found that normal Zimm-type plots could be obtained as low as 0.0055 ionic strength. Under this condition we found a molecular weight of 5.0 million and a radius of gyration of 2500 Å. The difference from the 0.165 ionic strength measurements showed up in the value of the second virial coefficient. At the lower ionic strength it had a value of 13.5×10^{-4} (mole-cc./g.). This is about 10 times the value found at the usual ionic strength. Since the molecular size is unchanged this difference in excluded volume is presumably the result of the increased intermolecular electrostatic repulsion.

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Equilibria in the Fibrinogen–Fibrin Conversion. IV. Kinetics of the Conversion of Fibrinogen to Fibrin Monomer[1,2]

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The kinetics of the proteolytic action of thrombin on fibrinogen (*i.e.*, step 1 of the fibrinogen–fibrin conversion) have been studied in 1 *M* NaBr, *p*H 5.3, at 0, 15 and 25°. No polymerization occurs under these conditions. The reaction was followed by analysis for fibrin monomer, using *p*-toluenesulfonyl-L-arginine methyl ester as a thrombin inhibitor. Step 1 was found to be initially first order in both thrombin and fibrinogen. The apparent disagreement between this result and those from previous kinetic studies has been resolved in a separate publication. Hence, previously postulated thrombin-inactivation reactions need not be introduced to explain the kinetic data. Step 1 thus appears to be a simple bimolecular reaction to form the enzyme–substrate complex under conditions where the enzyme is not saturated. The effects of *p*H and solvent on the rate constants have been reconciled with the expected behavior of the kinetic constants k_3 and K_m. Finally, in carrying the kinetic runs to their equilibrium position, it was found that step 1 does not go to completion in 1 *M* NaBr at *p*H 5.3. The failure to attain completion is not due to inactivation of any of the protein components present in the reaction but to a reversible equilibrium which will be discussed in greater detail in a forthcoming paper.

Introduction

The conversion of fibrinogen to fibrin may be considered to occur in three reversible steps, only the first of which involves thrombin.[4] In step 1 thrombin catalyzes the hydrolysis of two arginyl–glycine bonds[5–11] in fibrinogen leading to the liberation of at least two peptides and also carbohydrate material.[12] In this process the molecular weight of fibrinogen (330,000)[13,14] is reduced by about 3%, the remaining protein being designated *fibrin monomer*.[15] Under appropriate conditions[4] fibrin monomer polymerizes (step 2) and ultimately forms the fibrin clot (step 3). The three reactions may be written as

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

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

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

where T is thrombin, F is fibrinogen, f is fibrin monomer, P represents the peptide and carbohydrate material, f_n designates the intermediate polymers and n and m are variable numbers.

Considerable information already has been obtained about the mechanism of step 2.[4] This paper, and several to follow, report on attempts to understand the mechanism of step 1. In this initial paper the kinetics of the thrombin–fibrinogen reaction are investigated under conditions where only step 1 occurs.[16] If the fibrinogen concentration is less than about 4%, step 1 will occur in 1 *M* NaBr at *p*H 5.3, without the subsequent polymerization of fibrin monomer.[18,19] Hence, this solvent, in which the stability of purified F and T has been fully established,[18,20] was employed in the present study. By using relatively purified thrombin the uncertainties involving the inactivation of fibrin monomer by commercial thrombin preparations[20]

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(2) Presented before the Division of Biological Chemistry at the 131st meeting of the American Chemical Society, Miami, Florida, April, 1957.

(3) Research Fellow of the National Heart Institute, United States Public Health Service, 1952–1956.

(4) For a recent review of the fibrinogen–fibrin conversion see H. A. Scheraga and M. Laskowski, Jr., *Advances in Protein Chem.*, **12**, 1 (1957).

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

(6) L. Lorand, *ibid.*, **167**, 992 (1951); *Biochem. J.*, **52**, 200 (1952).

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

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

(9) J. A. Gladner and K. Laki, quoted by K. Laki and C. Kitzinger, *Nature*, **178**, 985 (1956).

(10) S. Sherry and W. Troll, *J. Biol. Chem.*, **208**, 95 (1954).

(11) K. Laki, J. A. Gladner and D. R. Kominz, Abstracts of the Seventh Annual Symposium on Blood, Wayne State University, Detroit, Michigan, Jan. 17, 18, 1958, p. 18.

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

(13) S. Shulman, THIS JOURNAL, **75**, 5846 (1953).

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

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

(16) The kinetics of step 1 at neutral *p*H, where steps 2 and 3 also occur, has been considered in a separate paper.[17]

(17) S. Ehrenpreis and H. A. Scheraga, *Arch. Biochem. Biophys.*, in press.

(18) T. H. Donnelly, M. Laskowski, Jr., N. Notley and H. A. Scheraga, *ibid.*, **56**, 369 (1955).

(19) M. Laskowski, Jr., T. H. Donnelly, B. A. Van Tijn and H. A. Scheraga, *J. Biol. Chem.*, **222**, 815 (1956).

(20) S. Ehrenpreis and H. A. Scheraga, *ibid.*, **227**, 1043 (1957).

have been eliminated. The synthetic substrate *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAMe) has been used for the quantitative determination of thrombin concentrations.[10,20] The kinetics of the reaction have been determined by analysis of mixtures of F, f and T for the concentration of f at various times using TAMe as a thrombin inhibitor.[10] All kinetic data pertain to the forward reaction, no data being reported here for the reverse reaction. The reversal of step 1 and an evaluation of the thermodynamic parameters for this equilibrium will be discussed in a forthcoming paper.[21]

In contrast to the present study, several kinetic investigations of step 1 have been carried out under conditions leading to the production of fibrin, *i. e.*, steps 1, 2 and 3 proceeded essentially simultaneously.[22,23] Because of the different conditions, these results may not be directly comparable with those reported here. However, in a separate publication,[17] we have reported kinetic results obtained[16] under conditions similar to those used by Waugh and Livingstone.[23] This paper[17] should be consulted for a further discussion of the kinetics of step 1 as a function of *p*H.

Experimental

Materials.—Purified fibrinogen was prepared from Armour Bovine Fraction I, lot P2803, using a modification of Laki's procedure[15] involving refractionation between 21 and 25% saturation with ammonium sulfate, as previously described.[24] The resulting precipitate was dissolved in NaBr (final concentration about 1 *M*, *p*H 5.3 to 5.7) and dialyzed at 3° against 1 *M* NaBr containing 0.05 *M* acetate buffer at *p*H 5.3 until free of sulfate.[25] The per cent. clottability of such solutions could not be determined by Laki's procedure[15] because of the high ionic strength (1 *M* NaBr). However, similar preparations of fibrinogen, when dissolved in 0.3 *M* KCl, were found to be about 93% clottable by Laki's method.[15]

Purified fibrin monomer was prepared by a method previously described.[18] A small thrombin impurity amounting to about 0.1 TAMe unit per ml. of 3% monomer solution was always found to be present.[26] This has been attributed[20] to the carrying over in the mother liquor of some of the thrombin used initially to clot the fibrinogen from which the fibrin monomer was prepared. As will be evident from the data to be presented, this small amount of thrombin in no way affected the results.

