There are ten single bonds for the $n = 11$ case and to a first approximation one may consider rotation to be equally likely about any one of them. Whichever single bond is involved, the energy of the molecule goes up by ΔE. The configurations reached after a single rotation will be lumped together and called "the unsymmetrical isomer." The ratio of molecules in the unsymmetrical and symmetrical isomeric forms is given by

$$n_u/n_s = 10e^{-\Delta E/RT}$$

The intensities for configurations reached after a rotation may be calculated approximately by assuming the coefficients c_ν remain constant. The intensities of the bands for the unsymmetrical isomer are the simple averages of intensities calculated for all configurations reached in a single rotation.

For the case of all s-*cis*, the intensities for the isomers are (units of $\bar{x}^2/4$)

Isomer	Excited State 1st	2nd	3rd
Symmetrical	9.625	0	1.057
Unsymmetrical	9.920	0.365	1.305

It is seen that assuming $\Delta E/RT$ is appreciable there will be developed a *cis* peak less than one third the 3rd band intensity, and concomitantly the 1st and especially the 3rd intensities will *increase* slightly.

For the case of all s-*trans* the intensities are (units of $\bar{x}^2$)

Isomer	Excited State 1st	2nd	3rd
Symmetrical	9.617	0	0.971
Unsymmetrical	5.364	3.208	.609

In this case the *cis* peak grows much faster as the amount of unsymmetrical isomer present at equilibrium increases, and for high T or small ΔE may surpass in intensity that of the third transition. As the *cis* peak grows the intensities of the 1st and 3rd bands *decrease*.

Examination of experiments[6] shows that there is an equilibrium value for *cis* peak development, reached from above (Figs. 19, 32)[6] and below (Figs. 7–9).[6] The observed behavior can be qualitatively reproduced by the theory, provided the configuration of the symmetrical isomer is chosen to be all s-*trans*. For example, the main band and third band are observed to *decrease* in intensity as the *cis* peak grows. The equilibrium mixture at room temperature corresponds roughly to the case where $n_s = {}^2/_3$ and $n_u = {}^1/_3$. This gives the following intensities, which compare favorably with the experimental values

Isomer	Excited State 1st	2nd	3rd
Symmetrical	9.617	0	0.971
Equilibrium mixture	8.201	1.068	.850

We may therefore now take $n_u/n_s \approx {}^1/_2$ giving for the heat of isomerization at 300°K. the estimated value $\Delta E = 3.2$ kcal./mole.

The intensity calculations are for a pure resonance force model, and are based on the approximation that the coefficients c_ν remain constant after isomerization. Nevertheless the agreement with experiment is probably not accidental, serving as evidence that the ground state configuration is all s-*trans*, and supporting the explanation of the *cis* peak effect given here.

The writer wishes to thank D. L. Peterson for suggesting that the all s-*trans* ground state configuration was not considered carefully enough in the early stages of the preparation of this manuscript.

SEATTLE, WASHINGTON

[CONTRIBUTION NO. 1315 FROM THE DEPARTMENT OF CHEMISTRY OF YALE UNIVERSITY, AND THE DEPARTMENT OF CHEMISTRY OF CORNELL UNIVERSITY]

Equilibria in the Fibrinogen–Fibrin Conversion. III. Heats of Polymerization and Clotting of Fibrin Monomer[1]

