.3.

The total protein concentration in the fibrin preparations was determined from the optical density at 280 mμ of a sample diluted with 1.0 M NaBr, pH 5.3, using the factor 0.625 mg/ml per OD unit (Ehrenpreis and Scheraga, 1957). To determine the concentration of nonclottable protein, a sample (0.5 ml) was diluted to 10.0 ml with clotting buffer (0.0947 M KCl, 0.0158 M Na_2HPO_4, 0.0316 M KH_2PO_4, pH 6.4) and allowed to stand at room temperature overnight. The clot was

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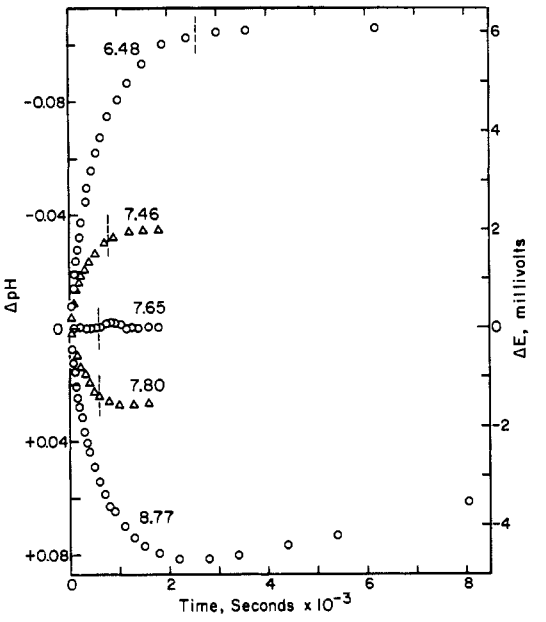

FIGURE 1: pH change during polymerization of fibrin (7.1–7.4 mg/ml) in 1.0 M NaBr at 25.0°, initial pH as indicated. The clotting times are indicated by the broken lines, except for initial pH 8.77, where the clotting time was later than the above time scale.

squeezed out and removed, and the concentration of nonclottable protein in the supernatant was determined from the optical density as above. All results reported in this paper are calculated on the basis of clottable fibrin concentration; the preparations used were 97–99% clottable.

The standard base was *ca.* 0.25 N NaOH in 1.0 M NaBr, made up carbonate free, and accurately standardized *vs.* potassium acid phthalate. It was stored in a Machlett Auto-Buret protected by a tube of Indicarb (Fisher Scientific Co.) to prevent absorption of carbon dioxide. The concentration was such that no significant dilution of the fibrin solutions occurred upon addition to raise the pH.

Measurement of pH. The electrode system consisted of a Beckman General Purpose glass electrode (Type 40498) and a calomel reference electrode with an open liquid junction (Radiometer Type K100). The reference electrode was filled with 2.6 M KCl, rather than the usual saturated solution, to avoid salting out of protein in the liquid junction. The junction was frequently blocked by the fibrin clot, but there was no indication that the results were materially affected. Cleaning of the junction was facilitated by breaking it just above the tip, and reconnecting by means of a short section of plastic tubing. The tip could then be readily removed and flushed out after use. A more serious problem appeared to be fouling of the glass electrode surface by the clot, which resulted in erratic readings and failure to approach a steady final reading after completion of reaction. We found it necessary to use magnetic stirring to avoid this; however, care must be taken not to stir excessively, since fibrin and fibrinogen are particularly susceptible to mechanical denaturation.

The instrument used for potentiometric measurements was designed and built in this laboratory by Mr. Gary Davenport, and has already been described (Schrier *et al.*, 1964). With the above electrodes, the precision of a single measurement was ±0.05 mv (0.001 pH unit) at 25.0°.

Measurements were made in a glass vessel with jacket for circulation of water at constant temperature. The electrodes were inserted through a rubber stopper, which also carried connections for entrance and exit of nitrogen above the sample surface, and for addition of standard base as described below. Carbon dioxide was excluded by flushing with nitrogen (Airco Seaford Grade) which had been passed through solid sodium hydroxide and Indicarb, and bubbled through 1.0 M NaBr.

