TABLE V

PROVISIONAL VALUES OF THERMODYNAMIC INTERACTION PARAMETERS AT 25°. POLYSTYRENE IN VARIOUS SOLVENTS

Solvent	θ, °K.	$10^3 \times \psi_1 C_M$ at 25°C.	$\psi_1 C_M V_1$ at 25°C.	ψ_1	κ_1
Benzene	100	0.29	0.26	0.09	0.03
Bromobenzene	115	.25	.27	.09	.03
Toluene	160	.32	.35	.12	.06
o-Dichlorobenzene	220	.52	.54	.18	.13
Pyridine	161	.28	.23	.08	.04
Ethylbenzene	156	.27	.33	.11	.06
1,4-Dioxane	198	.36	.31	.10	.07
2,5-Dimethylfuran	145	.24	.26	.09	.04
Cyclohexanone	170	.23	.24	.08	.05
Methyl *n*-amyl ketone	210	.10	.14	.05	.04
Ethyl chloroacetate	255	.19	.23	.08	.07
Ethyl acetate	222	.09	.10	.03	.02
1-Nitropropane	272	.12	.11	.04	.04

umns 1 and 2 of Table V. The former multiplied by V_1, the molar volume of the solvent concerned, yielded values of $\psi_1 C_M V_1$ (column 3), whence values of ψ_1 (column 4) were obtained by dividing by the value of $C_M V_1$ (3.0) calculated for polystyrene at 25° by Fox and Flory.[6] Finally, the values of κ_1 (column 5) were calculated using the relationship $\kappa_1 = \psi_1 \theta / T$.[6]

Table V, then, constitutes an extension of Table V of the paper by Fox and Flory,[6] providing values for an additional 11 solvents. Our values of ψ_1 and κ_1 are subject to almost all the uncertainties mentioned by Fox and Flory. In other words, these values too are only approximate.

Nevertheless they are good enough to reinforce the conclusions that Fox and Flory drew from their more limited data, namely, that solvents of cyclic molecular structure have higher values of ψ_1 than those whose molecules are acyclic. If we include their data, the values of ψ_1 for the 10 cyclic solvents all lie above 0.08, whereas the values of ψ_1 for the 6 acyclic solvents are all below 0.09 (and all but one below 0.06).

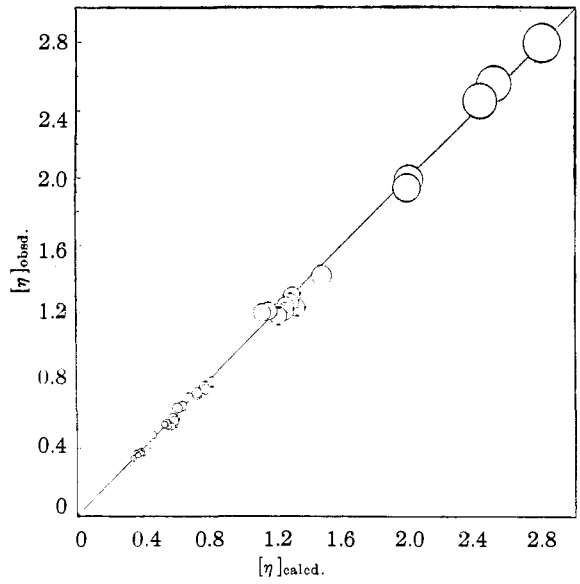

Fig. 1.—System polystyrene–solvent at 25° and 65°: 6 polystyrene fractions, mol. wt. 0.9 × 10^5 to 1.1 × 10^6; 13 solvents (ranging from good to poor).

Acknowledgments.—We acknowledge with thanks the assistance given us by Mr. L. J. Sharman in making some of the experimental measurements.

HAMILTON, ONTARIO, CANADA RECEIVED NOVEMBER 15, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Flow Birefringence in Arrested Clotting Systems[1]

BY HAROLD A. SCHERAGA AND JOHN K. BACKUS

Flow birefringence measurements have been made on fibrinogen–thrombin mixtures to which hexamethylene glycol had been added at various stages of the clotting reaction. The extinction angle and birefringence data indicate that the early stages of the clotting process involve end-to-end aggregation, which is reversible upon dilution with solvent containing hexamethylene glycol.