BY JULIAN M. STURTEVANT, MICHAEL LASKOWSKI, JR.,[2] THOMAS H. DONNELLY AND HAROLD A. SCHERAGA

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The heat evolution during the polymerization and clotting of monomeric fibrin *f*, with no thrombin added, was studied calorimetrically. The heat evolution on polymerization at *p*H 6.08 in 1 *M* NaBr followed first-order kinetics with $k = 0.090 \pm 0.010$ min.$^{-1}$ and $\Delta H = -21 \pm 1$ kcal./mole of monomer. The heat evolution on clotting at *p*H 6.88 in 1 *M* NaBr was first order with $k = 0.13 \pm 0.01$ min.$^{-1}$ and $\Delta H = -54 \pm 2$ kcal./mole of monomer; when this process was essentially complete there appeared to be an onset of a slower endothermic reaction, the existence of which, however, may have been due to experimental difficulties. The ΔH values for the initial first-order process should be corrected since a small exothermic reaction occurs in pure fibrinogen F. The corrected values for step 2 of the fibrinogen–fibrin conversion at *p*H 6.08 and 6.88 are −19 and −44.5 kcal./mole, respectively, and are compatible with a mechanism in which the polymerization of step 2 is regarded to take place by formation of intermolecular hydrogen bonds between approximately 19 histidyl acceptors and 19 tyrosyl (or amino) donors.

Introduction

The solubility of fibrin increases with increasing temperature[3,4] suggesting that the association of fibrin monomers, f (previously designated "activated fibrinogen"[5]) into intermediate polymers f_n,

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(2) U. S. Public Health Service Fellow of the National Heart Institute, 1952–1954.

(3) M. Laskowski, Jr., D. H. Rakowitz and H. A. Scheraga, THIS JOURNAL, **74**, 280 (1952).

(4) K. Laki, personal communication.

(5) (a) T. H. Donnelly, M. Laskowski, Jr., N. Notley and H. A. Scheraga, *Arch. Biochem. Biophys.*, **56**, 369 (1955). This paper should also be consulted for a more detailed discussion of the mechanism of the fibrinogen–fibrin conversion and for a more extensive bibliography. (b) The term "fibrin monomer" has been suggested by Laki.[6]

(6) K. Laki, *Arch. Biochem. Biophys.*, **32**, 317 (1951).

and ultimately fibrin clots, should be accompanied by a measurable heat evolution. Recently it has been shown[5a] that preparations of fibrin dissolved in 1 *M* NaBr (free of thrombin T, fibrinopeptide P, and fibrinogen F) remain monomeric at *p*H 5.1–5.3, polymerize appreciably at *p*H 5.7–6.1, and form fibrin clots at *p*H values more alkaline than 6.2. These observations are compatible with the following steps, which are regarded as reversible.[3]

$$F \xrightleftharpoons{T} f + P \qquad \text{Proteolysis} \qquad (1)$$

$$nf \rightleftharpoons f_n \qquad \text{Polymerization} \qquad (2)$$

$$mf_n \rightleftharpoons \text{fibrin} \qquad \text{Clotting} \qquad (3)$$

where n and m are variable numbers. With the availability[5a] of solutions of f the heats of reaction of steps 2 and 3 can be obtained by direct calorimetric measurements without complications from the proteolytic reaction of step 1.

Further, in conjunction with ionization data,[7] evidence will be provided to show that intermolecular hydrogen bonds are formed in step 2; also the number of donor and acceptor groups involved will be computed. Finally, it is possible to identify these groups and to understand why extensive polymerization occurs in the *p*H range between 5.5 and 10.5.

Experimental

Bovine fibrinogen was prepared from plasma Fraction I (Pentex Laboratories, Lot No. C0503) by a modification of the method of Laki.[6] After filtering off the first precipitate formed in the cold, the filtrate was adjusted to 0.21 saturation with ammonium sulfate at room temperature. The resulting stringy, sticky precipitate was discarded. The filtrate was then adjusted to 0.25 saturation and the flake-like precipitate retained and treated as before.[8] The resulting preparations were 90% clottable when tested by the method of Laki.[6,8]

Solutions of fibrin monomer f in 1 *M* NaBr at *p*H 5.08 were prepared by the method described in the preceding paper.[5a] All other materials were analytical reagents.

All *p*H measurements were made with a Beckman model GS instrument, standardized at *p*H 4.00 and 7.00 with Beckman buffers. The ultracentrifuge runs were carried out with a Spinco Model E ultracentrifuge at 59,780 r.p.m. and room temperature.