The electrodes were standardized *vs.* the revised standard buffers (pH 4.008, 6.865, and 9.180 at 25.0°) of the National Bureau of Standards (Bates, 1962), prepared as specified. The pH response of a particular glass electrode was usually found to be stable, and was checked *vs.* all three standards only occasionally. After the pH of the fibrin stock solution was assigned by comparison with the standards, the stock solution itself was used as a secondary standard in the pH change experiments. The stock solution was checked every few days against a standard buffer, and was never found to change significantly. The ionic strength of solutions in 1.0 M NaBr is much higher than that of the standard buffers, introducing a possible inaccuracy in assignments of pH and derived values of p*K*. However, this error should cancel out in measurements of pH changes.

Since the pH changes in the polymerization of fibrin were quite small (up to 0.11 unit at 25.0°), and several hours were often required for completion of reaction, the time stability of the electrode system was of critical importance. It was found helpful to condition the glass electrode by overnight immersion in 1.0 M NaBr at the temperature of measurement. Electrodes thus treated showed drifts of <0.001 pH unit/hr in well-buffered solutions of 1.0 M NaBr. On the other hand, repeated use of a glass electrode in polymerization experiments appeared to slow its time response to such an extent that it could cause difficulty in the assignment of initial pH. This effect could be eliminated by rejuvenating the glass electrode after each day's measurements by soaking for 1 hr in 0.05 M potassium acid phthalate (pH 4) at room temperature, and then reconditioning the electrode overnight in 1.0 M NaBr at pH 5.3.

Polymerization Experiments. A sample (3–7 ml) of a stock solution of fibrin in 1.0 M NaBr (pH 5.3) was pipetted accurately into the reaction vessel, and the electrode tips were immersed in the solution. The solution was stirred magnetically with a micro stirrer bar, as slowly as possible, for a time sufficient to establish temperature equilibrium and obtain a steady reading of emf (1 hr at 25°, 2 hr at 10°). During the first 30 min the vessel was flushed with nitrogen at the rate of

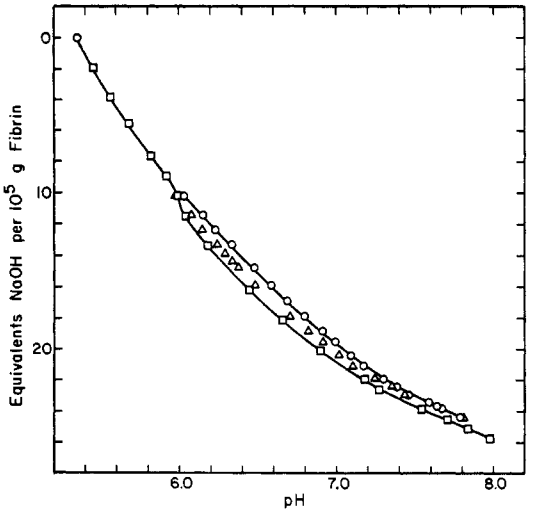

FIGURE 2: Titration curves of fibrin (7.4 mg/ml) in 1.0 M NaBr, at 25.0°. O, monomer; Δ, monomer plus polymer at equilibrium, from pH change experiments; □, monomer plus polymer at equilibrium, by continuous titration.

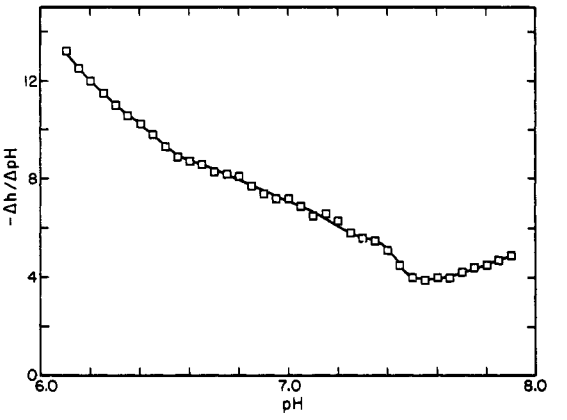

FIGURE 3: Slope of equilibrium titration curve of fibrin in 1.0 M NaBr, at 25.0°.