The concentration of fibrinogen and fibrin monomer in the stock solutions was determined both spectrophotometrically and by clotting as previously described,[20] making use of Morrison's procedure.[28] 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,[29] adjusted from 6.8 to 6.3. Then 1 ml. of a thrombin solution was added to the resulting mixture (final concentration = 1 unit/ml.). The concentration of NaBr was thus reduced to 0.045 *M* so that the salt did not interfere in the Morrison assay. The clots were collected after 4 hours.[20]

The thrombin used for most of the experiments was citrate thrombin,[30] kindly supplied by Dr. W. H. Seegers. It was received in 50% glycerol which was removed by dialysis against the bromide solvent. The activity of this preparation, which was unaltered by dialysis,[31] was about 2500 TAMe units/ml. The stability of citrate thrombin under a variety of conditions has been discussed in a previous publication.[20]

For some runs at 0°, commercial Parke, Davis thrombin having an activity of about 13 TAMe units/mg. was used. It was dissolved in the bromide solvent prior to use.

The synthetic substrate *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (TAMe) was supplied by the H. M. Chemical Co., Ltd., Santa Monica, Calif., and had the properties previously reported.[20]

The other chemicals used (NaBr, phosphates, acetate) were of reagent grade quality.

All *p*H measurements were made with a Beckman model G *p*H meter standardized at *p*H 4 and 7 with Beckman buffers.

Thrombin Analysis.—The concentration of thrombin was determined by following its esterase activity toward TAMe (TAMe assay), using the *p*H-stat modification[20] of the method of Sherry and Troll.[10] In this procedure,[20] 0.01 *M* TAMe in 0.15 *M* KCl or in 1 *M* NaBr[32] is adjusted to *p*H 8.0 at 25°, the thrombin solution is added and the *p*H is maintained constant by the addition of dilute NaOH. The titrant solutions were 0.057 *N* NaOH in 0.15 *M* KCl and 0.132 *N* NaOH in 1 *M* NaBr for the determinations in the presence of KCl and NaBr, respectively. The hydrolytic reaction was followed for 10 min. to determine the thrombin activity.[26] By convention, we report thrombin concentrations as if they were determined under the standard conditions: *p*H 8 at 25° in *0.15 M KCl*. Hence, determinations done in NaBr have been corrected to values corresponding to KCl since, as previously reported,[20] 1 *M* NaBr decreases thrombin activity toward TAMe by about 50% in the *p*H range 5 to 9.

It should be noted that the actual thrombin activity in the bromide solvent is only a very small fraction of the activity of the same weight of thrombin under standard conditions. This reduction in activity arises not only because of the presence of 1 *M* NaBr but also because of the low *p*H (5.3 compared to 8.0).[20] As a rough estimate from the data of reference 20, 1 TAMe unit/ml. of thrombin activity at *p*H 8 in 0.15 *M* KCl at 25° corresponds to about 0.024 unit/ml. in the bromide solvent at 25° or about 0.0024 unit/ml. in the bromide solvent at 0°. For example, in a typical reaction carried out in the bromide solvent at 0°, thrombin activity was nominally called 100 TAMe unit/ml. even though its activity was, in reality, only 0.24 TAMe unit/ml. As a consequence of this lower activity, the rate of the thrombin-catalyzed conversion of fibrinogen to fibrin monomer is very low in the bromide solvent, even though large amounts of thrombin are used.

Thrombin activity also was checked by determining the clotting time of standard fibrinogen solutions in clotting buffer.[29]

TAMe Method for Following Step 1 in the Bromide Solvent.—It has been shown previously,[18,19] by means of flow birefringence and sedimentation experiments, that steps 2 and 3 are inhibited in the bromide solvent. Hence, the rate of conversion of F to f (*i.e.*, step 1) in this solvent could be followed by any one of several methods. The choice of method for following the course of step 1 is based on the following considerations. (1) An instantaneous thrombin

(21) M. Laskowski, Jr., S. Ehrenpreis, T. H. Donnelly and H. A. Scheraga, THIS JOURNAL, to be submitted.

(22) J. Lein, *J. Cellular and Comp. Physiol.*, **30**, 43 (1947).

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

(24) J. M. Sturtevant, M. Laskowski, Jr., T. H. Donnelly and H. A. Scheraga, THIS JOURNAL, **77**, 6186 (1955).

(25) This 1 *M* NaBr solution, buffered at *p*H 5.3, will hereafter be referred to as the "bromide solvent."

(26) The TAMe unit of thrombin activity has been defined elsewhere.[20] Our unit differs somewhat from those of Sherry and Troll[10] and Ronwin.[27]

(27) E. Ronwin, *Can. J. Biochem. Physiol.*, **35**, 743 (1957).

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

(29) The composition of this standard clotting buffer was 0.05 *M* KCl, 0.05 *M* KH_2PO_4, 0.05 *M* Na_2HPO_4, *p*H 6.8.

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

(31) The glycerol solution of thrombin could also be analyzed by the TAMe method since the solution was sufficiently diluted (about 250-fold) prior to assay so that the concentration of glycerol was reduced to a few tenths of a per cent. Therefore, the glycerol did not interfere with the analysis.

(32) Most of the TAMe assays in the experiments to be reported were performed in the presence of fibrinogen or fibrin monomer. In 0.15 *M* KCl at *p*H 8.0 clotting invariably occurred in the assay solution; adherence of the clot to the electrodes gave rise to erratic results. The presence of 1 *M* NaBr in the assay solution prevented clotting (at least during the 10 min. period required for the assay) and permitted very satisfactory duplication of results. As previously shown,[20] the presence of F, f or fibrin in a solution of thrombin does not affect its activity as determined by the TAMe method.

inhibitor could be added to the reaction mixture and the concentrations of F and f determined directly. While F and f cannot be distinguished by hydrodynamic measurements,[15,18] they can be by electrophoresis.[33] However, attempts to determine the relative areas of the peaks corresponding to F and f in electrophoretic patterns have thus far been unsuccessful; (2) F and f can be precipitated by trichloroacetic acid and the supernatant solution analyzed for P. Spectrophotometric analysis cannot be used because of the extremely low ultraviolet absorption[34] of P; although nitrogen analyses of the supernatant have been carried out[19] and do give information on the rate of production of P, the data cannot be converted to a molar basis because of difficulties in the determination of the stoichiometric value of P; (3) step 1 has been followed by observing the rate of appearance of the new N-terminal group of fibrin monomer[35]; however, until the exact rates of production of peptides A and B are determined,[36] it will be difficult to interpret kinetic data obtained by this method; (4) finally, if a reagent were available which could function as an instantaneous inhibitor of thrombin and if an aliquot of the reaction mixture consisting of F, f and T in the bromide solvent were added to a clotting buffer containing this inhibitor, then only f but not F would clot, since the clotting of f does not require thrombin (see eq. 1, 2 and 3). Thus, the weight of fibrin obtained would be a direct measure of the concentration of f. For the reasons discussed above, method 4 has been selected for following the kinetics of step 1.

