Equilibria in the Fibrinogen–Fibrin Conversion. VII. On the Mechanism of the Reversible Polymerization of Fibrin Monomer*

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ABSTRACT: An investigation was made of the mechanistic implications of the previously determined ionization and enthalpy changes in the reversible polymerization of fibrin monomer in 1.0 M NaBr at 25.0°. The mathematical treatment of the pH dependence of these quantities, originally derived for a polymerization model involving intermolecular hydrogen bonding between ionizable groups, was restated in more general terms applicable to other types of bonding between such groups.

Taking the maximum heat of formation of a single hydrogen bond between groups in water as *ca.* −1.5 kcal/mole (rather than the previous overestimate of −6 kcal/mole), it is not possible to account satisfactorily for the observed results with the simple hydrogen-bonding model. Consideration was also given without success to the possibility of hydrogen bonds buried in nonpolar regions and of hydrogen bonds competing with anion binding to the protonated form of the acceptor group. On the other hand, the results appear to be entirely consistent with the postulation of intermolecular coordinate covalent bonds, in which the electron donors are α-amino groups of the *N*-terminal amino acid residues, and the acceptors are imine-type functional groups. This view is supported by the known involvement of the α-amino groups in the subsequent irreversible clot-stabilization reaction. The possible relation between reversible polymerization and clot stabilization is discussed, and a mechanism is suggested for covalent bond formation. In this mechanism, the proposed imine-type acceptor groups are derived from carbohydrate-bound side-chain amide groups.

Previous papers in this series have described the results of investigations of the ionization changes (Endres *et al.*, 1966, preceding paper) and heat evolution (Sturtevant *et al.*, 1955) accompanying the reversible polymerization of fibrin monomer in 1.0 M NaBr at 25.0°. In the present paper, the mechanistic implications of the combined results will be considered.

Method of Data Treatment

In an earlier treatment of this problem, Sturtevant *et al.* (1955) postulated that the ionization and enthalpy changes in the reversible polymerization of fibrin monomer result from formation of numerous intermolecular hydrogen bonds between ionizable donor and acceptor groups during polymerization. A finite equilibrium constant was assumed to represent hydrogen bond formation in each of the equivalent donor–acceptor group pairs. From this model theoretical expressions were derived, giving the pH dependence of the ionization and enthalpy changes in terms of thermodynamic parameters of the system, *i.e.*, the equilibrium constants and standard enthalpy differences for hydrogen bond formation and for ionization of the donor and acceptor groups. However, the mathematical form of their treatment does not depend on the postulation of a particular mechanism of bonding. All that it requires is the interaction of pairs of ionizable groups, one group being in its protonated form and the other in its deprotonated form. The interaction must be sufficiently strong to displace the ionization equilibria of the groups involved. The observed release or absorption of protons during polymerization is interpreted as the result of the displacement of these equilibria, and the calorimetric heat of reaction as the algebraic sum of the enthalpy differences due to protonation, deprotonation, and bonding interactions.

With the above considerations in mind, the development of Sturtevant *et al.* (1955) will now be restated in more general terms, without specifying the mechanism of the bonding interaction or the relative magnitudes of the equilibrium constants. The following assumptions are made: (1) Each fibrin monomer molecule contains some fixed number r (>1) of ionizable, chemically equivalent functional groups G_1H, and an equal number of equivalent groups G_2H, which are potentially involved in intermolecular bond formation. The ionization constants of these groups are designated K_1 and K_2, respectively. The general case where G_1H and G_2H are different will be assumed in the following, but the case where these groups are identical can also be developed along similar lines. (2) Each intermolecular bond involving these functional groups is formed by the protonated form of G_1H and the deprotonated form of G_2H, *i.e.*, G_2. Reaction between G_1 and G_2H is assumed

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not to occur. (3) There is a unique conformational fit in polymerization, *i.e.*, the *i*th group G_1H on one monomer molecule can form a bond only with the *j*th group G_2 on another molecule. (4) The initial monomer concentration and pH of polymerization are such that, at equilibrium, the degree of polymerization is high. Thus, it is possible to regard the bonds formed as intramolecular with respect to the product polymer, although they are intermolecular with respect to the reactant monomer units. The equilibrium constant for bond formation in a given pair of functional groups is designated K_{assocn}, and is assumed to have the same value for each pair.

The relevant equilibria may be summarized as follows

$$G_1H \rightleftharpoons G_1 + H^+ \quad K_1 \tag{1}$$

$$G_2H \rightleftharpoons G_2 + H^+ \quad K_2 \tag{2}$$

$$G_2, G_1H \rightleftharpoons G_2G_1H \quad K_{assocn} \tag{3}$$

where possible charges are ignored, and the nature of the bond in G_2G_1H is unspecified for the moment. Since the mathematical development from this point is analogous to the previous treatment (Sturtevant *et al.*, 1955) the details will not be repeated here. However, the principal derived relations will be summarized and restated in the present context.

The fraction x_{ij} of each of the *r* group pairs in which a bond is formed at equilibrium is given by

$$x_{ij} = \frac{K_{assocn}}{1 + K_{assocn} + K_1/[H^+] + [H^+]/K_2 + K_1/K_2} \tag{4}$$

where $[H^+]$ is the hydrogen ion activity. The net number of protons released during polymerization per *ij*th pair is then

$$q = x_{ij}\frac{[H^+]/K_2 - K_1/[H^+]}{1 + K_1/[H^+] + [H^+]/K_2 + K_1/K_2} \tag{5}$$

The experimental quantity Δh, the net number of equivalents of protons released per 10^5 g of protein, is related to q as follows

$$\Delta h = rq/3.3 \tag{6}$$

if the molecular weight of fibrin monomer is taken as 3.3×10^5. By substitution from eq 5

$$\Delta h = \frac{rx_{ij}([H^+]/K_2 - K_1/[H^+])}{3.3(1 + K_1/[H^+] + [H^+]/K_2 + K_1/K_2)} \tag{7}$$