one vessel volume per minute; after that time the flow was discontinued and the vessel kept sealed from the atmosphere. To begin the polymerization reaction, the stirring rate was increased moderately, and the pH was raised by addition of the necessary volume of standard base. The base was measured from an Agla Micrometer Syringe (Burroughs Wellcome and Co.), with delivery through a capillary of finely drawn polyethylene tubing. The capillary was admitted to the vessel through a section of small-bore glass tubing, which fitted snugly when the capillary was fully inserted, and which was sealed off when not in use. To obtain the highest precision of delivery, the syringe was adjusted to fill the capillary, and the capillary inserted, just before the addition; the capillary was withdrawn as quickly as possible thereafter. An electric timer was started immediately after the alkali addition, and readings of emf *vs.* time were begun as soon as possible.

Results

At low pH (5.1–5.3) solutions of fibrin in 1.0 M NaBr are stable and monomeric, but appreciable polymerization occurs if the pH is raised to 5.7–6.1, and fibrin clots are formed at pH values more alkaline than 6.2 (Donnelly *et al.*, 1955). The method employed here was to suddenly raise the pH of a monomeric solution by the addition of alkali, and to follow the pH change during the subsequent polymerization with a sensitive potentiometer. The results of typical experiments at 25.0° are represented in Figure 1 as plots of the change in emf or pH *vs.* time. At this temperature the pH adjustment of the protein by the added base is complete before the first observation of emf is made (usually at about 50 sec). The initial emf and pH can be reliably assigned by linear extrapolation of the first few readings to 0 time, on an expanded time scale. (Some of the initial points were omitted from Figure 1 for the sake of clarity.) It is clear that most of the pH change occurs before the first appearance of clot. At or below initial pH 7.8 the pH approaches a steady value soon after this; the bump in the data at pH 7.65 appears to be characteristic of temporary fouling of the glass electrode. At higher initial pH, the data fail to approach a steady value, showing instead a maximum followed by an indefinitely prolonged downward drift of pH. It is unlikely that this is due to electrode fouling, since the effect is observed before the appearance of clot.

Experiments at 10.0° revealed an additional complication. Here, the initial observations showed a sharply falling pH after the addition of base, followed

TABLE I: pH Stability of Fibrinogen and Fibrin at 25.0°.

Solute	Solvent (M), pH[a]	$(pH)_0$	ΔpH[b]
Borax, 0.01 M	1.0 NaBr, 8.74	8.74	−0.001
Fibrin, 7.1 mg/ml	1.0 NaBr, 5.3	8.77	−0.017
Fibrinogen, 7.5 mg/ml	1.0 NaBr, 5.5	6.66	−0.0015
Fibrinogen, 7.5 mg/ml	1.0 NaBr, 5.5	7.70	−0.007
Fibrinogen, 7.5 mg/ml	1.0 NaBr, 5.5	8.68	−0.026
Fibrinogen, 7.5 mg/ml	1.0 NaBr, 10.0	8.75	+0.009
Fibrinogen, 7.5 mg/ml	0.3 KCl, 5.8	8.67	−0.035

[a] pH of the stock solution before addition of base or acid. [b] During the first hour after addition of base or acid, except for fibrin, where the quoted value is the drift in pH units per hour after completion of polymerization.

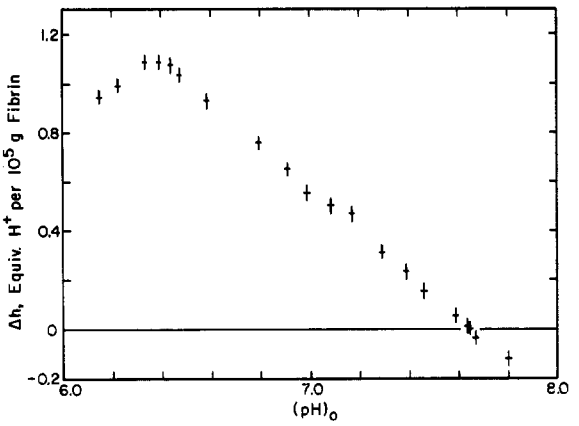

FIGURE 4: Proton release or absorption in the polymerization of fibrin (7.4 mg/ml) in 1.0 M NaBr, 25.0°, as a function of initial pH.

