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Equilibria in the Fibrinogen–Fibrin Conversion. V. Reversibility and Thermodynamics of the Proteolytic Action of Thrombin on Fibrinogen[1,2]

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The equilibrium of the proteolytic step (step 1) of the thrombin–fibrinogen reaction has been investigated in 1 *M* NaBr at *p*H 5.3 at three temperatures. Essentially the same equilibrium position for this proteolytic reaction is attained in both the forward and reverse directions. An analysis of the thermodynamic parameters derived from the equilibrium constant and its temperature dependence leads to the following mechanism for the step 1 equilibrium: Hydrolysis of the peptide bond, connecting the peptide P to the protein core, also involves the rupture of some secondary bonds, probably a carboxyl...carboxyl, acetic acid dimer type hydrogen bond. After hydrolysis, P is still bound to the protein core by additional secondary (hydrogen) bonds. At *p*H 5.3, essentially no dissociation of the peptide-core complex takes place. If the reaction mixture is dialyzed or if the *p*H is raised thereby breaking the hydrogen bonds, then the complex dissociates, and step 1 goes to completion. In this respect, this proteolytic reaction differs from that involving simple peptides, since enzymatic hydrolysis of the latter is generally complete. The groups unmasked in the dissociation of P from the protein core play a role in the subsequent polymerization of fibrin monomer in step 2.

Introduction

Paper IV[6] of this series was preliminary to the present one; the former considered the kinetics of the thrombin-catalyzed conversion of fibrinogen, F, to fibrin monomer, f, in 1 *M* NaBr at *p*H 5.3, while the present paper is concerned with the reversibility and thermodynamics of the same reaction. A review of the pertinent literature will be found in the Introduction of paper IV[6] and in ref. 5. Also, many of the experimental details pertaining to the work presented in this paper will be found in paper IV.[6] The reaction under consideration here is step 1 of the scheme

Proteolysis	$F \underset{}{\overset{T}{\rightleftharpoons}} f + P$	(1)[7]
Polymerization	$nf \rightleftharpoons f_n$	(2)
Clotting	$mf_n \rightleftharpoons$ fibrin	(3)

where T is thrombin, P represents peptide and carbohydrate material,[7] f_n designates intermediate polymers and n and m are variable numbers. The equilibrium of step 1 has been investigated over a range of concentrations and temperatures (under conditions where steps 2 and 3 are inhibited) by determining the concentration of f in the reaction mixture. Thermodynamic data have been obtained for the equilibrium and compared with corresponding data for the hydrolysis of a low-molecular-weight peptide. It would appear that the proteolysis catalyzed by thrombin is anomalous in the sense that the reaction does not go to completion under certain conditions. Similar observations have been made on the pepsinogen–pepsin system,[8] and a theory has been provided for such anomalous, limited proteolysis.[9] It would seem that step 1 does not go to completion because P is combined with f by side-chain hydrogen bonds which appear to stabilize the peptide bond against hydrolysis.

Experimental

Materials.—The fibrinogen used was Armour Bovine Fraction I, lot P2803, refractionated by Laki's procedure,[10] modified as previously described.[11] The clottability, by the method of Laki,[10] was 93%. Purified fibrin monomer was prepared as before.[4] Further details on the properties of F and f are given in paper IV.[6]

The thrombin was very kindly supplied by Dr. W. H. Seegers. Most experiments were carried out with citrate-activated thrombin having the properties previously described.[12,13] Use also was made of Seegers lyophilized bio-thrombin having an activity of 150–175 TAMe units[14] per mg. of material. This preparation was found to be free of the f-inactivating enzyme[12] contained in Parke, Davis thrombin, also a bio-thrombin. Very similar results were obtained for the equilibrium constants of step 1 with both Seegers preparations.

The other materials were the same as those of the previous paper.[6]

Concentration of F and f.—The concentration of fibrinogen and fibrin monomer was determined both spectrophotometrically and by clotting as previously described,[12] making use of Morrison's procedure.[15] One ml. of the stock solution containing fibrinogen (or fibrin monomer) in 1 *M* NaBr was added to 20 ml. of a standard clotting buffer,[16] adjusted from *p*H 6.8 to 6.3. Then 1 ml. of a thrombin solution was added to the resulting mixture (final concentration = 1 TAMe unit/ml.). Since the concentration of NaBr was reduced to 0.045 *M*, the salt did not interfere in the Morrison assay. The clots were collected after 4 hr.[12]

Thrombin Analysis.—Thrombin concentration was determined by its esterase activity toward TAMe.[6,12] In addition, a comparison has been made between the esterase and clotting activity of thrombin (see Table I below).

General Procedure.—A study of the step 1 equilibrium requires a suitable solvent in which the reaction can be con-

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(2) Various aspects of this work have been presented before the Division of Biological Chemistry at several meetings of the American Chemical Society: 122nd meeting, Atlantic City, New Jersey, September, 1952; 124th meeting, Chicago, Illinois, September, 1953; 128th meeting, Minneapolis, Minnesota, September, 1955; 131st meeting, Miami, Florida, April, 1957. A preliminary summary was given in paper II[4] of this series and also in a recent review[5] of the fibrinogen–fibrin conversion.

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(4) T. H. Donnelly, M. Laskowski, Jr., N. Notley and H. A. Scheraga, *Arch. Biochem. and Biophys.*, **56**, 369 (1955).

(5) H. A. Scheraga and M. Laskowski, Jr., *Adv. in Protein Chem.*, **12**, 1 (1957).

(6) S. Ehrenpreis, M. Laskowski, Jr., T. H. Donnelly and H. A. Scheraga, THIS JOURNAL, **80**, 4255 (1958).

(7) Since P may represent several species, equation 1 is an over simplified formulation of the proteolytic reaction. Further elaboration of this point will be presented below.

(8) R. M. Herriott, *J. Gen. Physiol.*, **22**, 65 (1938); **24**, 325 (1941).

(9) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **78**, 5793 (1956).

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

(11) J. M. Sturtevant, M. Laskowski, Jr., T. H. Donnelly and H. A. Scheraga, THIS JOURNAL, **77**, 6168 (1955).

(12) S. Ehrenpreis and H. A. Scheraga, *J. Biol. Chem.*, **227**, 1043 (1957).

(13) W. H. Seegers and N. Alkjaersig, *Arch. Biochem. Biophys.*, **61**, 1 (1956).

(14) TAMe is the abbreviation for *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride. The TAMe unit of thrombin activity has been defined elsewhere.[12]

(15) P. R. Morrison, THIS JOURNAL, **69**, 2723 (1947).

(16) The composition of this standard clotting buffer was 0.05 *M* KCl, 0.05 *M* KH_2PO_4, 0.05 *M* Na_2PO_4, *p*H 6.8.

veniently carried out, both in the forward and reverse directions, and an analytical procedure for determining the composition of the reaction mixture at any given time.

An ideal solvent system for the study should fulfill a number of requirements. Among these are:

(1) The reaction (step 1) must proceed in the absence of steps 2 and 3. Otherwise, if step 2 occurred to any extent, it would remove fibrin monomer from the reaction products and shift the equilibrium position of step 1 to the right.

(2) The system should allow high solubility of F, f and P in order to permit a study of the effect of wide variations in concentration on the equilibrium. In addition, the higher the concentration of clottable protein, the more the equilibrium position would be expected to be displaced to the left[17] and the greater the precision in the analytical procedure.

(3) All components of the system (F, f, T, P) should be stable over long periods of time at a maximal range of temperatures (for determination of ΔH).

(4) The equilibrium position of step 1 should be approachable from both directions.