The calorimetric method was described previously,[9] all the measurements being carried out at 25.00°. In the calorimetric runs 12-ml. volumes of fibrinogen F, or fibrin monomer f (the concentration of clottable protein[10] being 2.91%) in 1 *M* NaBr, buffered at *p*H 5.08 with 0.1 *M* acetate buffer, were mixed with equal volumes of appropriate 0.1 *M* phosphate buffer in 1 *M* NaBr to raise the *p*H of the solution to the desired value. The heat evolution in the resulting 1.46% solutions was then followed calorimetrically as a function of time. In all cases duplicate runs were made.

Results

On bringing fibrin monomer f from *p*H 5.08 to *p*H 6.08, as described above, a large, essentially instantaneous heat absorption was observed. Since the heats of ionization of phosphoric and acetic acids are quite low[11] this heat of mixing was probably due in large part to essentially instantaneous ionization of histidyl residues (see below) on the fibrinogen (or fibrin monomer, respectively). The very rapid heat absorption was then followed by a slower heat evolution accurately obeying first-order kinetics (Fig. 1), characterized by a rate constant $k = 0.090 \pm 0.010$ min.$^{-1}$, up to at least 90% completion. The heat *evolved* corresponded to a value $\Delta H = -21 \pm 1$ kcal./mole of monomer, taking 330,000 as the molecular weight of bovine fibrinogen.[12–15]

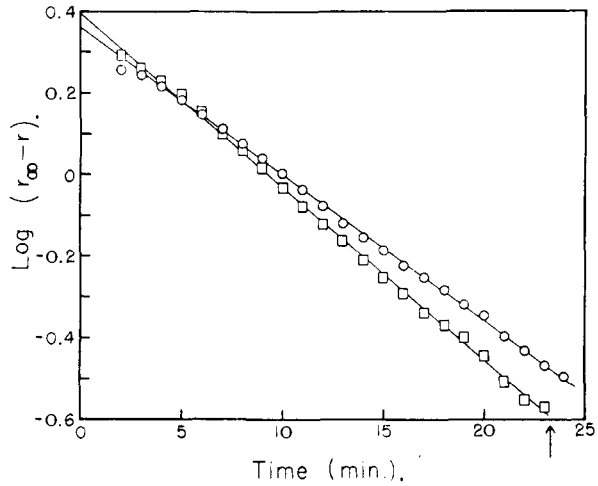

Fig. 1.—A plot of log ($r_\infty - r$) against time in minutes, showing the first-order character of the rate of heat evolution for duplicate runs on the polymerization of f at *p*H 6.08. The initial heat of mixing is not recorded. (The symbols are defined in reference 9.) The arrow indicates the time at which approximately 90% of the reaction, indicated by the squares, was complete.

Since no clots resulted at *p*H 6.08, even after a day, step 3 must not have taken place. However, a very high degree of polymerization in step 2 is indicated by the ultracentrifuge run (Fig. 2) and by the fact that an increase in the *p*H to only 6.2 resulted in clotting. Further, a very high degree of polymerization should be expected from the reported light scattering studies[5a] in 1 *M* NaBr at lower concentrations of fibrin monomer. Thus, it is felt that the degree of polymerization at the high concentration used here is sufficiently large so that the number of unreacted sites may be neglected compared to those which have reacted. Therefore, the heat evolved per monomer is essentially equal to the heat evolved per link formed. We shall discuss below the determination of the number of hydrogen bonds formed per link.

(7) E. Mihalyi, *J. Biol. Chem.*, **209**, 723, 733 (1954).

(8) Even though this modification causes a great loss in yield, and some loss in clottability, it was adopted since all of the fibrinogen preparations obtained by the Laki method from the recently available lots of Fraction I (Armour Laboratories N2510, P2602, P2803, P3004 and Pentex Laboratories C0503, C0504) developed precipitates in 1 *M* NaBr at room temperature and had undesirable clotting characteristics. This is in contradistinction to older lots such as Armour Laboratories L110 which yielded good preparations by Laki's method. With the modification of this method introduced here, Pentex lot C0503 and Armour lot P2803 gave satisfactory fibrinogen solutions.

(9) A. Buzzell and J. M. Sturtevant, THIS JOURNAL, **73**, 2454 (1951).