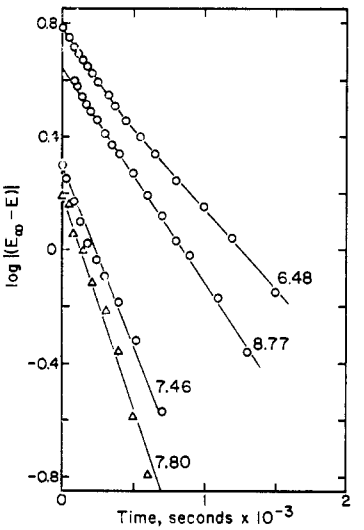

FIGURE 5: First-order kinetic plots of the data of Figure 1, initial pH as indicated.

by curves similar to those of Figure 1. This is opposite in direction to a slow electrode response, and appears to be due to very slow pH adjustment of the protein. In any event, it was not possible to make a reliable extrapolation to assign the initial pH of the polymerization.

In order to clarify the origin of the downdrift at relatively high pH, the pH stability of fibrinogen was examined under the conditions used for fibrin polymerization. Since fibrinogen does not polymerize under these conditions, the possibility of electrode fouling can be excluded. In Table I are summarized the pH changes observed during the first hour after raising or lowering the pH of fibrinogen solutions in 1.0 M NaBr or 0.3 M KCl, with data for a sodium borate buffer and fibrin. It is clear that the pH of fibrinogen solutions drifts downward when suddenly raised from low pH to neutrality or higher. At pH 8.7 the drift is comparable to the long-range drift observed in fibrin polymerization. The drift is not due to absorption of carbon dioxide, since the borate buffer shows good stability under the same conditions, even though its buffer capacity is about half that of the fibrinogen solution. Further, when the pH of fibrinogen is lowered from 10.0 (by the addition of dilute HBr in 1.0 M NaBr) a drift in the reverse direction occurs. Finally, the effect does not seem to be related to the specific properties of 1.0 M NaBr, since a comparable result was obtained in 0.3 M KCl.

For purposes of quantitative treatment, it is desirable to convert the observed values of ΔpH during polymerization to Δh, where h is the number of equivalents of hydrogen ion released per 10^5 g of fibrin. (Taking the molecular weight of fibrin monomer as 3.3×10^5, the number of equivalents of hydrogen ion released per mole of monomer is 3.3 h.) This requires knowledge of the equilibrium titration curve of fibrin over the pH range of the experiment. For small changes, Δh is given to a good approximation by the product of ΔpH and dh/dpH at the mean pH of the experiment. The latter quantity is the negative of the slope of the titration curve, when this is plotted as equivalents of base added per 10^5 g of fibrin *vs.* pH. Values of dh/dpH can be derived from the titration curve by means of a smoothed plot of $\Delta h/\Delta pH$ *vs.* pH, where ΔpH is a small increment (0.1 unit) in which the change Δh occurs, and the pH is the midpoint of the range over which Δh and ΔpH are computed.

The equilibrium titration curve of fibrin was determined at 25.0° in 1.0 M NaBr, and the data are plotted in Figure 2. Two independent methods were used to obtain this curve. First, the data of the individual pH change experiments were plotted *vs.* the equivalents of base added in each experiment, to obtain separate titration curves for fibrin monomer and fibrin at equilibrium. In the pH range of polymerization, the data for fibrin monomer were obtained by extrapolation to 0 time (at which time no polymers are present). The precision of these experiments can be judged from the smoothness of these curves. Second, a sample of fibrin monomer was titrated continuously, allowing time for pH equilibrium to be established after each addition of base. Figure 2 shows that the agreement between the two methods is very good, considering that the fibrin is largely clotted over much of the range when the second method is used.