The heat evolved per mole of monomer during polymerization is given by

$$\Delta H = r(x_{ij}\Delta H^0_{assocn} - q_1\Delta H^0_1 + q_2\Delta H^0_2) \tag{8}$$

where ΔH^0_{assocn} is the enthalpy of bond formation, q_1 is the number of protons absorbed by G_1 per *ij*th pair, q_2 is the number of protons released by G_2H per *ij*th pair, and ΔH^0_1, ΔH^0_2 are the standard enthalpies of ionization of the respective groups. (It is assumed as before that the enthalpies of ionization of the buffers employed in the calorimetric measurements are negligibly small.) By the methods previously employed it can be shown that q_1 and q_2 are given by

$$q_1 = x_{ij}\frac{K_1/[H^+] + K_1/K_2}{1 + K_1/[H^+] + [H^+]/K_2 + K_1/K_2} \tag{9}$$

$$q_2 = x_{ij}\frac{[H^+]/K_2 + K_1/K_2}{1 + K_1/[H^+] + [H^+]/K_2 + K_1/K_2} \tag{10}$$

Substitution in eq 8 then yields:

$$\Delta H = rx_{ij}\left\{\Delta H^0_{assocn} + \frac{([H^+]/K_2 + K_1/K_2)\Delta H^0_2 - (K_1/[H^+] + K_1/K_2)\Delta H^0_1}{1 + K_1/[H^+] + [H^+]/K_2 + K_1/K_2}\right\} \tag{11}$$

Equations 4, 5, 7, and 11 differ from the corresponding relations derived by Sturtevant *et al.* (1955) in the inclusion of the term K_1/K_2. This term can be ignored if K_1 is much smaller than K_2, which was assumed in their treatment. It will be shown below that this assumption is justifiable in the case of the hydrogen bonding model, but we will consider the more general case where the relative magnitudes of K_1 and K_2 are not restricted.

Equations 7 and 4 give the pH dependence of the ionization change due to polymerization in terms of the four parameters *r*, K_{assocn}, K_1, and K_2. These can be most conveniently related to the experimental data by analysis of the 0 point and extrema as follows. It is evident from eq 5 and 6 that Δh is 0 when $[H^+] = [H^+]_x$, where

$$[H^+]_x = (K_1K_2)^{1/2} \tag{12}$$

It can be shown that Δh is at a maximum when $[H^+] = [H^+]_{max}$, where

$$[H^+]_{max} = K_2[(1 + K_{assocn} + K_1/K_2) \times (1 + K_1/K_2)]^{1/2} \tag{13}$$

and at a minimum when $[H^+] = [H^+]_{min}$, where

$$[H^+]_{min} = K_1/[(1 + K_{assocn} + K_1/K_2) \times (1 + K_1/K_2)]^{1/2} \tag{14}$$

The magnitudes of Δh at the maximum and minimum are given by

$$\Delta h_{max} = -\Delta h_{min} = \frac{rK_{assocn}}{3.3[(1 + K_{assocn} + K_1/K_2)^{1/2} + (1 + K_1/K_2)^{1/2}]^2} \tag{15}$$

(While eq 12 is an exact relation from the theory, eq 13–15 are close approximations). If $K_1 \ll K_2$, these re-

TABLE I: Summary of Experimental Results in the Polymerization of Fibrin Monomer in 1.0 M NaBr at 25.0°.

Quantity	Exptl Value	Ref
Δh_{max}	1.10 ± 0.03 equiv/10^5 g	Endres *et al.* (1966), preceding paper
$(pH)_{max}$	6.39 ± 0.05	Endres *et al.* (1966), preceding paper
pH_x	7.65 ± 0.015	Endres *et al.* (1966), preceding paper
ΔH (pH 6.08)	−19 ± 1 kcal/mole	Sturtevant *et al.* (1955)
ΔH (pH 6.88)	−44.5 ± 2 kcal/mole	Sturtevant *et al.* (1955)

lations reduce to the simpler forms given by Sturtevant *et al.* (1955).

Table I summarizes the values of the experimental quantities of eq 12–15 in 1.0 M NaBr at 25.0° (Endres *et al.*, 1966) and the experimental heats of reaction in the same medium (Sturtevant *et al.*, 1955). It was not possible to obtain a reliable value for $(pH)_{min}$ under these conditions, since steady final values of pH were not observed in this region. Unfortunately, it is not possible to derive a unique set of values for the four parameters of interest by application of eq 12–15 to the experimental pH dependence of Δh even if $(pH)_{min}$ is accurately known, since together they constitute only three independent equations. As in the treatment of Sturtevant *et al.* (1955) it is necessary to assume a value for one parameter in order to define the other three. It is convenient in the present case to use r as a parameter and solve eq 12, 13, and 15 simultaneously. Solution of eq 12 for K_1 yields

$$K_1 = [H^+]_x^2/K_2 \tag{16}$$

which, upon substitution in eq 13 and 15, leads to two equations in two unknowns, K_2 and K_{assocn}. Simultaneous solution of these two equations is facilitated by the following substitutions

$$1 + K_{assocn} + [H^+]_x^2/K_2^2 = y \tag{17}$$

$$1 + [H^+]_x^2/K_2 = x \tag{18}$$

which lead to a quadratic equation in the single variable x. The real solutions of the quadratic equation give, in general, a pair of possible values of K_2 for each assumed value of r; for each value of K_2 there are corresponding values of K_1 and K_{assocn}. (Examples are shown in Table II, for r = 4–6, corresponding to the covalent bonding model to be described below.) In each such pair of solutions, the values of K_1 and K_2 are found to be interchanged, and the values of K_1/K_2 are mutually reciprocal. The larger value of K_{assocn} pertains to $K_1/K_2 > 1$, and the smaller value to $K_1/K_2 < 1$. The physical meaning of this dual solution for each value of r is very interesting. Consider, *e.g.*, the situation at relatively low pH. If $K_1/K_2 < 1$, groups G_1H and G_2H tend to be in the state of ionization required for bond formation, *i.e.*, G_2, G_1H, and a relatively low K_{assocn} suffices for a given extent of bonding. On the other hand, if $K_1/K_2 > 1$ the form G_2, G_1H is less favored, and a relatively high K_{assocn} is required.