The amount of thrombin which must be inhibited can be estimated as follows. In most of the experiments to be described a 1-ml. aliquot of a reaction mixture of T + F contains 100 units/ml. of thrombin. Since this aliquot is added to 20 ml. of clotting buffer,[29,37] the concentration of thrombin which must be inhibited is about 5 units/ml.[38]

It has been shown elsewhere[17] that formaldehyde is unsuitable for following step 1 in the *absence* of steps 2 and 3, since it does not inhibit thrombin completely and it also prevents f from clotting. However, the synthetic substrate TAMe has been found to be satisfactory as a thrombin inhibitor. It has been shown by Sherry and Troll[10] that, at high concentrations of this ester, thrombin hydrolyzes TAMe in a zero-order reaction (complete coverage of the enzyme) and that no clotting takes place until most of the TAMe is hydrolyzed. Thrombin then becomes available to act on fibrinogen. Making use of this observation, we have found, for example, that 0.04 *M* TAMe in clotting buffer[29] will prevent 5 units/ml. of thrombin from clotting F (1 mg./ml.) for about 4.5–5 hr. This inhibition applies only to step 1; *i.e.*, it will be shown below that TAMe does not interfere appreciably with steps 2 and 3. If TAMe were absent, the clotting reaction would start immediately under the same conditions. Therefore, the concentration of f in the presence of F and T can be determined with the use of TAMe.

To follow step 1, the concentration of f is determined by adding an aliquot of the reaction mixture to clotting buffer[29] containing TAMe. The addition of a similar aliquot to clotting buffer containing no TAMe gives the total clottable protein concentration, F + f; since thrombin is fully active in the absence of TAMe, all of F, as well as f, is converted to fibrin.[37] All clotting assays were performed at room temperature even though the kinetic runs were carried out at other temperatures. It is thus possible to measure the degree of reaction, α, of step 1 at any time, where α is defined as

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

the parentheses indicating molar concentrations. For this purpose, we have neglected the 3% difference in molecular weight between F and f. The kinetic data are expressed in terms of the variation of α with time.

While simple in principle, the TAMe method has certain difficulties. First, and perhaps most important, is the fact that TAMe functions only as a *temporary* thrombin inhibitor. Hence, the analysis for f must be completed before the major fraction of the TAMe is hydrolyzed in order to prevent the thrombin from attacking the fibrinogen. A convenient concentration of TAMe considered satisfactory from this point of view was 0.04 *M*. From the data quoted above, it can be seen that the inhibitory properties of TAMe can be maintained for 5 hr. Hence all analyses for f have been carried out by clotting for 4 hours.[39]

A second factor is that hydrolysis of TAMe leads to the production of protons; therefore, the solution must be sufficiently buffered to prevent large *p*H changes but still not affect the clotting of f. Standard clotting buffer[29] was used for this purpose. When the solution is made 0.04 *M* in TAMe by adding the solid ester, the *p*H falls slightly to 6.7. If thrombin (5 units/ml.) is added to such a solution, the *p*H falls to about 6 in 4 hr. despite the presence of the buffer. However, the clotting of f proceeds satisfactorily[40] (see below) in the *p*H range 6.7 to about 6. The same buffer,[29] adjusted to *p*H 6.3 and containing no TAMe, was used to determine the concentration of total clottable protein, F + f.

The effect of a variation in thrombin concentration on the clot yield from f in the presence of TAMe is shown in Table I. Since these data were all obtained in 4 hr., they actually indicate the effect of a change in *p*H on the 4 hr. clot yield.[42]

TABLE I

EFFECT OF VARIATION IN THROMBIN CONCENTRATION ON 4 HOUR CLOT YIELD FROM f

(Solvent is clotting buffer[29] plus 0.04 *M* TAMe)

Thrombin concn. (units/ml.)	Clot yield (mg.)
0	15.8
1.1	15.6
2.2	14.8
4.4	14.8

In addition to the *p*H effect indicated in Table I, there is a slight effect of TAMe on the *rates* of steps 2 and 3 but not on the ultimate clot yield. While the clotting of f is complete within 4 hr. in clotting buffer,[20] the presence of TAMe *in the absence of thrombin* reduces the 4 hr. clot yield as indicated by the data for solution A in Table II. Since no thrombin was added to solution A, the clotting reaction could be carried beyond the usual 4 hr. period. Whereas the 4 hr. clot yield in 0.04 *M* TAMe (23.4 mg.) was less than the 4 hr. yield in the absence of TAMe (25.2 mg.), the two 24 hr. values were nevertheless identical (25.2 mg.); this shows that although TAMe affects the clotting rate slightly (*i.e.*, the 4 hr. clot yield), it does not interfere with the 24 hr. clot yield, *i.e.*, with steps 2 and 3. In the presence of thrombin and TAMe, both effects are present (*i.e.*, the *p*H effect and the reduced clotting rate). The magnitude of the combined effect is shown by the data for solution B in Table II.

(33) E. Mihalyi, *Acta Chem. Scand.*, **4**, 351 (1950).

(34) F. R. Bettelheim, THIS JOURNAL, **76**, 2838 (1954).

(35) B. Blomback and I. Yamashina, *Acta Chem. Scand.*, **11**, 194 (1957).

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

(37) The 1 *M* NaBr must be diluted to a very low concentration in order that it should not interfere with the clotting assay. In the procedure used here it is diluted to 0.045 *M*.

(38) In a few kinetic runs to determine the order in thrombin the enzyme concentration was as high as 135 units/ml. in the reaction mixture, corresponding to a concentration of about 6.5 units/ml. in the clotting buffer.

(39) It was observed that, if the fibrinogen were dialyzed against the bromide solvent immediately after being fractionated, addition to TAMe resulted in marked precipitation. Dialysis of the F first against 1 *M* NaBr (*i.e.*, without acetate buffer) and then against the bromide solvent almost completely eliminated the precipitate. No explanation for this effect of acetate can be offered.

(40) If the concentration of the phosphate were increased to achieve greater buffer capacity, poor clots would have been obtained.[41] If the TAMe concentration were increased to extend the period of thrombin inhibition from 5 to 24 hr., the *p*H would have dropped considerably below 6 in the standard clotting buffer[29] in 24 hr. Therefore, the concentrations of TAMe and buffer chosen represent a compromise.

(41) J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 388 (1947).

(42) Most of the kinetic runs to be described were carried out at a single thrombin concentration (100 units/ml.). However, in a few experiments (designed to determine the order in thrombin and also the rate at temperatures other than 0°) the thrombin concentration was varied. Despite the *p*H effect shown in Table I, no significant error was introduced because the thrombin concentration in the clotting buffer was always in the range of 2 to 6 units/ml. where the clot yield is not very *p*H dependent.