In recent physical chemical studies of the fibrinogen–fibrin reaction attention has been directed toward the early stages of the thrombin-induced polymerization of fibrinogen. It is important to have information about the nature of the polymers produced in the initial part of the reaction, before gelation, where the degree of polymerization is very low. From sedimentation and viscosity studies of inhibited fibrinogen–thrombin systems Shulman and Ferry[2] suggested that an elongated polymer is produced in the clotting reaction in the presence of hexamethylene glycol. The flow birefringence technique is especially suitable for the study of the structure of asymmetrical molecules such as fibrinogen.[3] It is applied here in an investigation of the nature of the polymerized products formed in the early stages of the reaction when bovine thrombin is added to bovine fibrinogen solutions and the clotting process arrested, after a time, by the addition of hexamethylene glycol. It has been shown[2] that this inhibitor does not destroy fibrinogen or thrombin.

Experimental

Materials.—Armour bovine fraction I was refractionated according to Laki's procedure[4] yielding solutions containing 10–15 mg./ml. of fibrinogen as determined by Morrison's method.[5] The protein, analyzed by Laki's method,[4] was

(1) This work was carried out in connection with project N6-onr 26414 supported by the Office of Naval Research.

(2) S. Shulman and J. D. Ferry, *J. Phys. Colloid Chem.*, **55**, 135 (1951).

(3) C. S. Hocking, M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **74**, 775 (1952).

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

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

95% clottable. Parke, Davis bovine thrombin (18 μ/mg.) was used to effect clotting and hexamethylene glycol, recrystallized from ether, was used to "stop" the clotting reaction.

Method.—Thrombin, dissolved in KCl, was added to fibrinogen solutions containing KCl and sodium citrate and the mixture allowed to stand for a given length of time at 25.0°. The standard clotting conditions used throughout this work were: fibrinogen concentration, 4.5–6 mg./ml.; thrombin concentration, 0.1 μ/ml.; pH 6.5; ionic strength, 0.66.

At various times before the gel point a solution of hexamethylene glycol in 0.3 M KCl was added to "stop" the reaction. The addition of the inhibitor solution produced approximately a twofold dilution of the original solution, the final hexamethylene glycol being 0.47 M. The pH did not change by more than 0.05 unit.

Dilutions of this inhibited reaction mixture were made with solvent of the same composition. The resulting solutions were immediately evacuated to remove dissolved air and placed in the flow birefringence apparatus. The elapsed time between the addition of hexamethylene glycol and the first measurements was approximately 10 minutes.

In several experiments the solutions were allowed to stand for several hours after adding the hexamethylene glycol to determine the extent of reaction after addition of inhibitor.

Flow Birefringence Measurements.—Measurements of the extinction angle, χ, and birefringence, Δn, were carried out at 25.0° in the flow birefringence apparatus described previously.[6] Since an inter-cylinder gap-width of 0.12 mm. was available it was possible to attain relatively high gradients in the region of laminar flow without requiring the addition of glycerol to increase the viscosity of the solutions. The solvent used in these experiments had a viscosity of 0.0110 poise at 25.0° and an index of refraction of 1.343 at 546 mμ and 25.0°.

Results

Purified Fibrinogen.—Flow birefringence results have been reported previously for purified bovine fibrinogen[3] solutions containing glycerol. In the present work, measurements were made on aqueous solutions of fibrinogen without glycerol. The behavior of the extinction angle with velocity gradient for these latter solutions is shown in Fig. 1. The curves are theoretical ones using the Peterlin–Stuart theory[7] with recently computed numerical solutions.[8] Since a strong concentration dependence of the extinction angle curves is evident, the empirical extrapolation procedure used previously[9] was applied in this case. The dependence of apparent length on concentration is shown in Fig 2. The length of bovine fibrinogen, extrapolated to infinite dilution, is 660 Å. in agreement with the previously reported value of 670 Å. for glycerol–water mixtures.

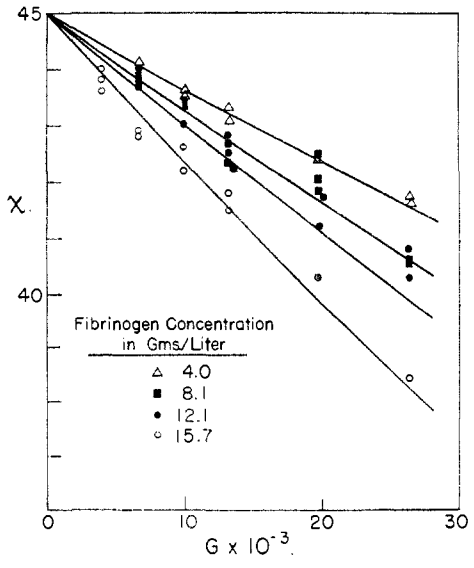

Fig. 1.—Extinction angle curves for aqueous solutions of purified bovine fibrinogen at various concentrations.