(5) The activity of thrombin should be sufficiently large in order that the reaction should not be too slow.

Unfortunately, all of these requirements cannot be simultaneously realized by any system, since some of them are clearly contradictory. For example, there is an upper limit[4] to the initial concentration of fibrinogen, $(F)_0$, beyond which the resulting f would polymerize. Further, in order to eliminate the polymerization, systems at either low or high *p*H's must be used; however, these conditions offer both poorer stability and solubility characteristics than the neutral region and lie outside the optimal *p*H range for thrombin activity.[4,12] To keep the solubility high and the extent of polymerization low, a number of mild hydrogen bond breaking reagents such as urea could be added, but these are also denaturing agents. The use of low temperatures to increase stability decreases the solubility, increases the extent of polymerization (at least at some *p*H's) and reduces thrombin activity. Thus, any system used for the study must necessarily be a compromise among these various considerations.

Of the many solvent systems tried, 1 *M* NaBr, *p*H 5.3, 0.05 *M* in acetate (referred to as the "bromide solvent") has proved to be the most satisfactory. Absence of polymerization of the resulting f up to about 4% initial F concentration has been demonstrated at 0° by viscosity[6] and at 25° by flow birefringence[4] and sedimentation.[4] Furthermore, although step 1 proceeds very slowly at 0° in the bromide solvent (requiring up to 10 days for the attainment of equilibrium despite very high thrombin concentrations),[6] the stability of F, f and T over this time period has been established.[6,12] This stability is maintained at 25°, where equilibrium is attained in less than one day at the thrombin concentration used. Further details on the properties of thrombin are provided below in the section under "Stability of Thrombin."

Analytical Procedure.—The method used for determining the concentration of f in the presence of F and T has been fully described.[6] It is applicable to both forward and reverse reactions as will be described below. By analyzing for f, the degree of reaction, α, defined in equation 4, may be computed at any time during the course of the reaction.

$$\alpha = \frac{(f)}{(F) + (f)} \qquad (4)$$

where the parentheses designate molar concentrations. A brief description of the analytical procedure follows.

At any given time the reaction mixture consists of F, f, P and T and thus the problem resolves itself into an analysis which can differentiate between F and f. From a consideration of the various methods available,[6] it is apparent that the selective clotting of f in the presence of an efficient thrombin inhibitor (TAMe) can provide the most unambiguous information about the relative concentrations of F and f in a given mixture. Thus aliquots of the reaction mixture were pipetted into a clotting buffer containing sufficient TAMe to inhibit all the thrombin. Since TAMe is only a temporary thrombin inhibitor, the clotting period must be reduced from the usual 16 hr. to about 4 hr., after which standard procedures for handling the clots were employed.[6] As previously discussed,[6] a number of factors inherent in this method may result in errors in clot weight, *e.g.*, *p*H changes during clotting due to TAMe hydrolysis by the thrombin, incomplete clotting of f in TAMe in 4 hr., and occlusion of fibrinogen in the fibrin clot. To account for such effects, known F-f mixtures were clotted under conditions identical with those used in the runs, and the resulting data were plotted in a form which directly relates a given clot weight in TAMe to the correct value of α. These calibration curves have been published previously.[6]

Forward Runs.—Fibrinogen at a final concentration of about 3, 2 and 1%, respectively, was mixed with thrombin at a final concentration of 70–100 TAMe units/ml.,[18] depending on the temperature. The temperatures were kept constant to ± 0.1° in thermostats. At various times, three to five 1 ml. aliquots were pipetted into 20 ml. each of standard clotting buffer[16] and into standard clotting buffer which was 0.04 *M* in TAMe, respectively, as previously described.[6] The weights of the resultant clots give the concentration in the step 1 reaction mixture of total clottable protein and of f, respectively. The calibration curves[6] set up for determining the kinetics of step 1 were used for all equilibrium data.

Reverse Runs.—Although f remains monomeric in the bromide solvent at *p*H 5.3, polymerization and gelation do occur in the same solvent at *p*H's above about 5.9. Thus, step 1 goes to completion under these conditions. By adjusting the *p*H back to 5.3 and depolymerizing the gel, the reaction may be studied from the reverse direction because the solution would now contain an excess of f and P which should lead to a synthesis of F by the T present. Accordingly, the following procedure was adopted for approaching the equilibrium from the right.

In a typical experiment, a 6% solution of F in 1 *M* NaBr was adjusted to *p*H 5.9 at 0°. A concentrated solution of T in 1 *M* NaBr, *p*H 5.9, was mixed with the F giving a final F concentration of about 5% and thrombin concentration of about 150 units/ml.[18]. The reaction mixture was kept in an ice-bath throughout.[19] Gelation occurs in about 3 days, although the reaction is not complete until about 5–6 days.[22]

When the reaction at *p*H 5.9 was complete (α near unity), 0.5 *M* acetate in 1 *M* NaBr, *p*H 4.9, was added dropwise to the gel until the *p*H was adjusted to 5.3. Complete solution was effected in about 1 hr. at room temperature using a magnetic stirrer to agitate the gel. The volume of the solution was measured, the acetate concentration adjusted to 0.05 *M* with 0.5 *M* acetate in 1 *M* NaBr, *p*H 5.3, and the approximate concentration of clottable protein was determined by ultraviolet absorption at 280 mμ.[12] Dilutions to final clottable protein concentrations of 3 to 1.5% were made with the bromide solvent, thrombin was added when necessary to make the final concentration about 100 TAMe units/ml.[18] and the temperature lowered to 0°. Clotting assays were then performed in both standard clotting buffer and in standard clotting buffer containing TAMe to establish the zero time values for the reverse runs. The procedure thereafter was the same as for the forward runs.

Stability of Thrombin.—The stability of thrombin during the time period of a run was monitored by its esterase activity toward TAMe.[6,12] For all results reported in this paper the maximum loss in thrombin-esterase activity was 10–15% over a two-week period at 0°. For the shorter duration runs at 15 and 25°, there was essentially no loss in esterase activity.

(17) This expectation was not fulfilled (see below).

(18) As determined by TAMe assay at *p*H 8 in 1 *M* NaBr at 25°. Even though the thrombin concentration is nominally designated as 100 TAMe units/ml., its activity in the step 1 reaction mixture at 0°, is, in reality, only 0.24 TAMe unit/ml. For an explanation, see the section "Thrombin Analysis" and the accompanying footnote 32 of paper IV.[6]

(19) Attempts at reversal by carrying out this reaction at *p*H 5.9 and room temperature followed by dropping the temperature to zero were unsuccessful, possibly due to the lability of the peptides[20] or to the lysine peptidase action of thrombin[21] on the peptides.

(20) F. R. Bettelheim, *Biochim. Biophys. Acta.*, **19**, 121 (1956).

(21) S. Ehrenpreis, S. J. Leach and H. A. Scheraga, THIS JOURNAL, **79**, 6086 (1957).

(22) The extent of conversion of F to gel was determined on *separate* solutions as follows: Pieces of the gel were removed and added to 2–3 ml. of 0.2 *M* acetate in 1 *M* NaBr, *p*H 4.9 in a 10 ml. cylinder until the volume indicated that about 100 mg. of material had been added. A few minutes' stirring resulted in complete solution of the gel. One ml. aliquots were clotted in standard clotting buffer and in standard clotting buffer containing TAMe; from the TAMe calibration curve, the extent of reaction readily could be determined.