TABLE II
CLOT YIELD FROM f IN CLOTTING BUFFER IN THE PRESENCE AND ABSENCE OF TAMe

Soln. of f	TAMe concn., *M*	Thrombin concn. (units/ml.)	Reaction time, (hr.)	Clot yield (mg.)
A	0	0	4	25.2
			24	25.2
A	0.04	0	1	22.1
			2	22.4
			3	23.0
			4	23.4
			24	25.2
B	0	4.5	3	19.5
			4	19.5
B	0.04	4.5	1	16.5
			2	16.8
			3	17.3
			4	17.4

A third problem in the TAMe method is that F may be occluded or copolymerized[18] in the fibrin clot. The magnitude of this effect is demonstrated in Fig. 1. These data were obtained from the calibration curves to be described

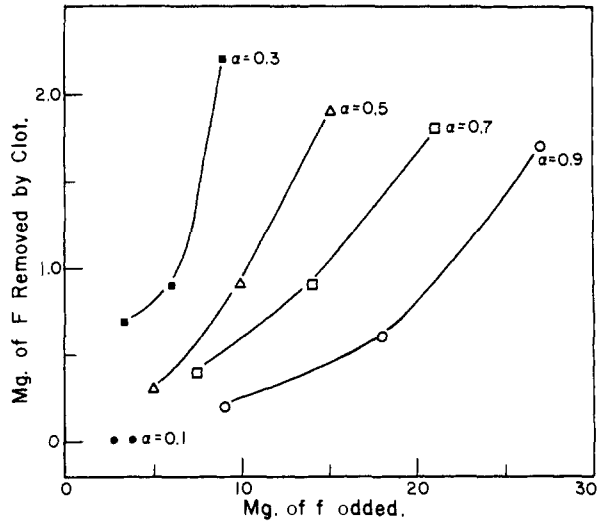

Fig. 1.—Adsorption of F by clot formed from f in clotting buffer in the presence of 4.5 units/ml. thrombin and 0.04 *M* TAMe (final volume = 22 ml.). The quantity α is defined in eq. 4.

in the next section. In the absence of occlusion or copolymerization of F, no F would have been removed by the clot. It is seen that the amount of occluded F increases with increasing concentration of f for each value of α. At low values of α, occlusion is large and becomes maximal around $\alpha = 0.3$ because F is in excess over f. As α increases, the relative amount of F decreases; hence the amount occluded decreases. It is apparent from Fig. 1 that the amount of F occluded when f is converted to a fibrin clot in the presence of F can be significant. The data of Fig. 1 fit a Langmuir adsorption isotherm over about $^3/_4$ of the range of concentrations of adsorbate F.

In summary, when f is converted to a fibrin clot in the presence of F, T and TAMe, errors can arise from: (1) *p*H changes, (2) retardation of clotting, making the 4 hr. clot yield lower than the true concentration of f and (3) adsorption of F by the clot. While these all appear to be small effects, they may give rise to erroneous conclusions, as shown below. Therefore, they have been taken into account by an appropriate calibration procedure described in the next section.[43]

(43) There is a fourth potential source of error which is independent of the presence of TAMe, namely, that the clotting rate of f, as well as the extent of clotting (and, therefore, the 4 hour clot yield), are dependent on the concentration of f. However, the calibration procedure automatically compensates for this source of error. Further elaboration of this point has been given elsewhere.[17]

Calibration Procedure for TAMe Method.—In essence, the calibration data were obtained by clotting mixtures of F and f under conditions identical with those used in analyzing aliquots from the reacting solutions in the kinetic runs. Thus, by determining the clot weight as a function of the known concentrations of F and f, correction was made for the potential sources of error mentioned in the last section. The calibration curves required for this correction were obtained as follows. Stock solutions of F and f (in the bromide solvent), corresponding to α-values of 0 (pure F) to 1 (pure f), were prepared at fixed total clottable protein concentrations (*i.e.*, F + f) of 30, 20 and 10 mg./ml., respectively. These particular concentrations were chosen to correspond with those used in most of the kinetic runs. Thus, a series of solutions of varying α-values, *at each of the three total protein concentrations*, was obtained. These various solutions simulate the conditions existing during the course of a kinetic run, *i.e.*, α increases progressively from zero at the start of the reaction to its final value when equilibrium is attained. All solutions were stored at 0° and the F–f mixtures were used immediately after their preparation because of the presence of the trace of thrombin contamination in solutions of f, as already mentioned. One ml. of citrate thrombin (100 units/ml.) in clotting buffer[29] was added to 20 ml. of 0.04 *M* TAMe in clotting buffer[29]; to this was added a 1-ml. aliquot of the particular F-f mixture. Triplicate analyses were carried out. Similar 1-ml. aliquots were clotted in clotting buffer[29] which contained no TAMe but sufficient thrombin to clot all of the F. *After 4 hours* all clots were removed[44] and washed for 15 minutes, first in 0.15 *M* KCl and then in distilled water. After drying overnight at 110°, the clots were weighed to ±0.1 mg.

In order to obtain the calibration curves in a form which would be useful for treating the data from a kinetic run, the calibration data were first plotted as clot weight in the presence of TAMe *vs.* clot weight in the absence of TAMe. The latter quantity represents, of course, the *total* clottable protein F + f. These plots are shown in Fig. 2, the several curves corresponding to various values of α. The curves of Fig. 3 were obtained from the curves of Fig. 2 by interpolation. The dashed curves represent the expected behavior if none of the effects, for which these calibrations correct, were present. It may be noted that the solid curves move upward across the dashed ones as the total clottable protein concentration increases. This behavior is a consequence of a balance between the adsorption of F and retardation of clotting of f. Since the kinetic runs were carried out at concentrations of approximately (but not exactly) 10, 20 and 30 mg./ml. of F, curves similar to those of Fig. 3 were constructed for each run.

With the aid of Fig. 3, it is possible to convert the TAMe clot weights from, say, a 1% protein solution to values of α (in reality concentration of f) in the original solution, thereby correcting for the sources of error mentioned in the previous section.

Kinetic Runs on Step 1 in the Bromide Solvent.—All the kinetic runs on step 1 in the bromide solvent were made at initial fibrinogen concentrations (F_0) of approximately 10, 20 or 30 mg./ml. Analyses were carried out under conditions identical with those described for the calibration curve.[45] The thrombin concentration, except in those runs

(44) The clots formed in the presence of TAMe were generally very friable and difficult to remove without loss when they were set up in butter dishes as suggested by Morrison.[28] An alternative procedure was therefore adopted: The clot was formed directly in 40-ml. centrifuge tubes cut down to a length of 7 cm.

After 4 hours the clot was first reamed with a spatula and then centrifuged for 5 minutes at about 1500 × *g*. Since these clots retained almost no fluid, they did not have to be synerized and could be placed directly into the wash water. The clots formed in the absence of TAMe were handled in a similar way, except that these clots required synerizing before washing.

(45) These conditions were not strictly identical with those of the calibration experiments in that most of the calibration data were obtained with *purified f* and thus in the absence of fibrinopeptide, carbohydrate, etc. In order to ascertain what effect, if any, these minor constituents might have on the clot weight, the following experiment was performed: 5% F and citrate T (150 units/ml.) were adjusted

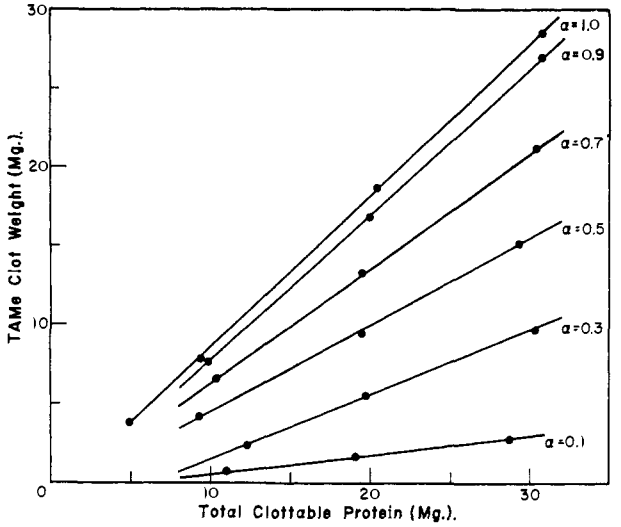

Fig. 2.—Calibration data plotted as TAMe clot weight *vs.* total clottable protein concentration in the original stock solution. The several curves correspond to various values of α. The curves were not extended below 1% total protein concentration because of the errors in clot weight determination below 1% and because no kinetic runs were performed at fibrinogen concentrations less than 1%. These curves would not be expected to extrapolate to the origin in view of the incomplete clotting of f under these conditions. The volume of original stock solution was 1 ml.