The slope of the equilibrium titration curve, evaluated as described above, is plotted *vs.* pH in Figure 3. The data from the continuous titration of fibrin were used, although the data from pH change experiments are closely similar, and yield essentially the same results. Finally, the calculated values of Δh are plotted *vs.* the initial pH in Figure 4, over the pH range where a steady final value is observed. The data at higher initial pH (not shown in Figure 4) are of no quantitative value, but qualitatively they indicate a minimum (maximum proton absorption) around pH 9.

The kinetics of the pH changes may also be con-

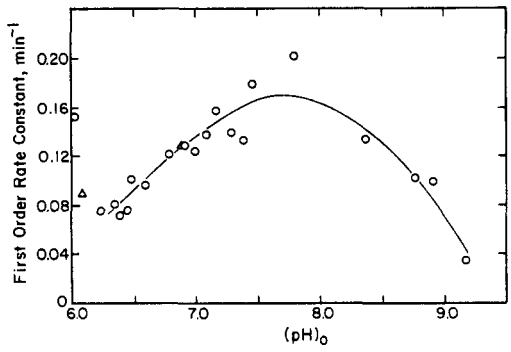

FIGURE 6: Dependence of the first-order rate constant for release or absorption of protons on the initial pH, at 25.0°. The two points indicated by triangles are from calorimetric data (Sturtevant *et al.*, 1955).

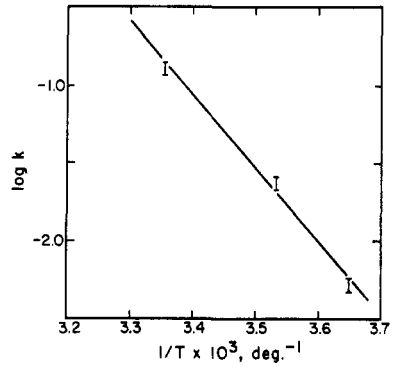

FIGURE 7: Arrhenius plot for proton release in the polymerization of fibrin, at initial pH 6.92 in 1.0 M NaBr.

sidered. The release or absorption of protons seems to be essentially a first-order process, as seen from the logarithmic plots of Figure 5, based on the data o Figure 1. Here E is the emf read at the time given on the abscissa, E_∞ is the value when change is essentially complete, and $|(E_\infty - E)|$ is the magnitude of the difference. Since E is proportional to pH, and ΔpH is proportional to proton release or absorption for the small changes observed here, the plots of Figure 5 should be linear for a first-order reaction. Above pH 6.9 at 25°, the first-order law is obeyed up to 90% completion of reaction. (For pH 8.77, where the reaction is complicated by drifting, E_∞ is taken as the minimum in Figure 1.) Below pH 6.9, the first-order plots show an abrupt change of slope part way through the reaction, corresponding to a decreased apparent first-order rate constant. The effect occurs at a progressively earlier extent of reaction with lowering of the initial pH.

The dependence of the first-order rate constant on the initial pH at 25.0° is shown in Figure 6, which indicates a maximum at pH 7.5–8, the region where ionization changes during polymerization are smallest. Below pH 6.9, the data correspond to the initial rate constant; the points for the subsequent rate constant would lie some 15–40% lower. The point near pH 6.0 is of doubtful significance, since it corresponds to a small extent of reaction. These data were all obtained at a fibrin concentration of 7.1–7.4 mg/ml. An experiment at 14.1 mg/ml of fibrin, initial pH 6.51, gave an initial rate constant of 0.108 min^{-1}, indicating no great dependence on monomer concentration in this range. Finally, an Arrhenius plot of kinetic data for temperatures 1.0, 10.0, and 25.0° at pH 6.92 is shown in Figure 7. The apparent activation energy for proton release at this pH is 22 ± 2 kcal/mole. (The uncertainty of the initial pH at low temperatures does not affect the calculation of rate constants from data plotted as in Figure 5.)