TABLE I

COMPARISON OF ESTERASE AND CLOTTING ACTIVITY OF THROMBIN DURING THE COURSE OF STEP 1 AT 0° IN THE BROMIDE SOLVENT

React. time of step 1 (days)	Esterase act.,[a,b] (TAMe units/ml.)	Clotting act.,[a,b] k, (min.$^{-1}$)	Ratio of clotting to esterase act.
2	3.00	0.018	0.0060
7	2.30	.016	.0070
11	2.15	.012	.0056

[a] The esterase activity represents the thrombin concentration in a $^1/_{38}$ dilution of the original reaction mixture. The clotting activity is expressed in terms of a first order rate constant using the same solution on which the esterase activity was determined. [b] The data of this table represent a greater decline in thrombin activity than the 10–15% which usually was observed. However, the important point is that esterase and clotting activities paralleled each other.

TABLE II

EFFECT OF DIALYSIS ON α_{eq} FOR FORWARD RUNS ON STEP 1 IN BROMIDE SOLVENT AT 0°

Thrombin[a] concn., (TAMe units/ml.)	$(F)_0$ (mg./ml.)	α_{eq} Dialyzed	α_{eq} Undialyzed control
100	21.3	0.96	0.84
90	26.0	0.92	..

[a] Seegers' bio-thrombin was used in these experiments.

It has been reported that the esterase and step 1 proteolytic (*i.e.*, clotting) activities of thrombin may not parallel each other under conditions where the thrombin molecule is altered.[23] Since all our thrombin assays were in terms of esterase activity, it was necessary to establish that no significant loss in proteolytic activity occurred during the runs, *i.e.*, that esterase and proteolytic activity parallel each other *under the conditions of our experiments*. However, since conventional clotting time assays are not precise, we have used a more elaborate clotting assay than is usually employed, *viz.*, we have followed the kinetics of clotting of a standard solution of F by the thrombin present in the step 1 reaction mixture. Since the kinetics of the F–T reaction at *p*H 6.8 in dilute buffer (0.15 ionic strength) have been investigated,[24] these conditions were used for the clotting assay. Four tenths ml. of the F, f and T mixture in the bromide solvent was added to 15 ml. of 0.15 *M* KCl, thereby diluting the thrombin to a final concentration of about 3 units/ml. After the F and f present in the 0.4 ml. aliquots had clotted, 0.1 ml. aliquots of the supernatant solution then were added to 5 ml. of 0.2% F in 0.15 ionic strength buffer at *p*H 6.8 and the kinetics of the F–T reaction accurately determined by stopping the reaction with TAMe and analyzing for unreacted F in the supernatant by the procedure previously described.[24] By this method, it could be demonstrated that over a period of 11 days, the clotting activity in this particular experiment declined to some degree (see Table I). However, the decrease in clotting activity in 11 days paralleled the decrease in esterase activity.

Results

Data were obtained for the values of α, the fraction of fibrinogen converted to fibrin monomer, at various times during the course of the forward and reverse runs. In paper IV,[6] a kinetic analysis was made of the data obtained during the initial part of the forward runs. The same runs of paper IV, plus additional ones reported here, when carried to equilibrium, provide the data of interest in this paper, namely, the values of α_{eq}. As shown in paper IV, the concentration of total clottable protein [(F) + (f)] remained essentially constant during the course of the runs. A small decrease in clottability, amounting to between 0.3 and 1.5 mg./ml., depending on $(F)_0$, occurred in each experiment regardless of the temperature. In this respect, the slight decline in clottability differs from that encountered when Parke, Davis thrombin was used,[12] the latter thrombin preparation causing losses in clottability which were much larger and highly temperature dependent. Despite the slight instability of F, the correct values of α are determinable.[25]

Figs. 1a and 1b show plots of α *vs.* time at 0° for initial fibrinogen concentrations of 30 and 20 mg./ml., respectively. Both forward runs and reverse runs are shown. Two important observations are immediately apparent: First, the values of α in the forward runs do not reach unity and, secondly, the values of α decrease in the reverse experiments, approaching the equilibrium values of the forward runs. These results provide strong evidence that step 1 is reversible, since essentially the same equilibrium position is reached from both directions.

If step 2 were to occur, step 1 would go to completion, *i.e.*, α would approach 1, as already indicated. Even if step 2 does not occur, it is still possible to obtain a value of α near unity by removing P from the reaction mixture. This was accomplished by performing a series of experiments in dialysis bags through which P could be removed as it was formed. As a control, the same reaction was carried out in a test tube in the usual fashion so that P was not removed. The data of Table II show that α_{eq} was near unity in the dialysis experiment and had a significantly lower value in the test tube experiment.

The equilibrium value, α_{eq}, is temperature dependent, as can be seen from Figs. 2a and 2b in which additional data at 15 and 25° are presented.

While an equilibrium is indicated for step 1, an anomaly is immediately apparent if one tries to characterize the equilibrium by the equation

$$K = \frac{\alpha_{eq}^2(F)_0}{1 - \alpha_{eq}} \qquad (5)$$

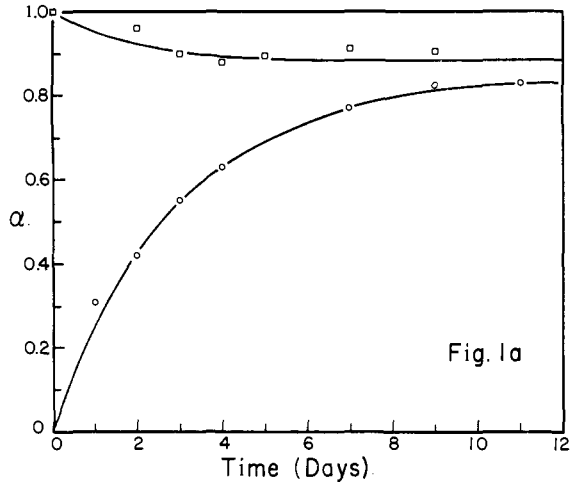
If eqs. 1 and 5 are to hold, the values of α_{eq} at a given temperature should be dependent on $(F)_0$, increasing toward unity as $(F)_0$ approaches zero.

(23) W. H. Seegers, G. Casillas, R. S. Shepard, W. R. Thomas and P. Halick, *Can. J. Biochem. and Physiol.*, **37**, 775 (1959).

(24) S. Ehrenpreis and H. A. Scheraga, *Arch. Biochem. Biophys.*, **79**, 27 (1959).

(25) The small amount of non-clotting F (or f) constitutes a problem inasmuch as it may be occluded in TAMe clots and thus give rise to errors in α. No provision for such material is made in the calibration procedure.[6] However, appropriate corrections may be applied if it is assumed that the non-clotting protein (evidently derived from F or f) is occluded in a manner identical with F itself.[26] With this assumption, and realizing that $(F)_0 = (F) + (f) + (I)$, where (I) is the concentration of inactivated material, the following procedure is used to obtain α. First, the value of (f) is determined from the calibration curve[6] corresponding to $(F)_0$ rather than from one corresponding to [(F) + (f)] since the $(F)_0$ curve contains the corrections for both non-clotting species present (when the clotting solution contains TAMe), *i.e.*, F and I. The calibration curve really gives α', which is the correct value only if (I) = 0. However, the correct value of (f) is still obtainable if α' is multiplied by $(F)_0$. The correct value of α is then computed from (f) and from the clot weight in the absence of TAMe [(F) + (f)] by means of eqn. 4.

(26) This may be justified by the following experiment: Fibrinogen was incubated with Parke, Davis thrombin at 25° for 5 days until inactivation by the fibrin-monomer-inactivating enzyme (E_{IM}) present in this preparation was complete, *i.e.*, clotting no longer occurred. Both native fibrinogen and this preparation were separately mixed with f and clotted in TAMe. The clots thus obtained were of identical weight. Presumably, some inactivated material becomes occluded in clots formed in clotting buffer without TAMe but experiments designed to estimate this were not carried out.