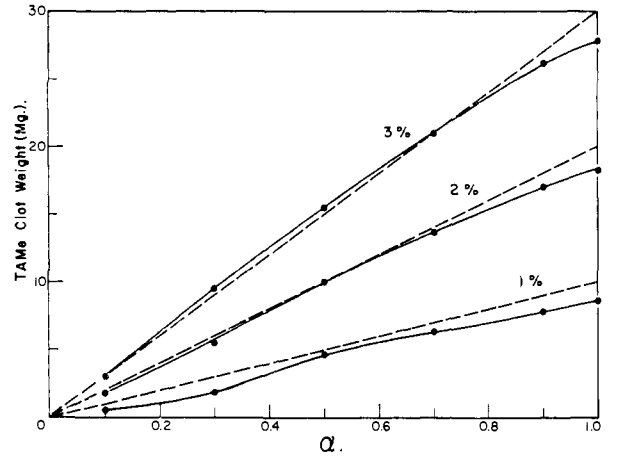

Fig. 3.—Calibration curves to convert clot weights (in the presence of TAMe) to values of α at three different total protein concentrations. The solid curves were obtained by interpolation from the curves of Fig. 2.

designed to determine the order in T, and also the rates at 15 and 25°, was always 100 units/ml. in the reaction mixture, corresponding to approximately 5 units/ml. in the clotting buffer.[29,33] In any run F and T were mixed at a given temperature. At various times during the run, 1-ml. aliquots of the reaction mixture were removed and added to 20 ml. of clotting buffer[29] containing 0.04 *M* TAMe; similar 1-ml. aliquots were added to 20 ml. of clotting buffer[29] containing no TAMe. Analyses were carried out in triplicate. The clotting reaction was allowed to proceed for 4 hours at room temperature, and the resulting clots were treated as described in the previous section. With the aid of the appropriate calibration curve of Fig. 3, the value of α in the reaction mixture at the time of sampling was determined.

The thrombin concentration was determined by the *p*H-stat, TAMe method[20] at the beginning and end of each run. Loss of enzyme activity generally amounted to about 10% in 2 weeks at 0°, while complete activity was retained during the shorter duration runs at 15 and 25°. Similar tests of the ability of aged reaction mixtures to clot fresh fibrinogen solutions confirmed the conclusions from the TAMe assay that there was no significant loss of enzyme activity.

Seegers citrate thrombin was used in most of the runs, except those in which the order in thrombin was determined at 0°. The latter data were obtained with Parke, Davis thrombin which, at this temperature, caused no side reactions during the time of the runs (6 days) and retained full activity throughout the experiment, as reported previously.[20]

In addition to previous criteria[18,19] for the absence of steps 2 and 3 in the bromide solvent at room temperature, the viscosity of a mixture of 3% F and 100 units/ml. T was checked to demonstrate that f is monomeric at 0°. No change in flow time was observed over a period of several days, indicating the absence of step 2. This finding was confirmed by ultracentrifugal observations on the same system at about 10°.

Results

Representative kinetic data for step 1 in the bromide solvent at 0° are shown in Fig. 4, where they are plotted as α *vs.* *t*. The total clottable protein concentration (F + f) remained constant over the 11 day reaction period, indicating no loss in clottability of F or f during the course of the run.

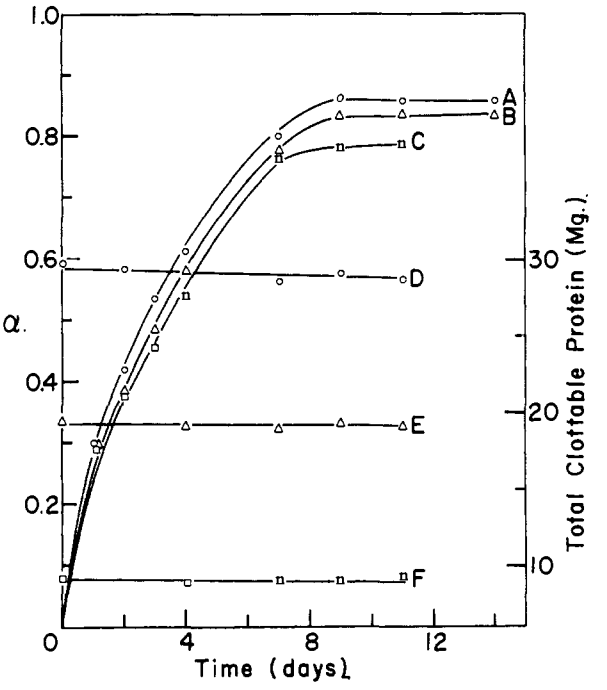

Fig. 4.—Variation of α with time for 3 typical runs on step 1 at 0° (1 *M* NaBr, *p*H 5.3) at a citrate thrombin (Seegers) concentration of 100 units/ml. Curves A, B, C correspond to approximately 30, 20 and 10 mg./ml., respectively (see Table III). Curves D, E, F are the corresponding plots for total clottable protein concentration (F + f), showing essentially no loss in clottability of F or f during the 11 day reaction period. It should be noted that the stated thrombin concentration is that determined in 0.15 *M* KCl at *p*H 8 at 25°; the actual activity in the reaction mixture is considerably lower (see text).

to *p*H 5.9 (where steps 2 and 3 proceed) and allowed to remain at room temperature until all the F was converted to f. The solution was adjusted to *p*H 5.3 with 0.5 *M* acetate, *p*H 4.8 in 1 *M* NaBr and all conditions adjusted to give α-values of 0.8, 0.6 and 0.4, total clottable protein concentrations 3 and 1.5%, T concentration 100 units/ml. The curves thus obtained were almost identical with those of Fig. 2.