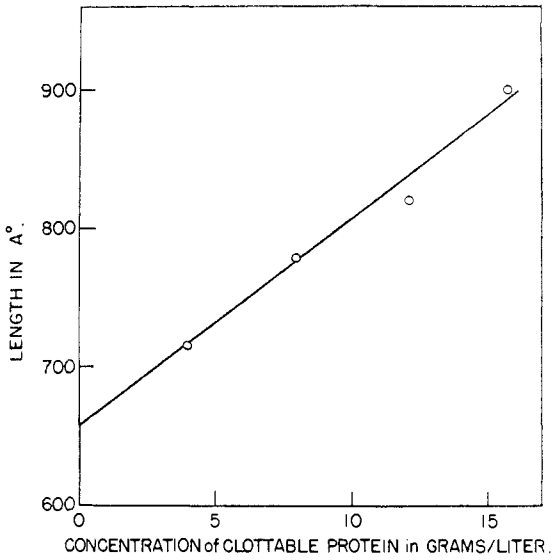

Fig. 2.—Dependence of the apparent length of the fibrinogen molecule on fibrinogen concentration.

Effect of Protein Concentration on Flow Birefringence Measurements of Arrested Clotting Systems.—The solutions which were examined had been allowed to react under normal clotting conditions for various times up to 25 minutes before adding the inhibitor. Gelation occurred at approximately 28 minutes in an uninhibited system under these conditions. Flow birefringence measurements were made immediately after the clotting process was arrested. A blank run on hexamethylene glycol in KCl showed no birefringence.

To study the effect of protein concentration on the flow birefringence measurements, solutions which had been allowed to react for 25 minutes in the normal manner were diluted with the same solvent immediately after the addition of the hexamethylene glycol. Measurements were begun within 10 minutes after dilution. The extinction angle curves of Fig. 3 and the birefringence curves of Fig. 4 show a marked concentration dependence and a significant degree of polydispersity.

To characterize the molecular dimensions the data at the lowest gradient (G = 6600 sec.$^{-1}$) were used to calculate a "maximum" particle length. As pointed out previously[6] the "maximum" length calculated depends on the value of the lowest gradient at which measurements are made. It would be desirable to have measurements at much lower gradients in order to perform an extrapolation to zero gradient. For purposes of the present discussion, the "maximum" length at 6600 sec.$^{-1}$

(6) H. A. Scheraga and J. K. Backus, *ibid.*, **73**, 5108 (1951).
(7) A. Peterlin and H. A. Stuart, *Hand. u. Jahrb. d. Chem. Phys.*, *Bd.*, 8, *Abt. IB*, (1943).
(8) H. A. Scheraga, J. T. Edsall and J. O. Gadd, *J. Chem. Phys.*, **19**, 1101 (1951).
(9) J. K. Backus and H. A. Scheraga, *J. Colloid Sci.*, **6**, 508 (1951).

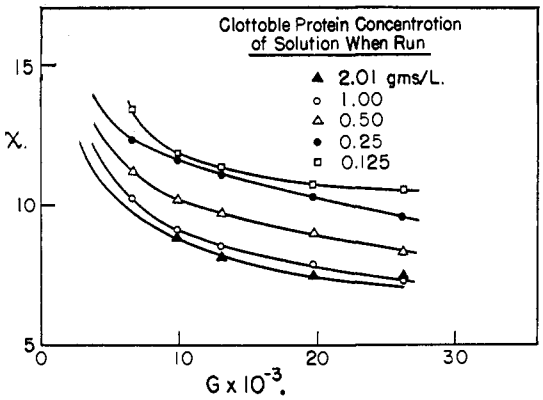

Fig. 3.—Concentration dependence of extinction angle curves for clotting mixtures arrested after 25 minutes and measured immediately.