(10) Determined by the method of P. R. Morrison, *ibid.*, **69**, 2723 (1947).

(11) S. A. Bernhard, *J. Biol. Chem.*, in press; H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, N. Y., 2nd Edition, 1950, pp. 583, 614.

(12) S. Katz, K. Gutfreund, S. Shulman and J. D. Ferry, THIS JOURNAL, **74**, 5706 (1952).

(13) S. Shulman, *ibid.*, **75**, 5846 (1953).

(14) H. A. Scheraga, W. R. Carroll, L. F. Nims, E. Sutton, J. K. Backus and J. M. Saunders, *J. Polymer Sci.*, **14**, 427 (1954).

(15) The slight expected difference (*ca.* 10,000) in molecular weights of fibrin monomer f, and fibrinogen F, is neglected.

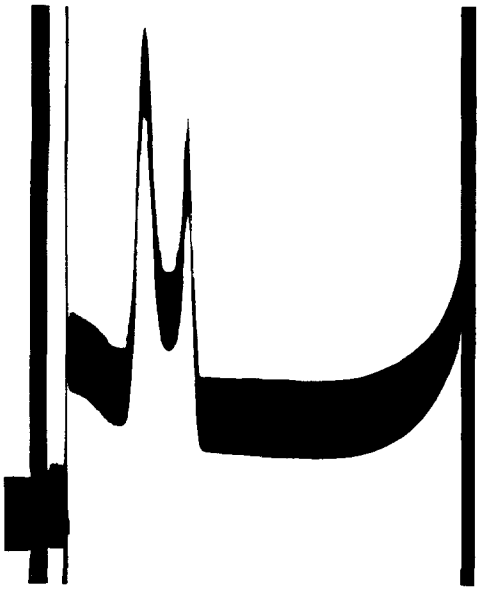

Fig. 2.—Sedimentation pattern of a 1.46% solution of fibrin monomer in 1 M NaBr at pH 6.08 showing the presence of intermediate polymers. The sedimentation patterns of such solutions at and below pH 5.3 show only the slow peak under the same conditions, as do those of F at all pH's studied.[5a]

A control experiment with fibrinogen F, which remains monomeric between pH's 5.08 and 6.08, led to a very similar heat absorption on mixing and then only to a slight, slow heat evolution of the order of -1 to -3 kcal./mole of fibrinogen. Thus, it appears that the heat evolution on changing the pH in the solution of fibrin monomer is almost entirely due to polymerization (step 2) and not to some intramolecular changes extraneous to the polymerization process. We can correct the value of ΔH from -21 to -19 kcal./mole at pH 6.08 to take account of this reaction in F.

Raising the pH of fibrin monomer f from 5.08 to 6.88 resulted in clotting in roughly 20 minutes after mixing. In the calorimeters there was an initial large heat absorption on mixing and then a heat evolution. The heat evolution was accurately first order, but after this first-order reaction (*i.e.*, step 2) was essentially complete it was complicated by a slower heat *absorption* which continued for at least 4 hr., and possibly longer, as a zero-order reaction. The possibility that the presence of a fibrin clot in the calorimeter may affect its response prevents us from identifying this latter endothermic reaction at present. Further study of this phenomenon appears to be in order. The quantitative values obtained for the first-order heat evolution at pH 6.88 are $k = 0.13 \pm 0.01$ min.$^{-1}$ and $\Delta H = -54 \pm 2$ kcal./mole of monomer (m.w. = 330,000).

A control experiment with fibrinogen F between pH 5.08–6.88 yielded, after the initial heat of mixing, a first-order heat evolution with $k = 0.19 \pm 0.02$ min.$^{-1}$ and $\Delta H = -9.5 \pm 1.0$ kcal./mole. The source of this heat evolution is unexplained. It may be due to either some slow intramolecular changes or to a slow polymerization reaction similar to, although much smaller in extent, than that of fibrin monomer. However, no evidence for such a polymerization of native fibrinogen in 1 M NaBr has been found from sedimentation and flow birefringence studies.[5a] It appears that -9.5 kcal./mole should be subtracted from -54 kcal./mole to give a value of -44.5 kcal./mole for step 2 at pH 6.88.