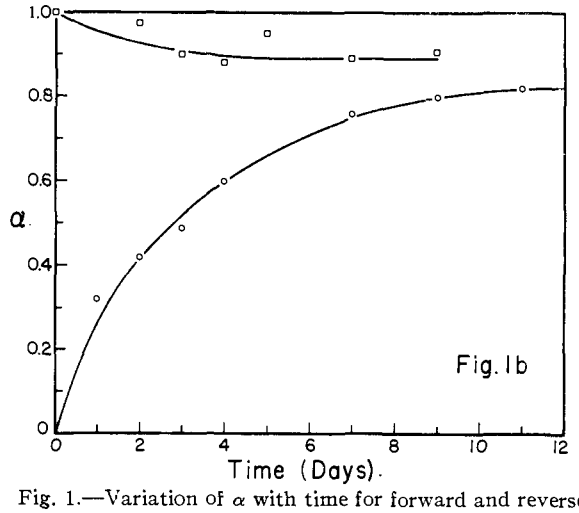
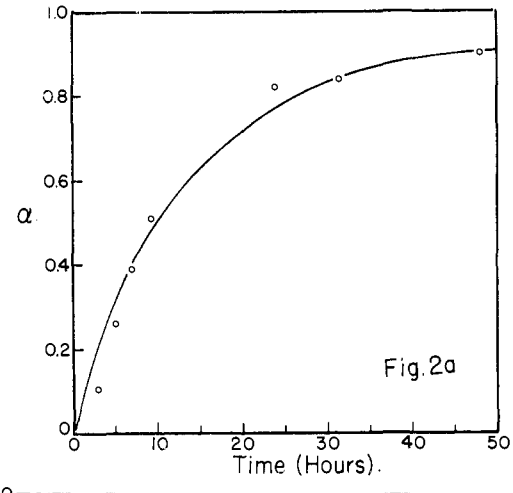


Fig. 1.—Variation of α with time for forward and reverse runs at 0° in the bromide solvent. The thrombin concentration was 100 TAMe units/ml., citrate thrombin being used in the forward runs and bio-thrombin in the reverse runs. Similar behavior in the reverse runs was obtained with citrate thrombin. $(F)_0$ was 30 mg./ml. in Fig. 1a and 20 mg./ml. in Fig. 1b.



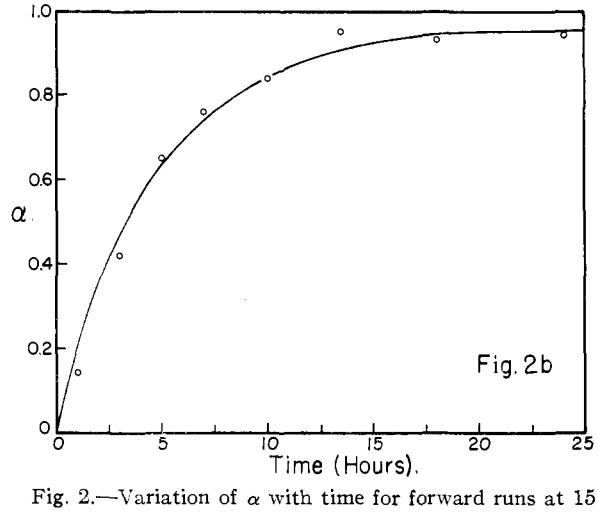


Fig. 2.—Variation of α with time for forward runs at 15 (Fig. 2a) and 25° (Fig. 2b) in the bromide solvent. The conditions were: $(F)_0$ = 19.7 mg./ml., $(T)_0$ = 80 TAMe units/ml. (Fig. 2a), and $(F)_0$ = 19.6 mg./ml., $(T)_0$ = 76 TAMe units/ml. (Fig. 2b).

However, as can be seen in Table III, the values of α_{eq} are essentially independent of $(F)_0$. (Actually, the values of α_{eq} at lowest $(F)_0$ depart slightly in a direction *opposite* from that predicted by eq. 1 and 5.) Therefore, we must modify our formulation of eq. 1, so that an equation of the form shown (6) will hold

$$K = \frac{\alpha_{eq}}{1 - \alpha_{eq}} \qquad (6)$$

The constancy of the K's computed from eq. 6 implies that step 1 should be formulated in a manner such that the total number of molecules does not change during the hydrolysis under the conditions employed here. If we assume that P and f are associated even after hydrolysis (*e.g.*, by hydrogen bonds present in the original fibrinogen molecule), then step 1 should be written as

$$F \underset{}{\overset{T}{\rightleftharpoons}} f \cdots P \qquad (7)$$

with an additional equilibrium for the dissociation of the f · · · P complex.

$$f \cdots P \rightleftharpoons f + P \qquad (8)$$

The possible influence of reaction 8 on the equilibrium of eq. 7 is explored below.

The temperature dependence of K, computed from eq. 6 at each temperature, is shown in Fig. 3, and leads to a value of ΔH° of 8 kcal./mole. Using this value of ΔH°, together with values of ΔF° (which is equal to $-RT \ln K$), a ΔS° of 32 e.u. was computed.

If the dissociation constant for the equilibrium in eq. 8 is very small (for purposes of calculation, assumed equal to zero), then, according to eq. 7, the addition of excess f at the start of the reaction should not affect the value of α_{eq}. On the other hand, if eq. 1 holds instead of eq. 7, then α_{eq} will depend on $(f)_0$, the concentration of added f, according to the equation

$$\alpha^2(F)_0 + \alpha[(f)_0 + K] - K = 0 \qquad (9)$$

which is derived from

$$K = \frac{[\alpha(F)_0 + (f)_0]\alpha}{1 - \alpha} \qquad (10)$$

If we assume, for the moment, that eq. 1 and 5 are valid, then a value of α_{eq} = 0.8 corresponds to $K = 2 \times 10^{-4}$ mole/l. at $(F)_0$ = 20 mg./ml. (taking 330,000 as the molecular weight of fibrino-

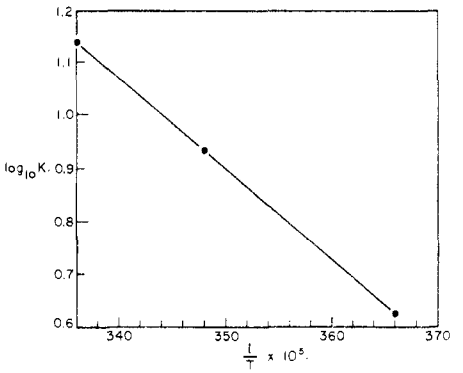


Fig. 3.—Van't Hoff plot of log K *vs.* $1/T$ for forward runs on step 1 in the bromide solvent.

gen). Using this value of K, the expected effect of added f on α_{eq} has been computed and is shown in the last column of Table IV. According to this calculation, α_{eq} should be affected by the addition of f. Experimental data, on the other hand, show no dependence of α_{eq} on $(f)_0$. Hence, the data of Table IV provide additional support for the formulations of eqs. 7 and 8, with the assumption of a negligibly small equilibrium constant for reaction 8.