The shape of the curve defined by eq 7 is not very sensitive to r, provided that the set of parameters satisfies eq 12–15. On the other hand, the analysis of calorimetric data according to eq 11 can provide a powerful independent test of any specific bonding mechanism proposed to account for the ionization changes. *E.g.*, if the identities of groups G_1H and G_2H are inferred from their pK values derived from eq 12 to 15, their expected enthalpies of ionization can be estimated from data for model compounds and other proteins (Tanford, 1962). Application of eq 11 to the calorimetric data will then yield the value of ΔH^0_{assocn}. Alternatively, assumption of reasonable values for the latter will yield predicted values of ΔH for comparison with experiment.

The Hydrogen Bonding Model

The arguments in favor of the hydrogen bonding mechanism for the reversible stage of fibrin polymerization have been summarized in detail in a review (Scheraga and Laskowski, 1957). The postulation of hydrogen bonding is consistent with the reversibility shown on simple dilution or on small increase of temperature. The fact that fibrin clots can be redissolved to form monomeric solutions in concentrated aqueous urea or concentrated solutions of certain salts (such as 1.0 M NaBr below pH 5.5) was taken as evidence for hydrogen bonding. Further, the possible identity of the donor groups was suggested by the effects of chemical modification on the clotting of fibrinogen or fibrin. Thus, the effects of iodination or tyrosinase treatment suggested that tyrosyl residues are involved in normal polymerization, while the effects of acetylation tended to implicate the ε-amino groups of lysyl residues. However, none of the evidence cited thus far can be taken as conclusive for hydrogen bonding. It was formerly believed that the dissociating and denaturing effects of aqueous urea on proteins are due to the rupture of inter- or intramolecular hydrogen bonds, but this view has been seriously questioned more recently (Levy and Magoulas, 1962). It seems quite possible that the depolymerizing effects on fibrin of concentrated aqueous salt or urea solutions are the results of effects on the water structure, with consequent rupture of intermolecular hydrophobic and/or electrostatic inter-

TABLE II: Derived Values of the Thermodynamic Parameters.[a]

r	Δh_{max}	K_{assocn}	K_1/K_2	pK_1	pK_2	ΔH^0_1 (Assumed)	$-\Delta H^0_{assocn}$ (Calcd)[b] pH 6.08	pH 6.88
4	0.97	159	1	7.65	7.65	1	18.6	22.7
4	0.97	159	1	7.65	7.65	7	18.4	21.8
4	0.97	159	1	7.65	7.65	13	18.3	20.9
5	0.97	285	13.5	7.08	8.21	1	18.6	22.0
5	0.97	285	13.5	7.08	8.21	7	18.1	19.7
5	0.97	285	13.5	7.08	8.21	13	17.5	17.4
5	0.97	21.1	0.074	8.21	7.08	..	...	...
5	1.10	268	6.04	7.26	8.04	1	18.0	21.8
5	1.10	268	6.04	7.26	8.04	7	17.6	20.0
5	1.10	268	6.04	7.26	8.04	13	17.6	18.3
5	1.10	44.1	0.17	8.04	7.26	..	...	...
6	0.97	281	27.6	6.93	8.37	1	18.6	21.2
6	0.97	281	27.6	6.93	8.37	7	17.8	18.3
6	0.97	281	27.6	6.93	8.37	13	17.1	15.5
6	0.97	10.2	0.036	8.37	6.93	..	...	...
6	1.10	286	17.6	7.03	8.27	1	18.0	20.6
6	1.10	286	17.6	7.03	8.27	7	17.3	18.1
6	1.10	286	17.6	7.03	8.27	13	16.7	15.6
6	1.10	16.2	0.057	8.27	7.03	..	...	...

[a] Polymerization of fibrin monomer in 1.0 M NaBr, 25.0°; ΔH in kcal/mole. [b] ΔH^0_2 is assumed to be 13 kcal/mole in all cases.

actions. Moreover, the results of experiments on proteins involving chemical modification or blocking of reactive groups must be interpreted with great caution. While of value as supporting evidence, the fact that destruction or blocking of certain functional groups changes the rate or extent of reaction does not prove that these groups are essential to, or even involved in, the normal reaction. As examples of possible complications, the normal charge pattern might be disrupted, the native conformation might be altered, or some other functional groups might be affected by side reactions.

Probably the strongest evidence for the hydrogen-bonding mechanism came from studies of the pH range of clotting (Shulman and Ferry, 1950), the ionization changes in the over-all fibrinogen–fibrin conversion in 0.3 M KCl (Mihalyi, 1954), and the heat evolution in fibrin polymerization in 1.0 M NaBr (Sturtevant *et al.*, 1955). These observations were interpreted by the latter authors on the basis of a model which is a particular case of the general mechanism represented by eq 1–3. If G_1H is the hydrogen-bond donor group DH, G_2 is the acceptor group A, and G_2G_1H is the hydrogen bonded pair DH···A, eq 1–3 may be rewritten as follows

$$DH \rightleftharpoons D + H^+ \quad K_1 \qquad (19)$$

$$AH \rightleftharpoons A + H^+ \quad K_2 \qquad (20)$$

$$DH, A \rightleftharpoons DH\cdots A \quad K_{assocn} \qquad (21)$$

where K_{assocn} is now identical with the previously employed K_{ij}, the equilibrium constant for formation of the ijth hydrogen bond (Laskowski and Scheraga, 1954). The results of this treatment will now be re-examined, taking into account th availability of data for the ionization changes in the isolated polymerization in 1.0 M NaBr (Table I) and current estimates of the enthalpy of formation of hydrogen bonds in aqueous solutions.