Discussion

The coagulation of the plasma protein fibrinogen by the enzyme thrombin *in vitro* is a multistep process, which may be summarized in the following reaction scheme. The symbols are as follows: F, fibrinogen; T,

$$\text{Step 1: Proteolysis} \quad F \xrightleftharpoons{T} f + P$$
$$\text{Step 2: Polymerization} \quad nf \rightleftharpoons f_n$$
$$\text{Step 3: Clotting} \quad mf_n \rightleftharpoons \text{fibrin clot}$$

thrombin; f, fibrin monomer; P, fibrinopeptides; f_n, a series of intermediate polymers; n, m, variable numbers. Much of the evidence concerning the details of this mechanism has been reviewed (Scheraga and Laskowski, 1957; Laki and Gladner, 1964). The gel-like fibrin clot which is produced in this system can be redissolved in certain mild reagents, such as 30% urea or 1.0 M NaBr at pH 5.3, with the complete reversal of steps 2 and 3. In the presence of calcium ions and an additional enzyme (fibrin-stabilizing factor) a further irreversible reaction occurs, in which the clot becomes insoluble in these reagents (Laki and Lorand, 1948; Loewy *et al.*, 1961; Lorand *et al.*, 1962). However, the present studies involve only the reversible stage, since the products were completely soluble in 1.0 M NaBr, pH 5.3, and sedimentation studies of similar redissolved fibrin samples showed only a single peak corresponding to the monomer (Donnelly *et al.*, 1955).

Understanding of the mechanism of fibrin polymerization was greatly advanced by investigations by Mihalyi (1954), who measured, as a function of initial pH, the pH change which accompanies the over-all process (steps 1–3) in 0.3 M KCl. The pH change in the proteolysis (step 1) alone was also measured, by inhibiting steps 2 and 3 by means of urea. By subtraction of Δh of step 1 from Δh for the over-all reaction (and correction for the medium effect of urea) it was inferred that protons are released or absorbed during polymerization; *i.e.*, in steps 2 plus 3, and that Δh depends on the initial pH. Kinetic studies of the over-all process (Mihalyi and Billick, 1963) gave consistent results. These changes in ionization were attributed to formation of intermolecular hydrogen bonds during polymerization.

The results of Mihalyi were reinterpreted quantitatively by Sturtevant *et al.* (1955), who also postulated the formation of intermolecular hydrogen bonds between ionizable groups during polymerization, but with a finite equilibrium constant for the formation of such bonds. From the derived pK values of the hydrogen bond acceptors and donors, these were inferred to be histidyl and either tyrosyl or lysyl residues, respectively.

In the present investigation, the ionization changes accompanying the polymerization and clotting steps were measured directly, through the use of solutions of fibrin monomer in 1.0 M NaBr. Possible complications due to the presence of other substances (fibrinogen, thrombin, and fibrinopeptides) and the correction for the urea effect were thus eliminated. The results also showed that most, if not all, of the ionization change is due to polymerization in solution (step 2) rather than to gelation (step 3).

On the other hand, it was not possible in the present work to measure quantitatively the ionization changes accompanying fibrin polymerization over the whole pH range of interest, owing to interference by a slow proton release which occurs at the higher initial pH values (Figure 1). The fact that a similar proton release is observed when fibrinogen is treated in the same way (Table I) indicates that the effect is not directly related to the occurrence of polymerization, but is probably a simultaneous side reaction. Further, this is not an artifact of the pH-measuring system, since similar complications were observed by Sturtevant *et al.* (1955) in their calorimetric studies of the polymerization of fibrin in 1.0 M NaBr, using the same method of suddenly raising the pH of a stock solution of fibrin monomer. They observed a slow zero-order endothermic reaction at initial pH 6.88, after the exothermic polymerization was complete; this appears to correspond to the long-range pH drift observed in the present work. The fact that the calorimeter contents were not agitated after the initial mixing rules out mechanical denaturation as a possible cause.