Additional evidence to support this latter formulation was obtained from various experiments on the comparative action of E_{In}[27] on F and f. These experiments were suggested by previous results on the action of E_{In} which indicated that the enzyme inactivates[12] (*i.e.*, renders non-clottable) f but not intact F. In the bromide solvent at 0° essentially no inactivation occurred in F–T mixtures.[12] In light of eq. 8, it may be that P must dissociate from the f core before E_{In} can inactivate f. If the f···P complex is only slightly dissociated, then no inactivation would occur, in agreement with the observations.[12] As a further test of this idea, identical solutions of F and Parke, Davis thrombin were set up in parallel experiments in test tubes and in dialysis tubes surrounded with the bromide solvent. The temperature was maintained near 0°. Aliquots were removed at various times, clotted and the optical density of the clot supernatants at 280 mμ determined. Control solutions of F and f, respectively, were similarly set up and optical density values from these clot supernatants subtracted from those for the F–PDT, and f–PDT mixtures, respectively, in order to assess the extent of net degradation, *i.e.*, the rate of appearance of non-clottable protein. Optical density measurements of the contents of the dialysis bags were also made and established that the concentration of ultraviolet absorbing material remained constant throughout the reaction. Thus, changes in clottability could not be attributed to altered concentration of the inside solutions due to passage of solvent. Results of this experiment (Fig. 4) show several pertinent features. In the test tube containing F and PDT, there was no appearance of non-clotting protein in 9 days (curve I). In the dialysis tube containing F and PDT, the rate of appearance of non-clotting protein was very large (curve III). Finally, the rate of attack of E_{In}

(27) E_{In} is the fibrin-monomer-inactivating enzyme found in Parke, Davis thrombin (PDT) preparations.[12]

TABLE III

SUMMARY OF THERMODYNAMIC DATA FROM FORWARD RUNS IN BROMIDE SOLVENT

Thrombin[a] concn., (TAMe units/ml.)	$(F)_0$ (mg./ml.)	α_{eq}	Equil.[b] const., $K \times 10^4$	Equil.[c] const., K	$-\Delta F^0$ kcal./mole
		0°			
93 C	9.2	0.74 ± 0.02	0.58	2.8	
122 C	9.7	.72 ± .01	.55	2.6	
100 C	9.9	.71 ± .01	.51	2.4	
98 C	19.5	.82 ± .02	2.2	4.6	
100 C	19.5	.81 ± .01	2.1	4.3	
102 B	19.7	.83 ± .02	2.4	4.9	
119 C	20.0	.81 ± .01	2.1	4.3	
100 B	21.4	.84 ± .01	2.8	5.3	
95 C	29.5	.83 ± .02	3.6	4.9	
100 C	30.2	.84 ± .02	4.1	5.3	
117 C	30.7	.84 ± .03	4.1	5.3	
98 B	30.7	.82 ± .01	3.5	4.6	
			Av. =	4.3	0.80
		15°			
70 B	15.5	0.87 ± 0.01	2.7	6.7	
80 C	19.7	.90 ± .02	4.8	9.0	
84 B	21.6	.89 ± .01	4.7	8.1	
70 B	26.1	.91 ± .03	7.3	10.1	
88 C	31.6	.90 ± .01	7.8	9.0	
70 B	34.2	.90 ± .02	8.4	9.0	
			Av. =	8.7	1.25
		25°			
76 C	19.6	0.94 ± 0.02	8.8	15.7	
84 B	21.6	.92 ± .01	6.9	11.5	
			Av. =	13.6	1.55

[a] B and C refer to Seegers' bio-thrombin and citrate-thrombin, respectively. [b] Computed from eq. 5. [c] Computed from eq. 6.

TABLE IV

EFFECT OF ADDED f ON α_{eq} FOR FORWARD RUNS ON STEP 1 IN BROMIDE SOLVENT AT 0°

Thrombin[a] concn., (TAMe units/ml.)	$(F)_0$ (mg./ml.)	$(f)_0$ (mg./ml.)	α_{eq}	α_{eq},[b] (if $(f)_0 = 0$)	α_{eq} (from eq. 9)
84 C	8.8	13.9	0.79	0.72 ± 0.02	0.74
80 B	21.5	13.9	.82	.82 ± .02	.68
80 B	15.7	19.8	.79	0.8	.69

[a] B and C refer to Seegers' bio-thrombin and citrate-thrombin, respectively. [b] These values are based on the most probable values of α_{eq} expected for the particular $(F)_0$ based on experiments listed in Table III.

on F (curves I and III) is distinctly lower than that on purified f (curves II and IV), in agreement with previous results.[12] This marked difference in the susceptibility of f produced by thrombin during the experiment (curves I and III) and that of previously purified f (curves II and IV) is accounted for on the basis that E_{In} does not attack the f-core when it is associated with P. Dialysis shifts the equilibrium of reaction 8 to the right by removal of P, and the resulting f-molecules are then attacked by E_{In}. In other words, aside from the peptide bond(s) broken in reaction 7, the f···P complex resembles F in that it is not inactivated by E_{In}.[28] Thus, ad-

(28) A plausible argument against this proposal might be that the P released by the action of T on F inhibits E_{In}, resulting in a slower rate of inactivation; dialysis removes P and thus reduces the inhibition. However, our previous finding[12] that the rate of inactivation is inde-

ditional evidence is provided for the formulation of eqs. 7 and 8. It is also suggested that E_{I_n} attacks f at the sites uncovered when P is removed. These are, presumably, the sites for the polymerization of f in step 2.

Discussion

Proof of Reversibility.—From the results obtained it is clear that step 1 does not go to completion in the bromide solvent. In this sense (*i.e.*, α measurably less than unity) we speak of step 1 as being reversible. Before discussing the thermodynamic parameters for this equilibrium it is worthwhile to examine several factors other than that of reversibility which might conceivably cause α to be less than unity. We shall show that these other factors are indeed not responsible for the observed results and that the reversibility arises because of the association of f and P, as indicated in eq. 8.

As possible factors which could prevent α from attaining a value of unity in the forward direction the following may be mentioned: 1. Inhibition of T by P, as reported by Bettelheim[20]; 2. Inhibition of T by f or by F, according to the mechanism described by Waugh and Livingstone[29]; 3. Progressive inactivation (denaturation) of T in the bromide solvent; 4. Instability of f, rendering it non-clottable and thereby resulting in clots of smaller weight in the presence of TAMe as the reaction proceeds; 5. Since P actually represents two peptides,[20,30,31] P_A and P_B, and peptide P_A is liberated faster[20,32] than peptide P_B, it is possible that our results reflect only the relatively fast liberation of peptide P_A (complete in 7 days at 0°), and that the slower release of peptide P_B was not observed, *i.e.*, our analysis may detect an intermediate fibrin monomer (one devoid of only peptide P_A, but still clottable), complete clottability being attained only when both peptides are removed; 6. Some systematic artifact in the calibration curve.

We can consider factors 1 and 2 together. If a complex of thrombin with, say, f could be removed from the reaction mixture, the remaining solution would have a lower thrombin activity which could be determined by the TAMe assay.[12] In fact, such a procedure was used previously[24] to check whether thrombin activity was reduced through complex-formation during the clotting of fibrinogen, the clot being removed and the supernatant solution analyzed. However, in the bromide solvent, complexes of T with P, F or f would be soluble and thus inseparable from the free T present. Therefore, a TAMe or clotting assay could not distinguish between complexed and free T since the complex would be dissociated upon dilution in the assay procedure.[12] On the other hand, the fact that the rate constant of step 1 is essentially independent of $(F)_0$[6,24] is indirect evidence for the absence of inhibition by P or f, inasmuch as the concentrations of these species are functions of $(F)_0$. Moreover, the appearance of these species would be progressive and, if they did indeed inhibit, the observed first order kinetics[6,24] could not result. Also, addition of f at the start of the reaction did not alter the initial rate or equilibrium position of step 1. Lastly, dialysis of F–T mixtures (which effectively removes P from the reaction mixture) had little effect on the initial rate constant of step 1. Thus no evidence for product inhibition of thrombin has been obtained.