In their treatment of the hydrogen-bonding model, Sturtevant *et al.* (1955) assumed that $K_1 \ll K_2$, since they found that if K_{assocn} (K_{ij} in their nomenclature) is of the order of unity, pK_2 and pK_1 are close to $(pH)_{max}$ and $(pH)_{min}$, respectively, and it is found experimentally that $(pH)_{min} \gg (pH)_{max}$. Trial solutions of the more general eq 12–15 using the data of Table I and various assumed values of r indicate that their assumption is correct for solutions where K_{assocn} is less than *ca.* 10. As before, $K_{assocn} = 1$ is taken as a reasonable estimate for the equilibrium constant governing formation of hydrogen bonds in aqueous solution (Laskowski and Scheraga, 1954; Némethy *et al.*, 1963). Assuming $K_1/K_2 \ll 1$, eq 15 then yields $r = 21$ for the experimental Δh_{max} (Table I) and eq 12 and 13 give $pK_1 = 8.75$ and $pK_2 = 6.54$. It can be shown by solution of eq 12–15 for $r = 21$ without this assumption that this is a correct solution. For comparison, the values calculated by Sturtevant *et al.* (1955) from the data of Mihalyi in 0.3 M KCl were $r = 19$, $pK_1 = 9.65$, and $pK_2 = 6.15$. The present value $pK_2 = 6.54$ is consistent with their assign-

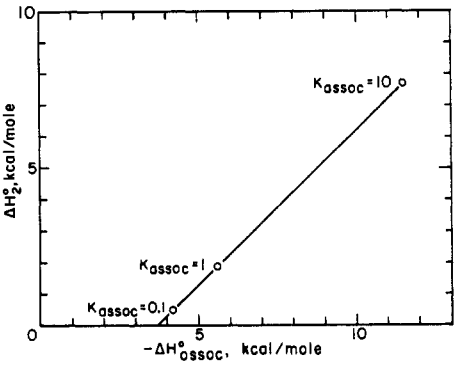


FIGURE 1: Dependence of theoretically possible values of the thermodynamic parameters of eq 22 on the assumed value of K_{assocn}, for the experimental data of Table I.

ment to histidyl residues as the hydrogen bond acceptor groups (A). The large difference in the pK_1 values is probably due mainly to the rather arbitrary assignment of $(pH)_{min}$ by Sturtevant *et al.* (1955). The present value pK_1 = 8.75 is rather low for their assignment to tyrosyl or lysyl residues as the donors (DH), since the intrinsic values (corrected to zero net charge on the protein) expected for these groups are in the range 9.4–10.8 and 9.6–10.4, respectively (Tanford, 1962). Since fibrin is probably well above its isoelectric point under the conditions of these experiments (see below), the correction would be negative, increasing the discrepancy further. Corrections for the high ionic strength of 1.0 M NaBr would be only of the order of 0.13 (Tanford, 1962). Assumption of a lower value for K_{assocn} would increase the derived pK_1, up to 8.91 in the limit $K_{assocn} \rightarrow 0$.

The analysis of the calorimetric data for the hydrogen bonding model is simplified by the small values of K_1/K_2, and by the fact that the term $K_1/[H^+]$ is small compared to $[H^+]/K_2$ in the pH range of measurement (6.08–6.88). Under these conditions, eq 11 is closely approximated by

$$\Delta H = rx_{tj}\left\{\Delta H^0_{assocn} + \frac{([H^+]/K_2)\Delta H^0_2}{1 + K_1/[H^+] + [H^+]/K_2}\right\} \quad (22)$$

in which ΔH^0_1 no longer appears. This is equivalent to the assumption that the observed proton release in this pH range is due almost entirely to release from G_2H, which can be verified by eq 9 and 10. If the acceptor groups are taken as histidyls on the basis of the derived pK_2, ΔH^0_2 should be about 7 kcal/mole (Tanford, 1962) and eq 22 requires *ca.* −8 kcal/mole for the standard enthalpy of formation of each hydrogen bond. Sturtevant *et al.* (1955) assumed the value −6 kcal/mole for the latter parameter, and successfully accounted for the measured ΔH. Recalculation with our experimental data with the same assumption would predict −4 and −35 kcal/mole for ΔH at pH 6.08 and 6.88, respectively, compared to the experimental quantities −19 and −44.5 (Table I). However, it has since become apparent that −6 kcal/mole is an overestimate of the heat of formation of a hydrogen bond between groups in aqueous solution, and that −1.5 kcal/mole would be a much more plausible estimate (Schellman, 1955; Némethy *et al.*, 1963). Taking ΔH^0_{assocn} = −1.5 kcal/mole, eq 22 predicts for ΔH the values +17 and +5 kcal/mole, in serious disagreement with experiment.

It is necessary at this point to analyze the possibility that the experimental and theoretical heats of reaction can be reconciled on the basis of the hydrogen bonding model by different assignments of the parameters ΔH^0_2 and K_{assocn} (which determines r by eq 15). The experimental heat of reaction is known at two values of pH. At each pH, assumption of a value for K_{assocn} yields by eq 22 a linear relation between the two remaining unknowns, ΔH^0_2 and ΔH^0_{assocn}. The simultaneous solution of these relations for the two different pH values then yields a point, corresponding to a unique pair of values for these parameters which will satisfy the ionization change data and the calorimetric data at both pH values, for a particular assumption or K_{assocn}. Repetition of the calculation for other values of K_{assocn} defines a curve (actually linear) which is the locus of all possible combinations of ΔH^0_2 and ΔH^0_{assocn} which will satisfy all of the data (Figure 1). It is clear that the maximum possible value for ΔH^0_{assocn} is −3.7 kcal/mole, as $K_{assocn} \rightarrow 0$ (and $r \rightarrow \infty$, by eq 15). If −1.5 kcal/mole is taken as the lower limit, the simple hydrogen bonding model will fail to account for the data, regardless of what other assumptions are made for the parameters.