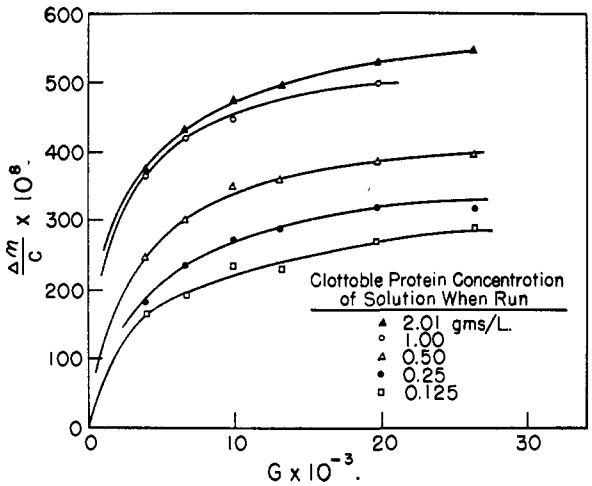

Fig. 4.—Birefringence curves corresponding to extinction angle data of Fig. 3.

is a sufficiently good quantity to characterize the system. To perform this calculation a knowledge of the minor axis, d, of the particle is required. Since the length is very insensitive to the value used for the minor axis, the calculations were performed using values of 38 Å., the value accepted for fibrinogen, and 76 Å., which is probably an overestimate of the thickness which the polymerized particles attain. The apparent "maximum" length is plotted against concentration of clottable protein in Fig. 5.

Effect of Reaction Time after Addition of Inhibitor.—The measurements described thus far on clotting solutions were carried out on systems which were still undergoing reaction after addition of the inhibitor, even though at a slow rate.[10] In order to determine the extent of reaction after the addition of hexamethylene glycol, a fibrinogen–thrombin mixture was allowed to react for 25 minutes under the above conditions; hexamethylene glycol was then added and measurements were made on aliquots at intervals up to 52 hours.

(10) Similar flow birefringence studies[11] have been carried out on solutions in which fibrinogen and thrombin were allowed to react for a long period of time in the presence of hexamethylene glycol.

(11) J. F. Foster, E. G. Samsa, S. Shulman and J. D. Ferry, *Arch. Biochem. Biophys.*, **34**, 417 (1951). We wish to thank Dr. Ferry for sending us a copy of the manuscript.

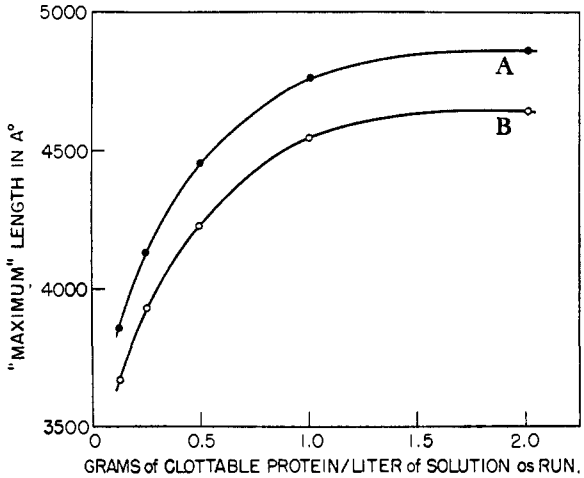

Fig. 5.—Effect of protein concentration on particle length calculated for two different values of the minor axis of the ellipsoidal particle: A, for d = 38 Å.; B, for d = 76 Å.

Figure 6 shows the effect of this time delay on extinction angle, the corresponding increase in apparent length being shown in Fig. 7. The poly-

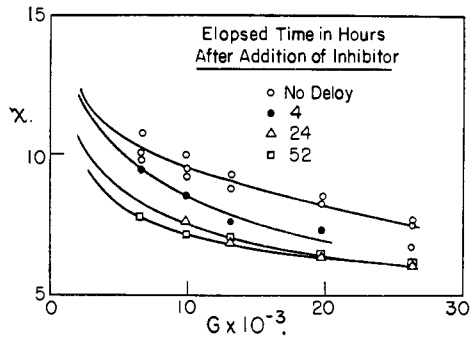

Fig. 6.—Extinction angle curves for solutions allowed to stand for various times after addition of hexamethylene glycol to solutions which had clotted for 25 minutes.