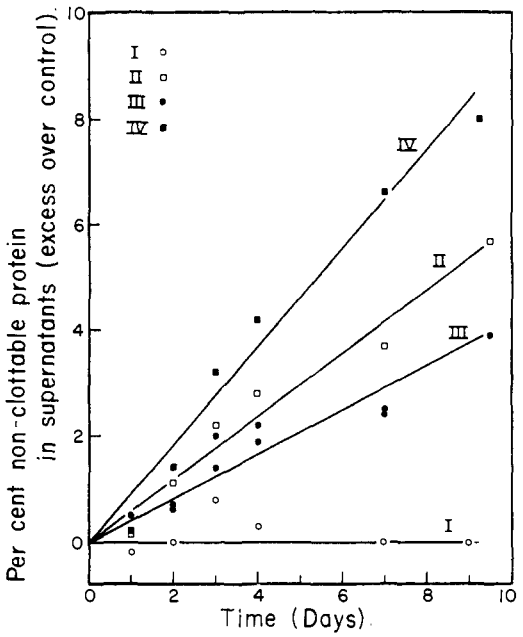


Fig. 4.—Increase in per cent. non-clottable protein (above corresponding F and f controls in test tube) in clot supernatants. The concentration of Parke, Davis thrombin was 90 TAMe units/ml. in all cases, and the bromide solvent at 0° was the medium. Curves I and II correspond to test tube experiments, and curves III and IV to dialysis tube experiments. The protein concentrations were: I, $(F)_0$ = 27 mg./ml.; II, $(f)_0$ = 20 mg./ml.; III, $(F)_0$ = 27 mg./ml.; IV, $(f)_0$ = 20 mg./ml.

As for factor 3, any irreversible destruction of thrombin would be detectible readily by the TAMe and clotting assays. Since the actual loss in TAMe-splitting and clotting activity of thrombin over the whole reaction time was but 10–15%, this possibility may be eliminated (see footnote b in Table I).

Concerning factor 4, it might be expected that any f-inactivation would be temperature-dependent, *i.e.*, α_{eq} would decrease with increasing temperature. The reverse behavior actually was observed. Moreover, the fact that almost 100% clottability is maintained throughout the course of the runs is evidence that both F and f are almost completely stable under these conditions.

The possible objection raised by factor 5 is difficult to counter since the calibration curve was set up with a fully clottable species of fibrin monomer, *i.e.*, f prepared by clotting F at *p*H 6.3. However, when F–T mixtures were dialyzed against the

pendent of $(F)_0$, and hence independent of (P), tends to refute this argument. Moreover, addition of F, which is a source of P, to a mixture of PDT and purified f failed to inhibit inactivation of f.

(29) D. F. Waugh and B. J. Livingstone, *J. Phys. and Colloid Chem.*, **55**, 1206 (1951).

(30) F. R. Bettelheim and K. Bailey, *Biochim. Biophys. Acta.*, **9**, 578 (1952).

(31) K. Bailey and F. R. Bettelheim, *ibid.*, **18**, 495 (1955).

(32) B. Blombäck and A. Vestermark, *Arkiv. Kemi.*, **12**, 173 (1958).

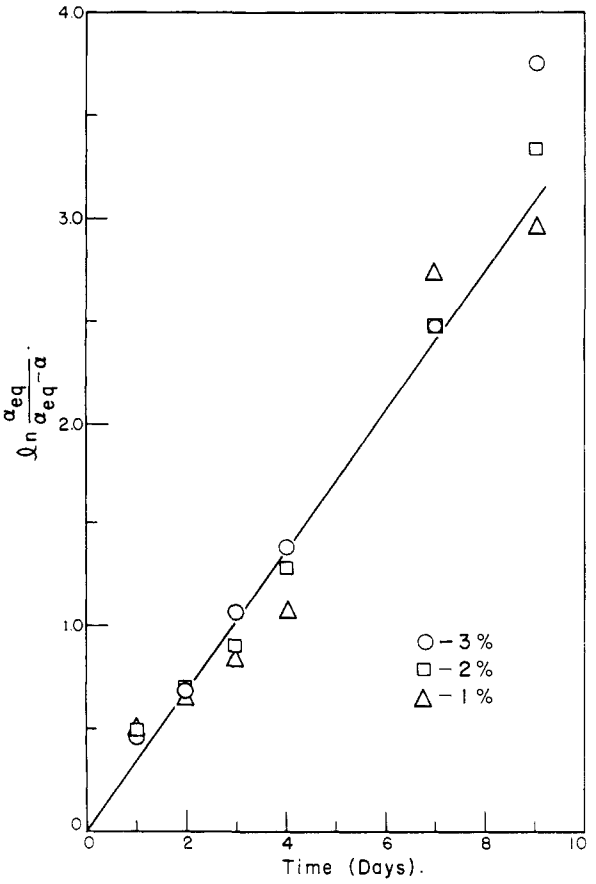


Fig. 5.—Plot of kinetic data for several forward runs on step 1 at 0° according to eq. 11. The values of $(F)_0$ are indicated in the diagram. The average concentration of thrombin is 95 units/ml. From the form of eq. 11, the experimental error in the data would increase as α approaches α_{eq}.

bromide solvent, values of α_{eq} were appreciably higher than those found in undialyzed samples. This could only result from a shift in an equilibrium, provided it is assumed that dialysis in no way affects the rate of release of the peptides. This is justified on the basis that the rate constant of step 1 in this particular experiment agreed well with previous data.

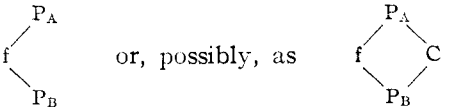
Finally, as for factor 6, the facts that a progression of α_{eq}-values is observed with increasing temperature, and also values of $\alpha_{eq} = 1$ result when step 1 is accompanied by step 2, suggest that the observed equilibria probably do not reflect some artifact present in the calibration curves.

Having disposed of other possible causes for the low values of α_{eq}, we can now summarize the evidence that the latter are due to reversibility. (1) The values of α in the forward runs level off at α_{eq} which is less than unity. In this connection, it may be noted that our experimental error is sufficiently small so that α-values of 0.8 or 0.9 are readily distinguishable from unity. (2) In accordance with the low values of α_{eq}, the kinetic data for the forward runs,[6] in which $-\log(1-\alpha)$ is plotted against time, fall off rapidly from linearity after about 70% conversion of F to f in the bromide solvent. At neutral *p*H,[24] on the other hand, where step 2 occurs and hence step 1 goes to completion, the log plot is linear up to about 90% conversion. Further, if the kinetic data at 0° are plotted in a manner to take the reverse reaction into account, *i.e.*, according to eq. 11 (applied to eq. 7)

$$\ln \frac{\alpha_{eq}}{\alpha_{eq} - \alpha} = (k_f + k_r)t \qquad (11)$$

where k_f and k_r are the forward and reverse specific reaction rate constants, respectively, then the data obey eq. 11 (see Fig. 5). Ignoring the concentration dependence of the slope of the line in Fig. 5, a value of 0.0142 $hr.^{-1}$ is obtained for $(k_f + k_r)$. Taking k_f for these same runs from paper IV[6] as 0.0092, values of $k_r = 0.0050$ and $K = k_f/k_r = 1.8$ are obtained. The latter is in as reasonably good agreement as can be expected with the average value $K = 4.3$ of Table III. (3) The value of α_{eq} becomes unity in the bromide solvent at 0 and 25° if the *p*H is raised from 5.3 to 5.9. This shift in the equilibrium of step 1 is due to the onset of step 2, *i.e.*, the polymerization of f. (4) Dialysis of F–T mixtures from the start of the reaction resulted in values of α which were distinctly higher than those obtained in any test tube experiments at the same temperature. Removal of P evidently results in a shift in the equilibrium position of step 1. (5) A distinct temperature dependence for the equilibrium position of step 1 is observed. If the experiments were merely reflecting an artifact, perhaps inherent in the calibration procedure, such a variation with temperature would most likely not be observed. (6) Finally, the fact that step 1 can actually be reversed (Fig. 1), with essentially the same values of α_{eq} being obtained in the forward and reverse runs, is perhaps the best evidence for reversibility.