The possibility must be considered that the simple model can be modified in order to explain the results, taking into account the likely complications. For this purpose ΔH^0_2 and ΔH^0_{assocn} may be regarded as apparent quantities, deviating from the values expected for the simple model of eq 19–21, but still of necessity satisfying the relation of Figure 1. One of the more obvious possible complications is ion binding to the charged forms of the functional groups in 1.0 M NaBr, about which nothing is known for fibrinogen or fibrin. It is possible, *e.g.*, that bromide ions are bound to the protonated (positively charged) forms of the imidazole groups of histidyl residues. Such binding would compete with hydrogen bond formation, which would cause ΔH^0_2 to appear greater or less than normal, if the binding is exo- or endothermic, respectively. However, Figure 1 shows that a reduced ΔH^0_2, while helpful, is insufficient to account for the data, as long as ΔH^0_{assocn} is limited to −1.5 kcal/mole. In order to rationalize the results, it is necessary to modify the model so as to lead to an expected value for ΔH^0_{assocn} more negative than −3.7 kcal/mole.

A high value of the enthalpy of formation of hydrogen bonds (∼−6 kcal/mole) might be expected if they are formed in a nonpolar environment, or in the present case if they are formed between groups which are surrounded by nonpolar side chains. Such burial of uncharged histidyl or tyrosyl side chains is conceivable,

although unexpected for charged lysyl amino groups. Since the present model requires that the bonding groups are ionizable, they would have to be in equilibrium with unburied forms; *i.e.*, they must be accessible to the aqueous solvent. Burial of polar side chains is expected to be a strongly endothermic process, through breaking of hydrogen bonds to water molecules and contributions from hydrophobic bonding. According to the treatment of Némethy *et al.* (1963), the enthalpy change associated with burial of groups of this type will be of the order of +7 to +8 kcal/mole. A large positive contribution would thus be made to $\Delta H^0_{\text{assocn}}$ or ΔH^0_2, depending on the extent to which burial took place during, or prior to polymerization, respectively. In either case, the discrepancy between theory and experiment would again be increased.

Another possible way to account for a high apparent enthalpy of hydrogen bond formation per ionizable group would be to assume additional hydrogen bonds between nonionizable groups, all bonds having $\Delta H^0_{\text{assocn}} = -1.5$ kcal/mole. These would contribute toward exothermicity without involving endothermic ionizations. *E.g.*, if ΔH^0_2 is taken as 7 kcal/mole, Figure 1 requires $\Delta H^0_{\text{assocn}} = -10.8$ kcal/mole and $K_{\text{assocn}} = 9$, from which the values $r = 7$, $pK_1 = 8.4$, and $pK_2 = 6.9$ are calculated. To account for this value of $\Delta H^0_{\text{assocn}}$, about six hydrogen bonds involving nonionizable groups are required for each hydrogen bond to a histidyl acceptor. As discussed earlier, however, there is no definitive independent evidence for an important role of hydrogen bonding in fibrin polymerization. A model which could account for the results with a single assumption of bond type would be preferable, and to this end consideration will now be given to another possibility.

The Covalent Bonding Model

The hydrogen bonding model represented an attempt to account for the exothermicity of fibrin polymerization on the basis of a large number of weakly exothermic interactions between ionizable groups. However, Figure 1 illustrates the fact that a relatively small number of more highly exothermic interactions can, in principle, account for the data, since increasing K_{assocn} corresponds to decreasing r by eq 15. As discussed earlier in connection with the hydrogen-bonding model, Figure 1 was drawn with the aid of eq 22, which is a good approximation only if K_{assocn} is less than *ca.* 10. For higher values of K_{assocn}, eq 11 must be used and a dependence on ΔH^0_1 is introduced. Trial solutions for various assumed values of r and ΔH^0_1, however, reveal that they lie on the line of Figure 1 or its linear extension even for $K_{\text{assocn}} > 10$, but the location of the point along this line is increasingly a function of ΔH^0_1, as K_{assocn} increases. It is clear, in any event, that the experimental data of Table I can be rationalized on the basis of a large positive ΔH^0_2 and a large negative $\Delta H^0_{\text{assocn}}$. This possibility will now be considered, since a large $\Delta H^0_{\text{assocn}}$ could be readily accounted for by covalent bonding between the ionizable groups.

Of the ionizable groups commonly found in proteins, amino groups are expected to be the most endothermic in ionization (~13 kcal/mole; Tanford, 1962). Assuming $\Delta H^0_2 = 13$ kcal/mole and a reasonable range for ΔH^0_1 (1–13 kcal/mole), trial solutions of eq 12–15 and 11 indicate that r is of the order of 5–6. The two solutions of eq 12–15 for $r = 6$, $\Delta h_{\max} = 1.10$ (Table II) correspond to $pK_2 = 8.27$ or 7.03 for $K_{\text{assocn}} = 286$ or 16.2, respectively. The expected intrinsic pK values (pK^0, corrected to zero net charge) for amino groups in proteins are ~7.5–8.0 for α-amino and ~10.4 for ϵ-amino. To correct the apparent pK values of Table II, use can be made of the following equation (Linderstrøm-Lang, 1924; Tanford, 1950)

$$K = K^0 e^{2wZ} \tag{23}$$

where Z is the average net charge per molecule. The isoelectric pH of fibrin is 5.6 in 10% urea at ionic strength 0.1 (Mihalyi, 1950). Taking this as an approximation in 1.0 M NaBr, and assuming no change in ion binding with increasing pH, Z can be estimated from the titration curve (Endres *et al.*, 1966) as −31 and −64 at $(pH)_{\max}$ and $(pH)_z$, respectively. The parameter w is calculated (Tanford, 1950) to be 0.0073 for a spherical protein of mol wt 3.3×10^5, at unit ionic strength at 25.0°. Taking this as a very rough estimate for the nonspherical fibrin molecule, eq 12–15 and 23 give the electrostatic corrections to be applied to the data in Table II as −0.61 and −0.20, respectively, for pK_1 and pK_2. The pK^0_2 values for $r = 6$, $\Delta h_{\max} = 1.10$ are thus estimated as 8.07 and 6.83 for $K_{\text{assocn}} = 286$ and 16.2, respectively. Neither of these values is consistent with ϵ-amino groups, but the former is quite reasonable for the α-amino groups of *N*-terminal amino acid residues. The set of parameters $r = 5$, $\Delta h_{\max} = 1.10$, $K_{\text{assocn}} = 268$, $pK_2 = 8.04$ is similarly consistent with α-amino groups (Table II).