Thrombin stability over this period has been discussed above. The long reaction time for step 1 in the bromide solvent at 0° is a consequence of the low thrombin activity under these conditions. The observation that the values of α became constant (even up to 18 days at 0°) at a value less than unity is attributed to a reversible equilibrium in step 1; as indicated above, the low final values of α cannot be attributed to the destruction of F, f or T. Moreover, at *p*H 5.9 and 0°, step 2 proceeds and the final value of α becomes equal to unity since the equilibrium position of step 1 is affected by step 2. A discussion of the *thermodynamics* of the equilibrium of step 1 will be presented in a forthcoming paper.[21]

The data of Fig. 4 are re-plotted for the first half of the reaction period in Fig. 5 as $-\log (1 - \alpha)$ *vs.* t and indicate that the reaction is initially first

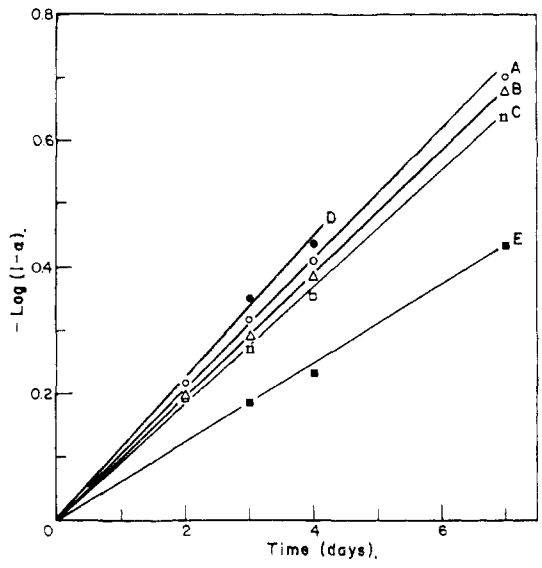

Fig. 5.—Re-plot of data of Fig. 4 to indicate that the initial part of the reaction is first order in F (curves A, B, C). Curves D and E are those that would have been obtained instead of curves A and C, respectively, if the corrections based on the calibration curve of Fig. 3 had not been introduced.

order in F at constant thrombin concentration (curves A, B, C). It may be noted that there is a slight positive dependence of the rate on the initial concentration F_0. If the corrections based on the calibration curves of Fig. 3 had not been introduced, the variation of the rate with F_0 would have appeared to be much greater. In such a case, curves D and E of Fig. 5 would have been obtained instead of curves A and C, respectively.[46] Hence, had we not used the calibration curves, we would have erroneously concluded that the rate is greatly dependent on F_0. Such a conclusion is, in fact, inherent in the data of Lein,[22] in which the dependence of the rate on F_0 resembles curves D and E, rather than A and C. Waugh and Livingstone,[23] on the other hand, also found a large dependence on F_0 but in the opposite direction. This point has been considered in detail in another paper.[15]

The maintenance of a first-order character for a long period of time in curves A, B and C of Fig. 5, for a reaction which is presumably reversible, will be discussed in the next paper[21] on the equilibrium in step 1.

The dependence of the reaction rate on thrombin concentration is shown in Fig. 6 where the first-order rate constant at 0°, in hours^{-1}, is plotted against the concentration of thrombin (Parke, Davis) at the constant value of F_0 of 29 mg./ml. From Fig. 6 it appears that step 1 is also first order in T. Waugh and Livingstone[23] found a similar first-order dependence on T for step 1 under conditions where steps 2 and 3 proceeded simultaneously.

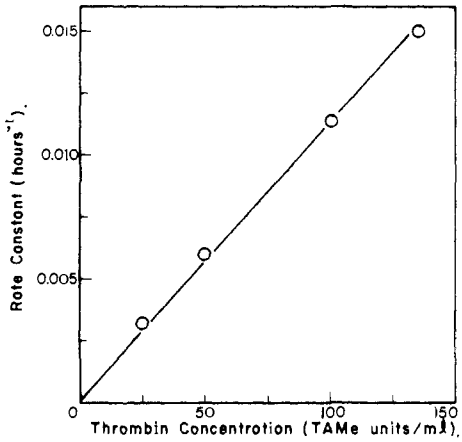

Fig. 6.—Dependence of the first-order rate constant for step 1 on thrombin (Parke, Davis) concentration at 0° (1 *M* NaBr, *p*H 5.3) at a fibrinogen concentration of 29 mg./ml. As in Fig. 4, the stated thrombin concentration is that determined in 0.15 *M* KCl at *p*H 8 at 25°, rather than under the conditions of the kinetics experiments.

Similar experiments were carried out at 15 and 25°. A summary of all of the kinetic data is given in Table III where $k_{obs} = [-\ln (1 - \alpha)]/t$. Since the observed rate constant k_{obs} is equal to the product of the first-order rate constant k and the thrombin concentration, the values of k_{obs} were divided by the thrombin concentration to obtain the values of k given in the last column of Table III. In obtaining the average values of k, the small dependence of k on F_0 has been neglected. However, the small variation in k at any temperature is apparent from an inspection of the table.

The values of log k have been plotted against the reciprocal of the absolute temperature in Fig. 7. The heat of activation, obtained from the slope of the line in Fig. 7, is 21 kcal./mole.

Discussion

Mechanism.—From the kinetic data the initial part of step 1 (forward reaction) in the bromide solvent appears to be first order in F and T. This conclusion applies to a range of thrombin concentrations of 25 to 135 units/ml. and a range of fibrinogen concentrations of 10 to 30 mg./ml. The range of thrombin concentrations was limited by the necessity of avoiding the complications ex-

(46) When the total protein concentration is 3%, the amount of F occluded by the clot can compensate to a great extent for the lack of complete clottability under these conditions. Hence, curve D does not differ from curve A as much as curve E differs from curve C.

TABLE III

SUMMARY OF INITIAL FIRST-ORDER RATE CONSTANTS IN 1 *M* NaBr, *p*H 5.3

Temp., °C.	F_0, mg./ml.	Thrombin concn.,[a] units/ml.	$k_{obs} \times 10^3$, hr.$^{-1}$	$k = k_{obs}$/(thrombin concn.) $\times 10^4$
0	29.3	25 (PD)	3.2	1.28
	29.0	50 (PD)	6.0	1.20
	29.2	100 (PD)	11.4	1.14
	29.2	135 (PD)	15.0	1.11
	29.7	104 (C)	10.5	1.01[b]
	30.2	100 (C)	10.1	1.01
	19.7	100 (C)	9.0	0.90
	19.5	100 (C)	8.7	.87
	19.5	98 (C)	9.5	.97[b]
	23.7	100 (PD)	10.8	1.08
	20.0	100 (PD)	9.5	0.95
	19.8	100 (PD)	9.0	.90
	19.4	100 (PD)	9.8	.98
	16.3	100 (PD)	9.8	.98
	9.9	100 (C)	7.4	.74
	9.3	95 (C)	7.6	.79[b]
			Av.	0.99×10^{-4}
15	31.6	74 (C)	42	5.7[c]
	22.0	84 (C)	44	5.2[c]
	9.0	88 (C)	52	5.9[c]
	19.6	80 (C)	65	8.1
			Av.	8.1×10^{-4}
25	19.1	55 (PD)	184	33
	19.6	76 (C)	205	27
			Av.	30×10^{-4}

[a] PD = Parke, Davis thrombin; C = Citrate (Seegers) thrombin. [b] Data for these three runs, which were performed concurrently, are plotted in Figs. 4 and 5. [c] These data are each based only on one point, obtained rather late in the run. They were obtained primarily for use in connection with equilibrium,[21] rather than kinetic studies and are therefore not included in the average. Even if they were included, the 15° point of the Arrhenius plot of Fig. 7 would be shifted only from −3.1 to −3.2.

bodied in Table I. The range of fibrinogen concentrations was limited by two factors. At low values of F_0 the clot weights in the initial part of the run were too small to obtain good precision. At concentrations above 30 mg./ml., complete inhibition of step 2 could not be achieved in the bromide solvent.