A clue to the nature of the side reaction appeared in the work of Hartley and Waugh (1960), who studied the solubility behavior of bovine fibrinogen, although at much lower ionic strength. At pH near isoionic (5.5) native clottable fibrinogen exhibited solubility behavior which indicated a stable heterogeneity in structural detail. At around pH 12 fibrinogen denatured (as shown by loss of solubility on rapid adjustment to pH 6.5–8.0), through both a fast reversible reaction and a slow, partially reversible one. Hydrodynamic and optical rotation studies indicated expansion of the molecule at high pH through loosening of large submolecular units. We suggest that under the conditions of our experiments, fibrin and fibrinogen undergo slow conformational changes after the pH is suddenly raised to a much higher value; this changes the environment of some ionizable groups in such a way that protons are released. A similar explanation is offered for the slow initial pH adjustment of fibrin observed at 10.0°. These conformational changes seem to be at least partly reversible, since a drift in the reverse direction occurs when the pH of fibrinogen is suddenly lowered from a high value (Table I). Drifting was also observed in the present work with fibrinogen in 0.3 M KCl, the medium used by Mihalyi (1954) and Mihalyi and Billick (1963). However, these workers were apparently able to allow the fibrinogen solutions to come to pH equilibrium before reaction was begun by the addition of thrombin.

In any event, it is clear from Figures 1 and 4 that protons are released during the polymerization of fibrin monomer in the lower range of initial pH, and absorbed in the upper range. Qualitatively, this confirms the inferences from the studies of the over-all fibrinogen–fibrin conversion (Mihalyi, 1954). The quantitative agreement is also good, considering that distinctly different media were employed. Thus Figure 4 indicates for 1.0 M NaBr a maximum proton release of 1.10 ± 0.03 equiv/10^5 g of fibrin at initial pH 6.39 ± 0.05 with 0 net change at pH 7.65 ± 0.015, compared to 1.0 equiv at pH 6.0 (Mihalyi, 1954) or 6.2 (Mihalyi and Billick, 1963) and 0 at 7.6 (Mihalyi, 1954) in 0.3 M KCl.

Attempts were made to determine the temperature dependence of the ionization changes in the polymerization of fibrin. By analysis of such data according to the treatment of Sturtevant *et al.* (1955) it would be possible to derive the heats of ionization of the groups involved and hereby obtain further information for their identification. It can be shown that a temperature range of at least 15° would be required, assuming precision in Δh comparable to that of the 25.0° data. However, higher temperatures are precluded by tendencies toward thermal denaturation and incomplete polymerization of fibrin in 1.0 M NaBr. Data were obtained at 0–1 and 10°, but with unsatisfactory precision, owing to relatively long reaction time, difficulty in establishing the initial pH, and the adverse effects of low temperature on glass electrode performance.

Since the mechanism of the polymerization of fibrin is certainly rather complex, it would probably not be realistic to attempt a detailed discussion of the kinetic behavior at this time. It is worth noting, however, that Sturtevant *et al.* (1955) observed that the heat evolution in the polymerization of fibrin in 1.0 M NaBr also followed first-order kinetics. Their rate constants are included in Figure 6 for comparison with the present results based on pH change. From kinetic studies of pH changes in the over-all fibrinogen–fibrin conversion in 0.3 M KCl, Mihalyi and Billick (1963) derived first-order rate constants for the proton absorption at pH 8–10. These would lie some two to three times higher than the data in Figure 6, probably due mainly to the considerably lower ionic strength.[1] In preliminary studies of the kinetics of fibrin polymerization, Scheraga and Ehrenpreis (1959) found an increase of rate with decreasing ionic strength. Mihalyi and Billick assumed that the rate constant for polymerization continues to rise with decreasing pH below pH 8. This is clearly not the case in 1.0 M NaBr, but this is not necessarily a

[1] The rate constants quoted by Mihalyi and Billick (their Figure 6) were multiplied by 2.3 before making the comparison, to convert from decimal to natural logarithms.

contradiction, owing to the different media employed.

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