It is known that bovine fibrin monomer has six N-terminal groups per mol wt 3.3×10^5, of which two are tyrosine and four are glycine residues (Blombäck and Yamashina, 1958). The glycine residues, at least, are known to be involved in the insolubilization of the fibrin clot in the presence of the enzyme fibrin stabilizing factor (Lorand *et al.*, 1962). Now, the simultaneous solution of eq 12–15 using the experimental data of Table I yields real solutions only for $r \geq 4.6$. However, a real solution is obtained for $r = 4$ if $\Delta h_{\max}$ is taken as 0.97, rather than the experimental value of 1.10 (Table II). Although the value 0.97 appears to be outside the limits of precision of the experimental value, it is probably not outside the limits of accuracy. The $\Delta h_{\max}$ calculated from the experimental pH changes during polymerization is dependent on the ultraviolet absorption coefficient used to estimate protein concentration, and is also subject to possible errors in converting ΔpH to Δh (Endres *et al.*, 1966). Further, the same real solution is obtained for $r = 4$, $\Delta h_{\max} = 1.10$ if the molecular weight is taken as 3.0×10^5 rather than 3.3×10^5. The value of pK_2 corresponding to $r = 4$ is 7.65 (Table II), which is still

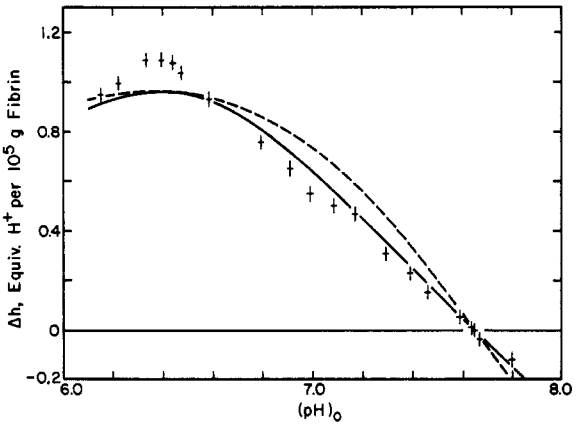


FIGURE 2: Comparison of experimental ionization changes with theoretical prediction according to eq 7, taking $\Delta h_{max} = 0.97$, $r = 4$ (dashed curve), and $r = 6$ (solid curve).

consistent with the assignment to α-amino groups. Recalculation of the results for $r = 5$ or 6, using $\Delta h_{max} = 0.97$ instead of 1.10, has little effect (Table II).

Table II also summarizes the values of ΔH^0_{assocn} calculated from the calorimetric data using eq 11 and assuming $\Delta H^0_2 = 13$ kcal/mole, the expected value for amino groups. For $r = 4$–6, $\Delta h_{max} = 0.97$–1.10, and $\Delta H^0_1 = 1$–13 kcal/mole, there are only small variations in the calculated ΔH^0_{assocn}, and the results at the two pH values agree reasonably well. The indicated magnitude of about -20 kcal/mole appears reasonable for covalent bonds, particularly of the coordinate type. Finally, Figure 2 compares the experimental dependence of Δh on $(pH)_0$ with theoretical prediction by eq 7, for $r = 4$ and 6, $\Delta h_{max} = 0.97$. The agreement is believed to be satisfactory, within the possible experimental error.

It is concluded that the ionization and enthalpy changes in the reversible polymerization of fibrin monomer in 1.0 M NaBr are satisfactorily accounted for by intermolecular covalent bonding between ionizable groups, and that the electron donors (G_2 in eq 3) can reasonably be taken as four–six N-terminal α-amino groups per monomer molecule. The possible identity of the acceptor groups (G_1) will be discussed below.

Discussion

It has been shown that the pH changes and heat evolution in the reversible polymerization of fibrin monomer *in vitro* can be more readily accounted for by covalent bonding between ionizable groups than by hydrogen bonding. On the other hand, the ready reversibility of many protein interactions under mild conditions has frequently been taken as an indication of noncovalent character. While this is very probably correct in many instances, it is not necessarily always true. In reactions of simple organic molecules in aqueous solution, covalent bond formation is frequently rapid, reversible, and pH dependent. This is particularly true of coordinate covalent bond formation by addition of electron donors to unsaturated functional groups (carbonyl, imino, etc.). In fact, covalently bonded intermediates are often postulated in enzyme–substrate interactions. If covalent bonding is indeed involved in reversible fibrin polymerization, it is probably of the coordinate type, since the derived enthalpy of bond formation is not very large ($\backsim -20$ kcal/mole, Table II).

As mentioned above in connection with the hydrogen bonding model, the nonenzymatic polymerization of fibrin can be reversed in 1.0 M NaBr by an increase of temperature, an increase or decrease of pH, or simply by dilution. The temperature effect is of course the result of the negative enthalpy of polymerization, which is readily accounted for by covalent bond formation. Dilution is expected to favor dissociation regardless of the mechanism by which it occurs. Likewise, the effect of pH is explained by any mechanism which accounts for the pH dependence of the ionization changes. In the low pH range of polymerization protons are produced, and addition of protons to the system will favor reversal, regardless of the mechanism. Similarly, at relatively high pH, protons are absorbed during polymerization, and their removal from the system will also favor reversal.