The small dependence of the rate on initial F concentration, indicated in Fig. 5, is negligible compared to the large effect found by Waugh and Livingstone,[23] who carried out kinetic studies on the fibrinogen–thrombin interaction in dilute phosphate buffer at *p*H 6.8. Also, our small dependence of the initial rate on F_0 is in a direction opposite to that found by Waugh and Livingstone. The Waugh–Livingstone results were interpreted in terms of additional reactions involving the inactivation of thrombin by fibrinogen and fibrin. Since the bromide solvent differs from the *p*H 6.8 buffer used by these workers, it may not be valid to compare the two sets of results, especially since the inactivation interactions considered by Waugh and Livingstone might well be suppressed at the low *p*H and very high ionic strength of the bromide solvent. Further, it is possible that the mechanism for the reaction of F and T in the bromide

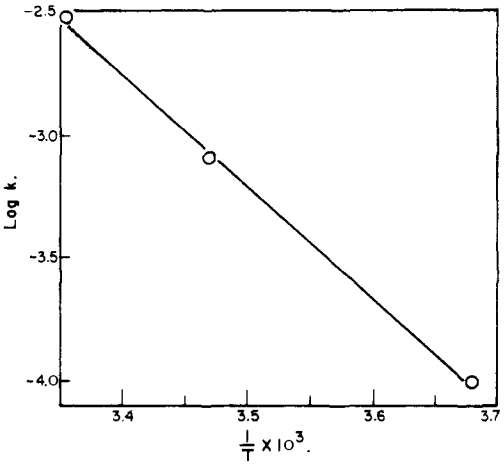

Fig. 7.—Plot of log k *vs.* $1/T$ for step 1.

solvent (*i.e.*, in the absence of steps 2 and 3) might differ from that at *p*H 6.8 where steps 2 and 3 occur. However, in a separate publication,[17] we have carried out kinetic studies of step 1 at *p*H 6.8 (in the presence of steps 2 and 3) and have shown that the rate law is the same at *p*H 6.8 as in the bromide solvent, contrary to the results of Waugh and Livingstone.[23] Further, some question has been raised[17] about the validity of the method used by Waugh and Livingstone to treat their data. It thus appears that the kinetics of the forward reaction of step 1 are compatible with a Michaelis–Menten bimolecular reaction to form the enzyme–substrate complex under conditions where the enzyme is not saturated. The kinetic data give no evidence for the presence of previously postulated[23] thrombin-inactivating reactions.

No attempt will be made here to interpret the activation energy. However, certain aspects of the reaction which conceivably could affect the activation energy will be discussed in the forthcoming paper[21] on the equilibrium in step 1.

Magnitude of k.—The kinetic data indicate that step 1 proceeds very slowly in the bromide solvent at 100 TAMe units/ml. (see Table IV). These results are in agreement with previous data on step 1, obtained by following the rate of production of fibrinopeptide[19] (*i.e.*, non-protein nitrogen). However, at *p*H 6.8 in dilute sodium chloride the reaction is much faster[17] at 25° at a much lower thrombin activity (0.045 unit/ml.). There is then an extremely large *p*H and/or solvent dependence of the rate constant of the fibrinogen–thrombin reaction. This, of course, implies a large *p*H and/or solvent dependence of k_3/K_m, where the Michaelis constant K_m is $(k_2 + k_3)/k_1$ and k_3 is the reaction rate constant for the formation of products from the enzyme–substrate complex.

The low rate in sodium bromide at *p*H 5.3 cannot be attributed to the reverse of step 1 since the *initial* rate of the forward reaction would not be affected by the reverse reaction. At *p*H 6.8, the reverse reaction is negligible since step 2 occurs. As shown here, and elsewhere,[17] the thrombin-inactivation by F or f is insignificant. Hence, the low rate of step 1 in sodium bromide at *p*H 5.3 cannot be attributed to such side reactions; also, the

TABLE IV

COMPARISON OF KINETIC CONSTANTS[a]

Temp., °C.	Solvent	pH	Thrombin concn. (units/ml.)	k_{obs}	$t_{1/2}$	k (per thrombin unit/ml.), min.$^{-1}$
0	Sodium bromide	5.3	100	0.01 hr.$^{-1}$	3 days	0.0000017
25	Sodium bromide	5.3	100	.30 hr.$^{-1}$	3.3 hr.	0.00005
25	Sodium chloride[b]	6.8	0.045	.07 min.$^{-1}$	10 min.	1.55
25	Potassium chloride	5.3	0.045	.007 min.$^{-1}$	100 min.	0.155

[a] The data at pH 6.8 were obtained from ref. 17. Those in potassium chloride at pH 5.3 were computed by assuming the rate constant to be 0.1 of that at pH 6.8. The factor 0.1 was estimated from a published curve of Mihalyi[47] on the pH dependence of the rate constant of step 1 in 0.3 M KCl. We are indebted to Dr. Mihalyi for sending us the data on which this curve is based. [b] 0.1 ionic strength NaCl, 0.05 ionic strength phosphate.

low rate in this solvent cannot be attributed to the destruction of F or T.

Having eliminated these other possibilities we may examine the pH and solvent dependence of k_3 and K_m. While it is at present not known how solvent and pH affect k_3 for step 1, we may assume these values to be the same as those for the thrombin–TAMe reaction, which can be studied under zero-order conditions. From the data of reference 20 at 25°, k_3 in 0.15 M NaCl is twice that in 1 M NaBr at all pH's in the range 5 to 9. Further, k_3 at pH 6.8 is 15 times that at pH 5.3 in either solvent. Hence, we may take k_3 for step 1 at pH 6.8 in 0.1 M NaCl as being 30 times greater than that at pH 5.3 in 1 M NaBr. The ratio of the values of k for step 1 under these two conditions is (1.55)/0.00005 or 30×10^3. Since only a factor of 30 is accountable for by k_3, the remaining factor of 1000 must be attributed to $1/K_m$. According to Mihalyi, a factor of 10 (see footnote in Table IV) is attributable to the pH variation of k_3/K_m between pH 5.3 and 6.8. Within the crude estimate made here, the pH variation of k_3/K_m arises essentially from the 15-fold dependence of k_3 on pH. Thus $1/K_m$ is not very dependent on pH. The 1000-fold variation of $1/K_m$ may then be attributed to a solvent effect; in other words a change in solvent from 1 M NaBr to 0.1 M NaCl decreases K_m by a factor of 1000. This implies that the association of F and T (at comparable concentrations) is much greater in 0.1 M NaCl than in 1 M NaBr at pH 5.3. The large effect of 1 M NaBr is probably due to its ability to break hydrogen bonds. The hydrogen bond breaking properties of 1 M NaBr already have been demonstrated in previous papers on step 2.[18,24] Hence, the reaction between F and T to form the enzyme–substrate complex seems to involve the formation of hydrogen bonds between specific polar side chain groups of fibrinogen and thrombin. From the fact that diisopropyl fluorophosphate inhibits thrombin,[48,49] one of these groups may be a seryl residue.[11] Further evidence for the mechanism of thrombin action may be forthcoming from studies on the pH and ionic strength dependence of the T-TAMe and T-F reaction with purified thrombin. For the present, the above discussion serves to reconcile the magnitudes of the rate constant k under conditions of varying pH and solvent.

(47) E. Mihalyi, quoted by J. A. Gladner and K. Laki, *Arch. Biochem. Biophys.*, **62**, 501 (1956).