It is further worth noting that the polymerization of f differs from other protein associations in that it is accompanied by a large negative heat of association. In several other systems the heats of association are either quite small or positive (*e.g.*, soybean trypsin inhibitor with trypsin,[16,17] insulin,[18,19] α-chymotrypsin,[20] T1 bacteriophage with E. coli bacteria,[21] and antigen–antibody reactions[22,23]).

Discussion

Having obtained values for ΔH of -19 and -44.5 kcal./mole for step 2 at pH's 6.08 and 6.88, respectively, we can interpret them in terms of intermolecular hydrogen bonding by accounting for the sign and the pH dependence of the magnitude of ΔH. No further discussion of step 3 will be given.

It has been suggested[5a,7,24] that steps 2 and 3 involve the formation of intermolecular hydrogen bonds between fibrin monomers f. Further, it was postulated several years ago[25] that histidyl groups are involved in the polymerization process. In the hydrogen bonding picture[5a,7,24] it has been suggested that these histidyl groups function as acceptors and that tyrosyl and amino groups function as donors. The Discussion given below adds evidence to the identification of the donors and acceptors as tyrosyl (or amino) and histidyl groups, respectively. Such donors should lose their protons above pH 10.5 while the acceptors should acquire protons below pH 5.5, thus enabling steps 2 and 3 to occur only in the pH region of approximately 5.5 to 10.5. On the basis of this model we can calculate the values of ΔH as a function of pH for step 2, for comparison with the observed values. Further, we can obtain information on the nature and number of the donor and acceptor groups involved in the polymerization.

In such calculations we shall assume that each donor site on a molecule of fibrin monomer is composed of r equivalent donors DH and that each corresponding acceptor site is composed of the same number r of equivalent acceptors A.[26] For the sake

(16) A. Dobry and J. M. Sturtevant, *Arch. Biochem. Biophys.*, **37**, 252 (1952).

(17) R. F. Steiner, *ibid.* **49**, 71 (1954).

(18) P. Doty and G. E. Myers, *Disc. Faraday Soc.*, **13**, 51 (1953).

(19) R. F. Steiner, *Arch. Biochem. Biophys.*, **44**, 120 (1953).

(20) R. F. Steiner, *ibid.*, **53**, 457 (1954).

(21) A. Garen, *Biochim. Biophys. Acta*, **14**, 163 (1954).

(22) R. F. Steiner, *Arch. Biochem. Biophys.*, **55**, 235 (1955).

(23) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3499 (1955).

(24) M. Laskowski, Jr., T. H. Donnelly and H. A. Scheraga, Abstracts of the 124th meeting of the American Chemical Society, Chicago, Illinois, p. 37C, Sept. 1953.

(25) S. Shulman and J. D. Ferry, *J. Phys. Colloid Chem.*, **54**, 66 (1950).

(26) For a more detailed discussion of these symbols and the definitions of the thermodynamic functions characterizing intramolecular hydrogen bonds see reference 27. Also, see reference 5a for more details on the mechanism of polymerization.

(27) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

of simplicity we shall further assume that polymerization requires a unique fit, *i.e.*, that the i^{th} donor on one monomer will be constrained to be hydrogen bonded only to the j^{th} acceptor on another monomer. The characteristic equilibrium constant[26,28] for this process will be called K_{ij}.