On the other hand, the reversal of polymerization by urea and the specific effects of concentrated salt solutions are not readily accounted for in terms of covalent bonding alone. It does not follow that covalent bonds are not formed, but it is implied that there must be additional factors contributing toward the favorable free energy of polymerization. It is even possible that the proposed covalent interactions are not the primary cause of the polymerization, but rather a result. That is, polymerization due primarily to interactions of a different nature may bring the appropriate ionizable groups into close proximity, permitting covalent bonding with its attendant heat evolution and proton release or absorption. Although ΔH is known from calorimetric measurements to be negative for fibrin polymerization, the magnitude of ΔF and consequently the sign of ΔS are unknown (Donnelly *et al.*, 1955). Regardless of the sign of the over-all ΔS, it may receive positive contributions from the liberation of restricted water molecules during polymerization, through intermolecular hydrophobic and/or electrostatic interactions (Scheraga, 1963). High concentrations of urea or certain salts, functioning as water structure breakers (Kavanau, 1964), may decrease this positive contribution to the entropy to the point where ΔF is no longer negative for polymerization, in spite of the negative ΔH of covalent bond formation. It is recognized that if interactions other than the postulated covalent bonds contribute to the free energy of polymerization, they may contribute significantly to the observed heat of polymerization as well. Hydrophobic interactions, *e.g.*, could make a positive contribution to ΔH (Némethy and Scheraga, 1962); in this case, the correct values of ΔH^0_{assocn} for covalent bonding would be even more negative than

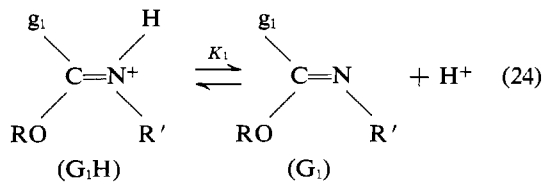
the apparent values of Table II. It is believed, however, that the possibility of other contributions to ΔH is not a serious drawback to the covalent bonding hypothesis at present, since it does not depend strongly on the numerical value of $\Delta H^0_{\text{assocn}}$.

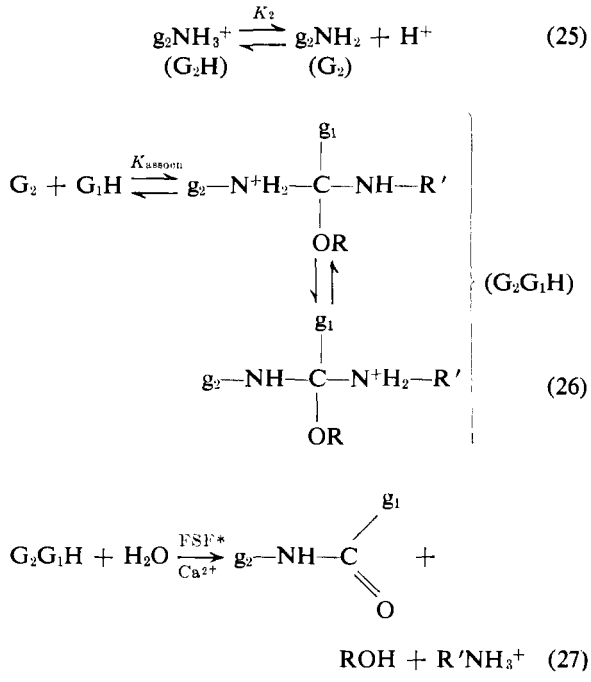
The standard entropy change for bond formation in a given pair of ionizable groups (eq 3) may be calculated for the covalent bonding model from the derived values of K_{assocn} and $\Delta H^0_{\text{assocn}}$ in Table II. The large negative values of $\Delta S^0_{\text{assocn}}$ which are obtained (~ -50 entropy units) appear plausible if an α-amino group is involved, since an N-terminus may have a large number of degrees of freedom in the unbonded state.

The question of the chemical identity of the electron acceptor groups (G_1H) and the proposed covalent bonds in reversible fibrin polymerization must be considered. In this connection the chemistry of clot stabilization (insolubilization) is highly suggestive, insofar as it is known at this time. The action of pure thrombin on pure fibrinogen or the polymerization of isolated fibrin monomer *in vitro* produces a fibrin clot which can be redissolved under mild conditions, as described above. However, the normal clot produced *in vivo* cannot be redissolved under these conditions, suggesting that irreversible covalent bond formation has occurred between the monomer units. This clot stabilization has been shown to be due to a further reaction in the clot, requiring the presence of calcium ions and an additional enzyme (fibrin-stabilizing factor) (Laki and Lorand, 1948; Loewy *et al.*, 1961) which is activated by thrombin (Lorand and Konishi, 1964). The stabilization reaction is accompanied by the release of ammonia and carbohydrate material, and is currently believed to be, in effect, a transamidation between α-amino groups of N-terminal amino acid residues and the side chain amide groups of asparagine or glutamine residues, resulting in stable intermolecular amide linkages (Chandrasekhar *et al.*, 1964; Loewy *et al.*, 1964; Lorand and Jacobsen, 1964). On the basis of the evidence presented here it is reasonable to infer that clot stabilization and the proposed covalent bonding in reversible polymerization involve the same functional groups, and are closely related chemically. We suggest that the function of the fibrin-stabilizing factor is not to form covalent bonds in the clot, but rather to prevent the rupture of covalent bonds formed originally in reversible polymerization, through cleavage of another bond (or bonds) which releases ammonia and the carbohydrate material.

The covalent bonding model as developed above requires that the bonds are formed between nonprotonated α-amino groups (G_2) and the protonated forms of another type of functional group (G_1H). It is reasonable to suppose that an α-amino group with its unshared pair of electrons functions as electron donor in coordinate covalent bond formation, and that G_1H is the electron acceptor. This is conceivable if G_1 and G_1H are unsaturated, and bond formation occurs by addition. The apparent pK_1 of G_1H for the covalent bonding model (Table II) is 7.65 for $r = 4$ or 6.9 for $r = 6$, indicating that G_1 is moderately basic. (Application of the approximate correction for the net protein charge, as described above, would give intrinsic values for pK_1 of 7.0 and 6.3, respectively.) Of the functional groups thought to occur in proteins, an imine or Schiff base type structure appears most reasonable for G_1. While the indicated pK is consistent with the imidazolyl side chains of histidyl residues we know of no evidence that such side chains can participate in covalent bond formation in proteins as electron acceptors. On the other hand, fibrinogen and fibrin were found (Mester *et al.*, 1963, 1965; Mester and Moczar, 1965) to contain six oligosaccharide chains, probably bound through the amide side chains of asparagine or glutamine residues. The release of part of this carbohydrate material during clotting in the presence of fibrin-stabilizing factor led Chandrasekhar *et al.* (1964) to propose that such residues are the acceptor groups in clot stabilization. The structure of the protein–carbohydrate linkage in fibrinogen and fibrin has not been reported. Chandrasekhar *et al.* (1964) suggested an *N*-glycosylamide structure by analogy with ovalbumin (Marks *et al.*, 1963), but it is known that other types of linkage can occur in glycoproteins (Grant and Simkin, 1964). In fact, an imidoester (iminoether) structure was also proposed for ovalbumin (Yamashina and Makino, 1962). The *N*-glycosylamide structure for ovalbumin was strongly supported by the isolation of a compound of that structure after enzymatic digestion and acid hydrolysis (Marshall and Neuberger, 1964; Yamashina *et al.*, 1963) but it seems possible that rearrangement or decomposition of the native structure might have occurred during this treatment. In any event, the protonated imidoester structure is an interesting possibility for the acceptor groups (G_1H) in fibrin, since the pK of this functional group is of the required order, and the enthalpy of ionization appears to be small. As examples, Hand and Jencks (1962) found $pK = 6.37$ for ethyl benzimidate (25°, ionic strength 1.0), and McCracken (1908) reported ~ 5.5 for ethyl phenylacetimidate (25°, low ionic strength). Hunter and Ludwig (1962) found $pK = 6.0$ for methyl benzimidate at both 22 and 39° in 30% dimethylformamide, indicating an enthalpy of ionization close to 0.