(48) J. A. Gladner and K. Laki, *ibid.*, **62**, 501 (1956).

(49) K. D. Miller and H. Van Vunakis, *J. Biol. Chem.*, **223**, 227 (1956).

Intermediates in the F–f Conversion.—It is known that P represents more than one species (*i.e.*, two peptides[50] and carbohydrate[12] material). It is thus conceivable that intermediate species are formed during the conversion of F to f. These intermediates might be devoid of only one instead of two peptides. Considering only the peptide liberation, step 1 conceivably could involve the following parallel reactions.

$$F \rightleftharpoons f_A + P_A \quad (5)$$

$$F \rightleftharpoons f_B + P_B \quad (6)$$

$$f_A \rightleftharpoons f + P_B \quad (7)$$

$$f_B \rightleftharpoons f + P_A \quad (8)$$

If reaction 6 is very slow compared to reaction 5 we could have, instead, a set of consecutive reactions.

$$F \rightleftharpoons f_A + P_A \quad (5)$$

$$f_A \rightleftharpoons f + P_B \quad (7)$$

Preliminary experiments of Bettelheim[36] indicate that equal amounts of P_A and P_B are released in the over-all reaction but that initially P_A is released about four times faster than P_B. The question may be raised as to whether f_A and f_B are polymerizable species, or whether both peptides must be released in order for step 2 to take place. Since we have analyzed for the clottable species, we cannot distinguish between the species f_A, f_B or f. Conceivably, other methods for following the kinetics might distinguish between the rates of production of P_A and P_B.

Other complications are also possible if the peptides are hydrogen bonded to the protein core *after* the peptide bond is hydrolyzed. This point will be explored in more detail in the next paper in this series.[21] For the present, it is worth pointing out that such a complication does not affect our definition of α. If f and P were associated to form an f-P complex, then we would have

$$F \overset{T}{\rightleftharpoons} f\text{–}P \quad (9)$$

$$f\text{–}P \rightleftharpoons f + P \quad (10)$$

Assuming only f clots, then α could be defined as

$$\alpha = \frac{(f\text{–}P) + (f)}{(F) + (f\text{–}P) + (f)} \quad (11)$$

since f–P complexes would dissociate so that the

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

free f, as well as the f originally bound in the f–P complex, would appear in the clot. The definition of α in eq. 11 is thus equivalent to that of eq. 4 and independent of the equilibrium constant of reaction 10.

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

ITHACA, NEW YORK

[CONTRIBUTION NO. 73 FROM THE DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES, UNIVERSITY OF COLORADO MEDICAL CENTER]

Effect of Binding of Ions and other Small Molecules on Protein Structure. IV. Two Electrophoretically Distinguishable Types of Interaction of Bovine Serum Albumin with Acidic Media[1]

BY JOHN R. CANN

RECEIVED JUNE 30, 1958

Bovine serum albumin undergoes two electrophoretically distinguishable types of interaction with acidic media. The first type of interaction is observed only in acetate-containing media at relatively high protein concentration, and is interpretable in terms of reversible complexing of the protein with undissociated buffer acid. The other type of interaction is best studied at low protein concentration, although with proper choice of electrolyte composition it can also be observed at high concentration. This type of interaction has been interpreted by Aoki and Foster in terms of a *p*H dependent protein transformation.

Introduction

A new effect of acetate buffer (NaAc–HAc) on the electrophoretic patterns of bovine serum albumin (BSA) and other proteins in acidic media has been described previously.[2–4] The patterns of 1% BSA at *p*H 4.7–4.0 show multiple moving boundaries, whose proportions and velocities depend upon the concentration of NaAc–HAc in the supporting medium and are very non-enantiographic even at ionic strengths as high as 0.3. These observations were interpreted in terms of reversible complexing of the protein with undissociated buffer acid.

Aoki and Foster[5–9] also have reported a new electrophoretic behavior of BSA. Their patterns of 0.2% BSA in NaCl–HCl solutions of ionic strength as low as 0.02, are fairly enantiographic and show two moving boundaries whose proportions depend upon *p*H. These observations have been interpreted in terms of a reversible, *p*H dependent protein transformation. These workers[6] also found that the proportions of the two boundaries are shifted by substitution of NaAc–HAc for NaCl–HCl. Unfortunately, this observation has led to some confusion since it would seem to suggest that the effect of NaAc–HAc on the patterns of BSA at high protein concentration is simply an effect of acetate on the same transformation postulated by Aoki and Foster to explain the electrophoretic behavior at low protein concentration. That this is not the case is demonstrated by the present experiments, which show that BSA actually undergoes two distinct processes in acid solution, one of which is observed only in NaAc–HAc at high protein concentration and has been described by us previously.[2–4] The other process is the one studied by Aoki and Foster[5–9] at low protein concentration.

(1) Supported in part by research grant No. E-1482(C6) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service; and in part by the Damon Runyon Memorial Fund for Cancer Research and the American Cancer Society.

(2) R. A. Phelps and J. R. Cann, THIS JOURNAL, **78**, 3539 (1956).
(3) J. R. Cann and R. A. Phelps, *ibid.*, **79**, 4672 (1957).
(4) R. A. Phelps and J. R. Cann, *ibid.*, **79**, 4677 (1957).
(5) K. Aoki and J. F. Foster, *ibid.*, 3538 (1956).
(6) K. Aoki and J. F. Foster, *ibid.*, 3385 (1957).
(7) K. Aoki and J. F. Foster, *ibid.*, 3393 (1957).
(8) J. F. Foster and K. Aoki, *J. Phys. Chem.*, **61**, 1369 (1957).
(9) J. F. Foster and K. Aoki, THIS JOURNAL, **80**, 1117 (1958).

Experimental

Electrophoretic analyses were carried out in the usual manner, using the Perkin-Elmer Tiselius apparatus, fitted with a current-regulating power supply and a potentiometer to measure the voltage drop across a standard resistance placed in series with the cell. Glass hypodermic needles were used to fill the electrophoresis cell in order to avoid contaminating the solution with the corrosion products of metal needles. Boundary velocities (10^5 x.v.) are shown above or beside the corresponding peaks in the electrophoretic patterns presented in Figs. 1 and 2.

The bovine serum albumin was Armour crystallized bovine plasma albumin.

Results and Discussion

Two kinds of electrophoretic experiments have been carried out on BSA. The results of the first kind of experiment are presented in Fig. 1. Electrophoretic analyses were carried out at different protein concentrations in an acetate–chloride buffer, ionic strength 0.02 and *p*H 4.0. (Essentially the same results were obtained in 0.01 and 0.02 *M* acetate buffers.) At 1% protein the rising pattern shows two moving boundaries: a diffuse, slow moving boundary and a sharp fast one which is actually composed of two poorly resolved peaks. As the protein concentration is decreased, the proportion of the slow boundary decreases until it disappears (or nearly so) at 0.2% protein. At this concentration the patterns are essentially the same as those described by Aoki and Foster, and the two rising boundaries are closely related to the two peaks constituting the fast boundary at 1% protein.

In the second kind of experiment, all electrophoretic analyses were carried out on 1% BSA but the composition of the supporting medium was varied. The results of this experiment are shown in Fig. 2. The patterns obtained in 0.01*M* NaCl–HCl are very similar to those of Aoki and Foster