When the pH is brought from 5.08 to some higher value at which it is desired to measure the heat of polymerization, some of the acceptors AH (and also some of the donors DH if the pH is high enough) ionize, this heat of ionization appearing in the initial heat of mixing. The distribution of species at equilibrium among the un-ionized forms DH and AH, and the ionized forms D and A, will be governed by the prevailing pH and by the ionization constants K_1 and K_2 of DH and AH, respectively. In the pH range in which the calorimetric measurements were carried out here (6 to 7) the donors will exist primarily in the un-ionized form DH. Polymerization can occur then by intermolecular hydrogen bond formation. After polymerization takes place the acceptors exist in one of three forms, (a) the hydrogen bonded (DH . . . A), (b) the ionized, non-hydrogen bonded A, and (c) the un-ionized, non-hydrogen bonded AH. Thus, in the pH range 6 to 7, the calorimetrically determined ΔH is a measure of two reactions: (1) some hydrogen bonds form between some donors DH and some ionized acceptors A, and (2) some protons are released from AH to form more A. This latter reaction takes place in order to maintain the equilibrium between AH and A, after some A is removed by hydrogen bonding. At a higher pH, perhaps about 10, all the acceptors would be ionized and some of the donors would be ionized. Polymerization at such a pH would remove DH and cause some D groups to pick up protons from the buffer to maintain equilibrium between DH and D.

These effects can be treated quantitatively at any pH by taking into account the degree of ionization of both AH and DH and the effects of hydrogen bonding thereon.[27] We shall neglect the factor e^{2wZ} arising from the electrostatic dependence of K on the net charge Z of the protein since it seems unwise to introduce an additional complicating parameter. The fraction x_{ij} of the molecules which contain a hydrogen bond between the i^{th} donor and j^{th} acceptor at any pH is[26]

$$x_{ij} = \frac{P_{(DH\ldots A)}}{P_{(DH\ldots A)} + P_{(DH,A)} + P_{(D,A)} + P_{(DH,HA)}} \quad (4)$$

if we neglect $P_{(D\ldots HA)}$ and $P_{(D,HA)}$, which are vanishingly small. By the method of Appendix I of reference 27 eq. 4 becomes

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \quad (5)$$

where $[H^+]$ is the hydrogen ion activity.

(28) Even though the process described here leads to the formation of *inter*molecular hydrogen bonds we shall use the constant K_{ij}, characteristic of *intra*molecular hydrogen bonds. This is in accord with our assumption (stated earlier) that the degree of polymerization is very high at all pH's of interest. Thus, the variation in ΔH cannot be due to a change in degree of polymerization with pH but must be due to a change in the average number of hydrogen bonds formed per link and to concomitantly induced changes in ionization. Such a theory must necessarily fail to apply at low concentrations and at extremely high and low pH's, where the degree of polymerization is sufficiently low to be a significant function of concentration.

The net number of protons released per ij^{th} pair, *after* the initial mixing but *during* the polymerization process is

$$q \equiv \{[P_{(DH,HA)}]_{initial} - [P_{(DH,HA)}]_{final}\} - \{[P_{(D,A)}]_{initial} - [P_{(D,A)}]_{final}\} \quad (6)$$

where the subscripts "initial" and "final" refer to the state of the system at the new pH before and after polymerization and the subsequently induced ionization have taken place. In the "initial' state no intermolecular hydrogen bonds exist whereas in the "final" state we have to take into account the presence of the species $P_{(DH\ldots A)}$. Therefore

$$q = \left\{\frac{[H^+]/K_2}{1 + K_1/[H^+] + [H^+]/K_2} - \frac{[H^+]/K_2}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2}\right\} - \left\{\frac{K_1/[H^+]}{1 + K_1/[H^+] + [H^+]/K_2} - \frac{K_1/[H^+]}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2}\right\} \quad (7)$$

$$= x_{ij}\,\frac{[H^+]/K_2 - K_1/[H^+]}{1 + K_1/[H^+] + [H^+]/K_2} \quad (8)$$

The quantity q will be positive or negative depending upon the pH. At low pH it will be positive since ionization of AH releases protons to the buffer; at high pH it will be negative since D will be taking up protons from the buffer. q will be zero when, according to equation 8

$$[H^+] = \sqrt{K_1K_2} \quad (9)$$

A plot of q against pH (*i.e.*, eq. 8) will have a maximum and a minimum which can be obtained as follows. At low pH (*i.e.*, $[H^+] \sim K_2$) we can neglect $K_1/[H^+]$. Recalling[27] that $K_{ij} \sim 1$, these approximations enable eq. 8 to be reduced to