Nature of P.—Having established a proof of reversibility and the notion that P and f are associated, we must now take cognizance of the fact that P may consist of two peptides,[20,30,31] P_A and P_B, and carbohydrate material,[33,34] C. We can discuss the observations on step 1 if we re-write F as



to show the carbohydrate explicitly, *e.g.*, as an ester link to the two peptides. The attachment of C to f or the peptides must not involve any linkage which is incompatible with the end-group analyses[36] of F and f. There are many alternative schemes to postulate how the carbohydrate is bound. However, since these are speculative, we shall not consider them here and shall write F in the first form shown, without introducing C explicitly. Step 1 can then be written as in Fig. 6. Form I is fibrinogen, forms II and III represent molecules in which a peptide bond has been hydrolyzed, but the resulting peptides P_A and P_B, respectively, are still associated with the remainder of the molecule.

(33) A. Szara and D. Bagdy, *Biochim. Biophys. Acta*, **11**, 313 (1953).

(34) It may be possible, as suggested by Lorand,[35] that the production of P_B is a separate reaction which is not even related to the clotting process.

(35) L. Lorand, Abstracts of 135th A.C.S. meeting, Boston, Mass., p. 16-I, April 1959.

(36) K. Bailey, F. R. Bettelheim, L. Lorand and W. R. Middlebrook, *Nature*, **167**, 233 (1951).

TABLE V
THERMODYNAMIC PARAMETERS FOR STEP 1 IN BROMIDE SOLVENT

t, °C.	ΔF^0, kcal./mole	ΔS^0, cal./mole deg.	ΔH^0, kcal./mole
0	−0.80	32	8
15	−1.25	32	8
25	−1.55	32	8
BTGA (25°)	−0.42	−3.8	−1.55
Step 1 (25°) (from eq. 5)	+4.3		

Form IV is a species in which two peptide bonds have been hydrolyzed but both peptides are associated with f. These associations are assumed to involve side-chain hydrogen bonds. Equations a, b, c of Fig. 6, with their corresponding equilibrium constants, represent dissociation reactions. According to Blombäck and co-workers,[32,37-39] species II is clottable in the absence of thrombin. We shall assume that species III is also clottable since α approaches 1 if P is removed by dialysis. Hence, we can consider all species II to VII as being clottable, and our experimental values of α are really defined by

$$\alpha = \frac{\text{II} + \text{III} + \text{IV} + \text{V} + \text{VI} + \text{VII}}{\text{I} + \text{II} + \text{III} + \text{IV} + \text{V} + \text{VI} + \text{VII}} \quad (12)$$

where the Roman numerals stand for concentrations of the respective species. Unfortunately, we have no way of determining the relative values of the concentrations of the various species[34] in eq. 12. Because of this difficulty, we shall assume in the remainder of this paper that the experimental values of α refer to the hydrolysis of a single peptide bond; therefore, α is represented by the equation

$$\alpha = \frac{\text{II} + \text{IV}}{\text{I} + \text{II} + \text{IV}} \quad (13)$$

with small equilibrium constants for the dissociation reactions of Fig. 6.

Thermodynamic Parameters.—Accepting the hypothesis stated in connection with eq. 13, the thermodynamic parameters of Table III are summarized in Table V together with those for the hydrolysis of a simple peptide. The latter pertain to the chymotrypsin-catalyzed hydrolysis of benzoyl-L-tyrosylglycinamide (BTGA)[40] in aqueous solution at 25°. However, these data are not directly comparable since the hydrolysis of BTGA presumably does not involve association of the hydrolysis products. In order to make a more illuminating even though less valid comparison, we shall temporarily ignore the lack of dependence of α_{eq} on $(F)_0$ and compute the value of ΔF^0 at 25° from eq. 5 (last line of Table V).[41] Comparing this value of ΔF^0 with that for BTGA it is seen that, whereas the equilibrium position in BTGA is in the direction of hydrolyzed products, the equilibrium position of step 1 is further in the direction of unhydrolyzed peptide bonds for all species in their standard state. If no association were involved in step 1, the apparently stronger peptide bond in this reaction could be accounted for by either of two models[9] in which the peptide bond in the protein is itself a normal one, *i.e.*, the same as a peptide bond in, say, BTGA. Either cyclic stabilization or hydrogen bonding between the side chains of P and f (with no association after hydrolysis) could account for the observed reversibility in step 1. Details for the calculations of the thermodynamic parameters based on these models have been presented elsewhere for limited proteolysis in general[9] and also for a preliminary treatment[5] of the above data on step 1. However, since step 1 has been shown here to involve a different mechanism (see Fig. 6), neither of these two models is applicable. We must, therefore, interpret the data on the first three lines of Table V in terms of the formulation of Fig. 6 and the assumption stated in connection with eq. 13.

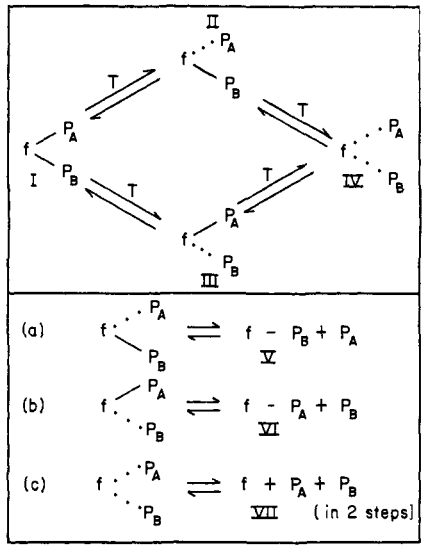


Fig. 6.—Schematic representation of step 1.

Having rejected the two models discussed in the previous paragraph, we shall now attempt to account for the observed thermodynamic parameters of step 1 in terms of a different model. In so doing, we assume that the hydrolysis of the peptide bond is accompanied by the rupture of some but probably not all of the side-chain hydrogen bonds in the formation of the f···P complex. If n is the maximum number of side-chain hydrogen bonds between the peptide and the f-core in the original F molecule and if a maximum of p bonds can be ruptured in step 1, then $n - p$ is the maximum number of hydrogen bonds which can be involved in the association reactions (a, b, c of Fig. 6). The thermodynamic parameters for step 1 then can be written in the simplified forms of eqs. II-18, II-19 and II-20 of reference 9, if all hydrogen bonds are equivalent and of the heterologous single bond type.

$$\Delta F^0_{obsd} = \Delta F^0_{pep} + p\,RT \ln 2 \quad (14)$$

$$\Delta H^0_{obsd} = \Delta H^0_{pep} - \frac{p}{2}\Delta H^0_{ij} \quad (15)$$

$$\Delta S^0_{obsd} = \Delta S^0_{pep} - \frac{p}{2}\Delta S^0_{ij} - pR \ln 2 \quad (16)$$

where the quantities on the left are the experimentally determined parameters for step 1, the

(37) B. Blombäck, *Arkiv Kemi*, **12**, 321 (1958).

(38) B. Blombäck and T. C. Laurent, *ibid.*, **12**, 137 (1958).