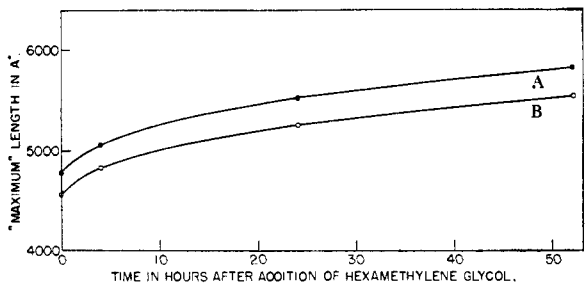

Fig. 7.—Increase in apparent particle length as a function of time after addition of the inhibitor: A, for d = 38 Å.; B, for d = 76 Å.

merized fibrinogen increases in length by about 1000 Å. within two days after the addition of hexamethylene glycol. The birefringence curves corresponding to the data of Fig. 6 are given in Fig. 8.

Effect of Reaction Time before Addition of Inhibitor.—Figure 9 shows the behavior of the extinction angle curves for solutions in which clotting was arrested at various times. In all cases the fibrinogen concentration was 5.9 g./liter during

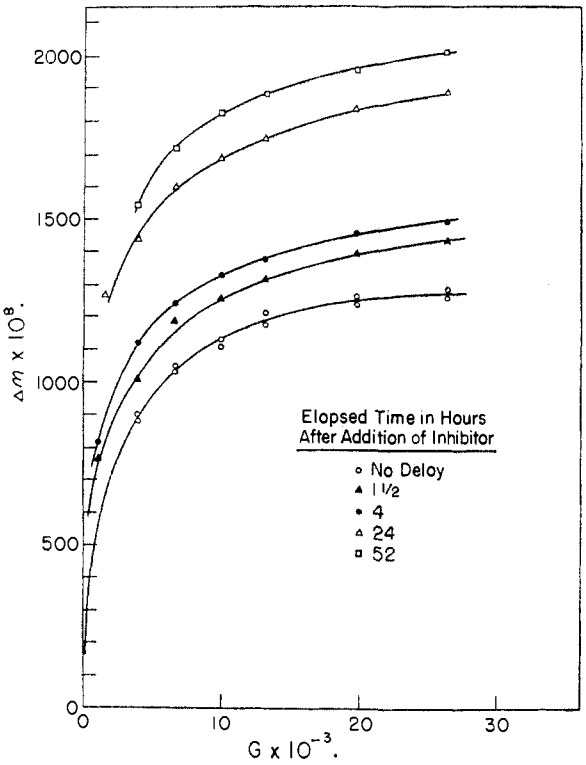

Fig. 8.—Birefringence curves corresponding to extinction angle data of Fig. 6.

clotting and 2.62 g./liter during the flow birefringence measurements. The data indicate that the apparent particle length is increasing as the reaction proceeds. The "maximum" length is plotted

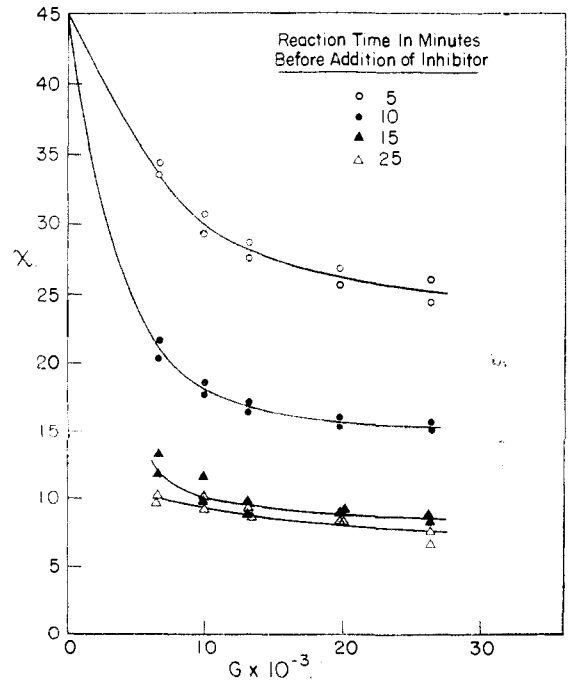

Fig. 9.—Extinction angle curves for clotting mixtures arrested at various times.

against reaction time in Fig. 10. It appears that some of the polymerized fibrinogen attains a particle length of about 5000 Å. or, an end-to-end aggregation of 7 to 8 fibrinogen molecules, before the gel point (28 minutes). It should be noticed that this maximum length before gelation is less than the value of 5800 Å. indicated in Fig. 7 when the solution had been allowed to stand for 52 hours with the inhibitor, *i.e.*, prolonged standing in the presence of the inhibitor appears to allow the polymerized material to attain a greater apparent length without gelation than occurs in the absence of inhibitor just prior to gelation under normal clotting conditions.