$$q = \frac{[H^+]/K_2}{1 + [H^+]/K_2}\left(\frac{K_{ij}}{1 + K_{ij} + [H^+]/K_2}\right) \quad (8')$$

at low pH. The maximum in this function occurs at

$$[H^+]/K_2 = \sqrt{1 + K_{ij}} \quad (10)$$

Similarly, at high pH (*i.e*, $[H^+] \sim K_1$), we can neglect $[H^+]/K_2$ and reduce eq. 8 to

$$q = \frac{-K_1/[H^+]}{1 + K_1/[H^+]}\left(\frac{K_{ij}}{1 + K_{ij} + K_1/[H^+]}\right) \quad (8'')$$

The minimum in this function occurs at

$$K_1/[H^+] = \sqrt{1 + K_{ij}} \quad (10')$$

The complete function, q *vs.* pH, is plotted according to eq. 8 in Fig. 3 with K_1 and K_2 obtained by applying the above theory to data of Mihalyi.[7] Mihalyi determined the number of protons liberated as a function of pH for step 1 and for the combined steps 1, 2 and 3. While our 1 M NaBr solvent differs from his we shall neglect such differences in solvent. Mihalyi subtracted out the results for step 1 and obtained data for the combined steps 2 and 3 which we shall assume to be characteristic of step 2. Mihalyi's resulting data, which can be deduced from Fig. 2 on p. 737 of reference 7, show a maximum at pH 6.0 and the suggestion of a minimum at a pH which we have estimated as 9.8. Applying eqs. 10 and 10′ to the

maximum and the minimum, respectively, and using a value of unity[26,28] for K_{ij} we obtain $pK_1 = 9.65$ and $pK_2 = 6.15$. From these data, we conclude that the acceptor is the imidazole group of histidine and that the donor can be either a tyrosyl or an ϵ-amino group. Additional work is in progress in our laboratory to identify further the donor group. Applying eq. 9 to these values of K_1 and K_2 we should expect q to be zero at pH 7.9. Inspection of Mihalyi's curve (Fig. 4 on p. 739 of reference 7) shows that this condition is met at pH 7.6, in good agreement with the theory. With the above values of K_1, K_2 and K_{ij} it is then possible to compute curves for the pH dependence of x_{ij} and q from eqs. 5 and 8, respectively. These are shown in Fig. 3. The agreement between the theoretical curve for q and the experimental data of Mihalyi[7] (not shown in Fig. 3) is striking. It is worth noting that x_{ij} approaches zero below pH 5 and above pH 10. Arrows are drawn in Fig. 3 at pH values where $x_{ij} = 0.1$ to indicate the approximate pH range for polymerization. These limits, outside of which clotting does not occur, represent the region within which the above theory is valid. Outside of these limits the approximation, that the degree of polymerization is high, breaks down.[28] Thus, the real curves should approach zero near these pH limits more rapidly than do the theoretical curves shown in Fig. 3.

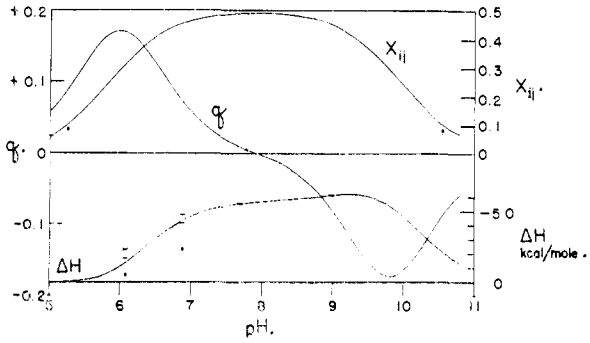

Fig. 3.—Curves showing the pH dependence of x_{ij}, q and ΔH for step 2, computed from parameters discussed in the text. The experimental points at pH 6.08 and 6.88, indicated by the rectangles, are included for comparison with the theoretical curve. The crosses are theoretical points obtained if the value of 8 instead of 1 is assumed for K_{ij} (see text).