(39) T. C. Laurent and B. Blombäck, *Acta Chem. Scand.*, **12**, 1875 (1958).

(40) A. Dobry, J. S. Fruton and J. M. Sturtevant, *J. Biol. Chem.*, **195**, 148 (1952).

(41) Note, however, that if a different $(F)_0$ were chosen to compute the last entry in Table V, a different answer would have been obtained. Thus, this entry should not be regarded seriously. It is introduced here only in order to show that, in contrast to BTGA, the hydrolysis of F, even at very low molar concentration, is relatively incomplete.

quantities ΔF^0_{pep}, ΔH^0_{pep} and ΔS^0_{pep} are the values to be expected for a simple peptide like BTGA,[4] and ΔH^0_{ij} and ΔS^0_{ij} are the enthalpy and entropy, respectively, of formation of an internal hydrogen bond.[42] Equations 14 and 16 cannot be applied in the present case since the standard states are chosen differently for step 1 and BTGA, *i.e.*, the hydrolysis of BTGA involves the formation of one more molecule than does the hydrolysis of a peptide bond in step 1 (due to the association reactions a, b, c of Fig. 6). The problem of standard states does not enter into ΔH and we can use eq. 15 to calculate p. Using the value $\Delta H^0_{pep} = -1.55$ kcal./mole and $\Delta H^0_{obsd} = 8$ kcal./mole, together with the value[42] $\Delta H^0_{ij} = -6$ kcal./mole, we obtain a value of $p = 3$ from eq. 15. The necessity to rupture these hydrogen bonds provides some stability to the peptide bond to prevent the hydrolysis from going to completion.

Since $\Delta H^0_{obsd.}$ is the key quantity of interest, a preliminary attempt was made to determine this value by an independent experiment. Accordingly, calorimetric measurements[43] were carried out. However, since the reaction was too slow at *p*H 5.3, it was run at *p*H 6.08 in 1 *M* NaBr, where it was accompanied by step 2. The known value[11] of ΔH of step 2 at this *p*H was subtracted, and a preliminary value of ΔH of approximately zero was obtained for step 1 at *p*H 6.08. A similar result for step 1 can be inferred from the data of Laki and Kitzinger[44] in the *p*H range 6 to 8.5 in 0.3 *M* salt. The two values of $\Delta H^0_{obsd.}$ may be reconciled[45] by assuming that this parameter is *p*H-dependent, being +8 kcal./mole (obtained from the Van't Hoff equation) at *p*H 5.3 and zero at *p*H's greater than 6. Such a *p*H dependence could arise if the p hydrogen bonds are carboxyl···carboxyl, acetic acid dimer type bonds[45] rather than heterologous single bonds. If we assume that only carboxyl···carboxyl double hydrogen bonds are ruptured when F is converted to f···P, we can write expression (17) for x_{lm}, the fraction of the molecules having such a hydrogen bond

$$x_{lm} = \frac{K_{lm}}{1 + K_{lm} + \left(\frac{K_2}{[H^+]}\right)^2} \qquad (17)$$

Taking K_2 for a non-hydrogen bonded carboxyl group as 2×10^{-5} (pK 4.7), and $K_{lm} = 100$,[42] we obtain values of 0.8 and 0.14 for x_{lm} at *p*H 5.3 and 6.08, respectively. By analogy with eq. 15, the value of $\Delta H^0_{obsd.}$ for one carboxyl–carboxyl double bond may be approximated[42] by

$$\Delta H^0_{obsd} = \Delta H^0_{pep} - x_{lm}\,\Delta H^0_{lm} \qquad (18)$$

Taking ΔH^0_{pep} as -1.55 kcal./mole and ΔH^0_{lm} as -12 kcal./mole,[42] we obtain for $\Delta H^0_{obsd.}$ the values +8 and +0.1 kcal./mole at *p*H 5.3 and 6.08, respectively. These are in excellent agreement with the experimental values of +8 and 0, respectively.[45] It is thus suggested that one carboxyl–carboxyl hydrogen bond is broken in the formation of the f···P complex.

Mechanism.—In light of the interpretation of the thermodynamic parameters, presented in the previous section, we regard step 1 as involving the mechanism indicated in Fig. 6, *i.e.*, proteolysis (involving the formation of f···P) is accompanied by the rupture of one carboxyl···carboxyl double hydrogen bond. The peptide remains associated to the core, even after proteolysis. The dissociation is negligibly small at *p*H 5.3, and no data are yet available for the magnitude of this dissociation constant. We can, however, make some reasonable statements about the nature of this association. Since step 2 involves the formation of intermolecular hydrogen bonds between 19 tyrosyl donors[11,46] and 19 histidyl acceptors,[11] we can postulate that the tyrosyl donors or histidyl acceptors are liberated in the dissociation reactions a, b, c of Fig. 6. The liberated groups are assumed to appear in an unmasking process (*i.e.* an uncovering of groups rather than a rupture of side-chain hydrogen bonds) when P is dissociated from the f···P complex. The dissociation reaction is highly *p*H-dependent since it involves the rupture of carboxyl···carboxyl double bonds; in other words, at *p*H's greater than 5.9, the peptide would not be associated to the protein core and would not influence ΔH (*i.e.*, ΔH would approach that for simple peptides above *p*H 5.9). The sole necessity for a thrombin-induced proteolysis (followed by dissociation of the peptide) appears to be to liberate the donors or acceptors, thus making them available for the polymerization (step 2).

Other Systems Showing Reversibility.—While many examples of limited proteolysis are known (trypsin on the B-chain of insulin,[47] zymogen activation,[48] ovalbumin–plakalbumin conversion,[49] etc.), a reversible equilibrium, as in step 1, has not yet been demonstrated except possibly in the pepsinogen–pepsin conversion.[8] It probably does not exist because the peptide is too short; the argument for a critical size of the peptide, in order to observe reversibility, has been presented elsewhere.[9] However, recent experiments of Richards on the subtilisin digestion of ribonuclease,[50] wherein the 20 residue N-terminal tail is removed by hydrolysis, may possibly involve an association through side-chain hydrogen bonding between the tail and the core. If so, a dialysis experiment may remove the tail.

Acknowledgments.—We are indebted to Dr. W. H. Seegers for his generous gifts of purified thrombin and to Miss Emilie Sullivan for skillful assistance with the experimental work.

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

(43) M. Laskowski, Jr., and W. W. Forrest, unpublished results (1955).

(44) K. Laki and C. Kitzinger, *Nature*, **178**, 985 (1956).

(45) In comparing these values of $\Delta H^0_{obsd.}$ two points should be kept in mind: (1) the experimental error in the calorimetric method may be several kcal./mole, and (2) the heat of dissociation of the f···P complex appears in the calorimetric value but not in the value obtained from the Van't Hoff equation. The thermodynamic parameters of both equilibria of eq. 7 and 8 seem to be strongly *p*H dependent, a fact which is reconcilable in terms of the postulated carboxyl··· carboxyl hydrogen bonds. A direct calorimetric determination of ΔH at *p*H 5.3 would provide a good test of this proposal.

(46) S. Ehrenpreis, E. Sullivan and H. A. Scheraga, Abstracts of the 133rd A.C.S. meeting, San Francisco, Calif., April, 1958, p. 26-C.

(47) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

(48) H. Neurath, *Adv. in Protein Chem.*, **12**, 319 (1957).

(49) M. Ottesen, *Compt. rend. trav. Lab. Carlsberg, Ser. Chim.*, **30**, 211 (1958).

(50) F. M. Richards, *Fed. Proc.*, **17**, 296 (1958).