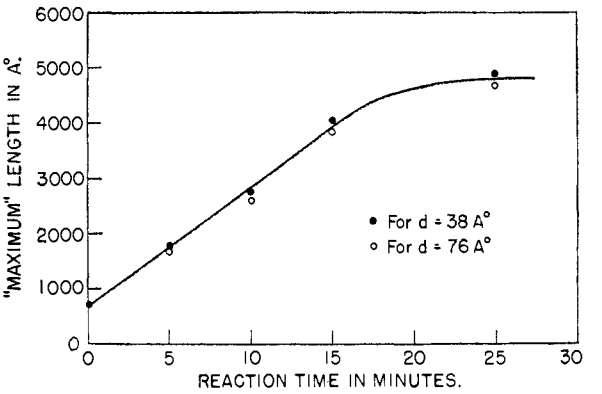

Fig. 10.—Increase in "maximum" particle length with time during the clotting process.

The birefringence curves corresponding to the extinction angle data of Fig. 9 are shown in Fig. 11.

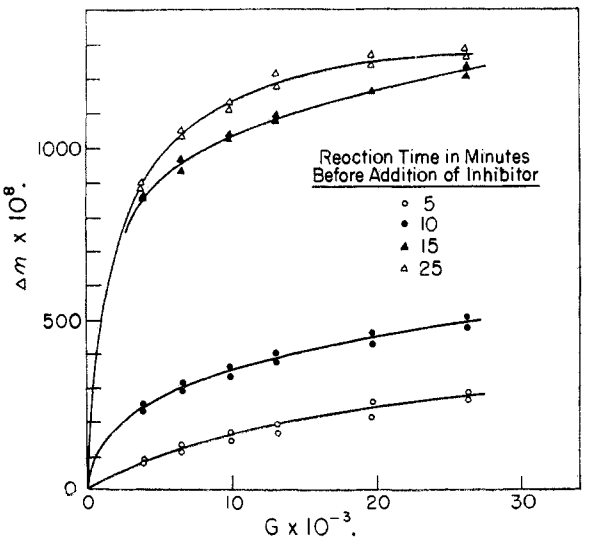

Fig. 11.—Birefringence curves corresponding to extinction angle data of Fig. 9.

Discussion

Figure 10 illustrates the change in apparent length of the polymers produced upon addition of thrombin to fibrinogen during the normal course of the clotting reaction, followed by addition of inhibitor. The increasing birefringence as the reaction proceeds (Fig. 11) is also indicative of the presence in solution of increasing amounts of long particles capable of a high degree of orientation. Whether

the effects measured here are present before the addition of hexamethylene glycol cannot be answered from these data. It is conceivable that the inhibitor produces changes in the polymerized material when it is added to the reaction mixture. The role of hexamethylene glycol in possible dissociation processes may become apparent from similar studies which we are carrying out without the addition of this inhibitor. The effect of *p*H and other variables on the clotting process is also under consideration.

The concentration dependence of the flow birefringence measurements, illustrated in Fig. 5, is different from the linear plots obtained for pure fibrinogen (Fig. 2) and detergent micelles,[9] *i.e.*, the decrease in length is much greater than would be expected from a decrease in solute–solute interaction. We are inclined to attribute this to a dissociation of the polymers into shorter particles upon dilution. Evidence for dissociation has also been obtained by Shulman and Ferry[2] from viscosity studies of inhibited clotting systems.

As the protein concentration decreases, the magnitude of the birefringence (Fig. 4) decreases considerably suggesting a significant decrease in particle length upon dilution.

Thus, the value of the flow birefringence measurements in conjunction with the empirical extrapolation procedure may be seen in that they show the character of the end-to-end aggregation during the course of the clotting reaction and also the reversibility of this aggregation process upon dilution.