In addition to the pK values we can evaluate the quantity r. The number of hydrogen ions released at the maximum (*i.e.*, the value of rq at pH 6) was found by Mihalyi to be 3.3 per monomer of molecular weight 330,000. Substitution of eq. 10 into eq. 8′ yields for $q_{max.}$

$$q_{max.} = -q_{min.} = \frac{K_{ij}}{(1 + \sqrt{1 + K_{ij}})^2} \qquad (11)$$

Again setting $K_{ij} = 1$, we obtain $q_{max.} = 0.17$. Since $rq_{max.} = 3.3$, we obtain $r = 19$ as the number of donors or acceptors per site.

We can now obtain a theoretical expression for ΔH as a function of pH. At any pH the total heat evolved per link (*i.e.*, per rx_{ij} hydrogen bonds) formed is

$$\Delta H = rx_{ij}\left\{\Delta H^0_{ij} + \frac{(\Delta H^0_2 - \Delta H^0_{buffer})[H^+]/K_2 - (\Delta H^0_1 - \Delta H^0_{buffer})K_1/[H^+]}{1 + K_1/[H^+] + [H^+]/K_2}\right\} \qquad (12)$$

where $r = 19$, x_{ij} is a known function of pH (Fig. 3), ΔH^0_{ij} is the heat of formation of the ijth hydrogen bond (previously estimated[27] as -6 kcal./mole), $(\Delta H^0_2 - \Delta H^0_{buffer})$ is the heat of transfer of a proton from AH to the buffer, and $(\Delta H^0_1 - \Delta H^0_{buffer})$ is the corresponding heat for DH. The value of ΔH^0_2 for histidyl groups is approximately[29] $+7$ kcal./mole, and the heats of ionization of phosphate and acetate are so low[11] that they can be neglected in this rough calculation. The theoretical curve computed from eq. 12 is shown in Fig. 3 together with the experimental points obtained at pH 6.08 and 6.88. These points, indicated by rectangles, are in good agreement with the theoretical curve. The reasonableness of the choice of $K_{ij} = 1$ (in addition to theoretical grounds[27]) is demonstrated by the fact that the above theory, with K_{ij} taken as 8, would lead to values at pH 6.08 and 6.88 indicated in Fig. 3 by crosses. Higher values of K_{ij} would increase the disagreement between theory and experiment. Having justified the choice of $K_{ij} = 1$ we become more confident in the values obtained for K_1 and K_2 (from eqs. 10 and 10′) and, therefore, in the identification of the acceptor groups as histidyls and the donor groups as tyrosyls (or lysyls). It should be pointed out that the agreement between the calculated and theoretical values of ΔH at pH 6.08 and 6.88 does not depend on a knowledge of the nature of the donor groups. Only the *high* pH portion of the curve depends on ΔH^0_1. This part of the curve was computed by *assuming* the donor group to be tyrosyl with a value of $+6$ kcal./mole for ΔH^0_1, and ΔH^0_{buffer} to be essentially zero.

In light of the theory discussed herein, and of the agreement between experimental and calculated values of ΔH it is suggested that the polymerization of fibrin monomer takes place through a hydrogen bonding mechanism involving about 19 histidyl acceptors and about 19 donors which are either tyrosyl or ϵ-amino groups or both.

Finally, it is worth noting that if, in keeping with recent experience, the molecular weight of fibrinogen continues to be revised downwards, then the agreement between our experimental and theoretical values of ΔH will be unaffected. The value of $rq_{max.}$ depends on molecular weight, and therefore the value of r does also, the value being 19 per monomer of molecular weight 330,000. From eq. 12, the value of ΔH is proportional to r. However, whereas a revised molecular weight would give a different theoretical value of ΔH, it would change the experimental value of ΔH correspondingly, keeping the experimental values in agreement with the theoretical ones, as shown in Fig. 3. The numerical values of only r and ΔH, but not K_1, K_2 and K_{ij}, depend on the choice of molecular weight.

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(29) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 445.