On the basis of the above considerations, a tentative mechanism is suggested for covalent bonding in reversible polymerization and clot stabilization. This is of course conjectural, but it is offered to show that the proposal of covalent bonding in reversible polymerization is chemically feasible, and in the hope that it will suggest further experimentation in this area. The proposed type of mechanism may be illustrated as follows, where G_2 is an N-terminal amino acid residue, G_1 is a carbohydrate-bound amide side chain, and FSF* is the activated form of the fibrin-stabilizing factor:





Reversible covalent bond formation is shown as addition of the free amino group to a protonated imine-type structure, which is a reaction type of recognized importance in biochemical mechanisms (Kosower, 1962). This mechanism is consistent with the second assumption made in the development of the mathematical treatment, *i.e.*, that bonding interaction can occur between G_2 and G_1H, and not between G_2H and G_1. The α-amino group in G_2 must be unprotonated in order to be nucleophilic, while protonation imparts electrophilic character to the imine group in G_1. The substituted ammonium ion in G_2H can function as neither electron donor nor acceptor in covalent bond formation. The final step, which is catalyzed by activated fibrin-stabilizing factor, is the effectively irreversible formation of an ordinary amide linkage. If G_1 has the imidoester structure, R′ = H, R is a glycosyl moiety, and the products liberated are NH_3 and a saccharide.

The imidoester formulation of the suggested mechanism is a particularly interesting possibility, since simple imidoesters like methyl benzimidate or methyl acetimidate have been used as specific blocking agents for amino groups in proteins and model compounds (Hunter and Ludwig, 1962; Ludwig and Byrne, 1962). Reaction occurs rapidly in aqueous solution at room temperature and moderate pH; in the region of neutral pH, glycylglycine is more reactive than ϵ-aminocaproic acid (Hunter and Ludwig, 1962). An extensive study of the mechanism using aromatic imidoesters (Hand and Jencks, 1962) indicated that the protonated imidoester and the free amine interact to form a tetrahedral intermediate of the type proposed above for G_2G_1H and that, in the low pH range, equilibrium is established between this intermediate and the reactants. The subsequent behavior of the intermediate in their experiments differs from the present proposal in that carbon–oxygen cleavage occurs readily, with formation of a stable amidine. It is reasonable to suppose, however, that the presence of the carbohydrate moiety or some other feature of the protein environment in the case of fibrin polymer might account for this difference.

In a series of important experiments, it has been shown (Lorand *et al.*, 1962, 1963; Lorand and Jacobsen, 1964) that the stabilization of the clot by fibrin-stabilizing factor in dilute salt solution is inhibited by glycine ethyl ester and certain other derivatives, and that labeled glycine ethyl ester is incorporated into fibrin in the process. Reversible clotting of vertebrate fibrin was reported in these experiments, and we have found that such compounds also do not prevent reversible clotting in 1.0 M NaBr (G. F. Endres and H. A. Scheraga, 1965, unpublished experiments). This is not inconsistent with the present proposals, since interactions other than covalent bonding may contribute to a favorable free energy of polymerization, as discussed above in connection with the reversal of fibrin polymerization by urea.

The proposed mechanism appears to be consistent with what is known of the stoichiometry of reversible polymerization and clot stabilization. As pointed out previously, there are four N-terminal glycines and two N-terminal tyrosines per fibrin molecule. (The molecular weight assumed throughout this discussion is 3.3×10^5.) The ionization change data require the involvement of at least four α-amino groups in reversible polymerization, but are also consistent with the involvement of all six. Analysis of the N-terminal amino acids indicated (Lorand *et al.*, 1962) that an average of 2.8 glycine residues and 0.85 tyrosine residue had reacted during clot stabilization by fibrin-stabilizing factor. On the other hand, experiments with model compound inhibitors indicated that only the glycine residues are involved in stabilization (Lorand and Jacobsen, 1964). It is, of course, quite possible that some groups involved in polymerization are not involved in stabilization, and this is suggested by other stoichiometric data for stabilization. It has been reported that 2 (Loewy *et al.*, 1964) and 2.7 moles (Chandrasekhar *et al.*, 1964) of ammonia/mole of fibrin and only 20% of its carbohydrate content (Chandrasekhar and Laki, 1964) are released. Finally, 1.4–1.8 moles of [1-^{14}C]glycine ethyl ester (Lorand and Jacobsen, 1964) and 4–5 moles of [^{14}C]glycylglycine (Loewy *et al.*, 1964) were found to be incorporated into fibrin in the presence of fibrin-stabilizing factor, with complete inhibition of stabilization reported in the former case.

We are currently investigating the effects of blocking the α-amino groups of fibrin on the ionization and enthalpy changes in the reversible polymerization, in order to obtain further evidence concerning their involvement.

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