ITHACA, NEW YORK RECEIVED NOVEMBER 15, 1951

[CONTRIBUTION NO. 1091 FROM STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

The Heat of Denaturation of Pepsin[1]

BY ANNE BUZZELL[2] AND JULIAN M. STURTEVANT

Pepsin undergoes an endothermic reaction, presumably a denaturation, in the same *p*H region as that in which it loses its proteolytic activity. The heat of this reaction increases from a value calorimetrically indistinguishable from zero to a maximum and decreases again to zero within less than one *p*H unit. This behavior is found at both 15 and 35°. The rate of the endothermic reaction is markedly less sensitive to *p*H and temperature changes than is the rate of the inactivation process.

Previous calorimetric study[3] of the denaturation of pepsin by alkali showed that the enthalpy change accompanying this reaction in *p*-nitrophenol buffers is strongly dependent on *p*H. We have now extended these measurements to cover a considerably larger range of *p*H, using an improved apparatus and a new calorimetric method.[4] In the present work, phosphate buffers have been employed.

Experimental

Pepsin.—Five different samples of pepsin were employed. Pepsins A and B were obtained from Armour and Company, and carried their lot numbers 80505 and 80802, respectively. Pepsin C was obtained from the University of Wisconsin. Pepsin D was prepared from Parke-Davis 1:10,000 pepsin essentially as described by Northrop.[5] After three crystallizations the pepsin was dialyzed against distilled water and then lyophilized. Pepsin E was Armour's lot 80802 material carried through three crystallizations from 20% ethanol,[6] followed by washing with *M*/500 HCl and solution in water by careful addition of dilute KOH to *p*H 4. Pepsins A, B, C and D were air-dried (approximately 40% relative humidity) and weighed amounts were dissolved in water (no alkali needed) to prepare solutions for experimentation. Analytical data obtained on solutions of these samples of pepsin are summarized in Table I. The concentrations of these solutions ranged from 0.2 to 2.0%, based on the weight of the air-dried protein used.

Determination of Peptic Activity.—The enzymatic activity of each pepsin stock solution was determined by a modification of the method of Anson.[7] Hemoglobin supplied for this purpose by Armour and Company was used as substrate, in 0.25% solution instead of 2.5% as specified by Anson, in order to obtain a smaller blank. The digestions were run for 10 minutes at 35.5°, and the trichloroacetic acid precipitate was removed by centrifugation rather than filtration. The digestion supernatants were examined without dilution at 280 mμ in a Beckman model DU spectrophotometer. It was found that if the pepsin concentration was adjusted to keep the optical density of the supernatant below 0.4 (1-cm. cell), the digestion rate was accurately zero order with respect to substrate and first order with respect to enzyme. A solution containing HCl, CCl_3COOH and formalin in the same concentrations as the digestion supernatants was used as spectrophotometric blank. Digestion blanks were prepared by adding the pepsin solution to the CCl_3COOH solution before mixing with the substrate solution, and were found to have optical densities close to 0.070.

Measurements on standard tyrosine solutions containing HCl, CCl_3COOH and formalin in the same concentrations as the digestion supernatants showed that the number of milliequivalents of tyrosine in 16 ml. (the volume of the digestion supernatants) of solution is obtained by multiplying the optical density by 0.0134. If we use the definition of a pepsin unit (PU) as the amount of enzyme which produces one milliequivalent of "tyrosine" per minute under the stated digestion conditions, it is evident that the number of pepsin units per ml. of enzyme solution is given by 0.00134 times the optical density of the digestion supernatant. The activity data given in Table I have been obtained using this calibration constant.

This definition of the pepsin unit is somewhat different from that employed by Northrop, Kunitz and Herriott,[8] who also examined the digestion product at 280 mμ, in that they referred their data to the original definition of Anson[7] based on the color developed by reaction between tyrosine and Folin phenol reagent.

Determination of *p*H.—All *p*H measurements were made at the temperature of reaction with a Beckman glass elec-

(1) Presented in part at the April, 1950, Meeting of the American Chemical Society, Philadelphia, Pa. The research reported in this paper was supported in part by a grant from the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council.

(2) Research fellow under a grant from the American Cancer Society.

(3) M. Bender and J. M. Sturtevant, THIS JOURNAL, **69**, 607 (1947).

(4) A. Buzzell and J. M. Sturtevant, *ibid.*, **73**, 2454 (1951).

(5) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948, p. 255.

(6) Reference 5, p. 257.

(7) M. L. Anson, *J. Gen. Physiology*, **22**, 79 (1938).

(8) Reference 5, p. 305.