A Polar Intermediate in the Conversion of Fibrinogen to Fibrin Monomer*

A. E. V. HASCHEMEYER†

From the Department of Chemistry, University of California, Berkeley

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A protein intermediate characterized by a large longitudinal permanent dipole moment has been found to occur in the conversion of fibrinogen to fibrin monomer by thrombin and by the snake venom extract Hemostase. The experimental method involved incubation of fibrinogen with the enzyme in a buffered solvent at *p*H 6.2, where enzymic activity is high and the reaction proceeds to the formation of a fibrin clot. At early times the reaction was stopped by the addition of acid, and the mixture was dialyzed into a solvent suitable for transient electric birefringence measurement. Analysis of the birefringence as a function of incubation time established the presence of the intermediate species and its kinetics of formation. Peptide release was determined by nitrogen analysis on the trichloroacetic acid–soluble fraction of the reaction mixture. The results are consistent with the identification of the polar intermediate as a fibrinogen molecule lacking one A peptide. Its dipole moment was obtained as a function of *p*H from birefringence measurements at saturating electric fields and was used to determine the site at which charge alteration had occurred. This, together with birefringence data for fibrinogen and fibrin monomer, led to the conclusion that the two A peptides of fibrinogen are located near the ends of the molecule, equidistant from the center. The presence of a transverse dipole moment in fibrin monomer suggests the sites may be on the same side of the molecule. The B peptides released by thrombin are apparently symmetrically located. In kinetic studies at early times in the thrombin-catalyzed reaction, both the formation of the polar intermediate and the release of peptide were linear; however, quantitative considerations indicated that the reaction at early times did not follow a simple mechanism. Direct determination of rotational diffusion coefficients during clotting at *p*H 8 established that the first step in the polymerization of fibrin monomer is end-to-end dimerization. End-to-end dimers also appeared at *p*H 4–5, where fibrin is largely monomeric. Polar dimers were observed along with the polar monomers in solutions of partially reacted fibrinogen. Comparison of rotational diffusion coefficients for the monomeric species of fibrinogen, the polar intermediate, and fibrin showed that little change in the length of the molecule occurs during peptide release, as is generally accepted.

The final stage of blood clotting, the conversion of fibrinogen to fibrin by thrombin, has been the object of considerable research for many years. In the first step of this conversion peptide material accounting for about 3% of the total nitrogen is released (Lorand, 1951, 1952). This material consists of two different species, designated peptides A and B (Bettelheim and Bailey, 1952). The stoichiometry is such that two A peptides and two B peptides are released from each fibrinogen molecule of approximately 340×10^3 mw (Blombäck and Yamashina, 1958).

In this research the reaction involving the release of the A peptides from bovine fibrinogen has been studied in two ways: (1) in the early stages of the reaction catalyzed by thrombin where almost all material released is peptide A (Blombäck, 1958; Blombäck and Vestermark, 1958); and (2) in the clotting of fibrinogen by a snake venom extract reported to release only peptide A (Blombäck, 1958).

EXPERIMENTAL

Materials.—Bovine fibrinogen obtained as Cohn Fraction I from Pentex, Inc. (Kankakee, Illinois) was purified according to Laki's (1951) procedure. The resulting clottability, determined by the method of Morrison (1947), was at least 95%. Total protein determinations were made by optical density measurement. The enzymes used were as follows: a purified citrate thrombin with an activity of about 800 N.I.H. units/mg which was generously provided by Dr. W. H. Seegers; a technical grade thrombin (Parke-Davis) at about 15 units/mg; and an aqueous solution of "Hemostase," an extract from the venom of *Bothrops jararaca*, obtained from Dr. W. T. Strauss of Schenlabs Pharmaceuticals Inc. (New York, N. Y.). Hemostase is also distributed under the name of "Reptilase" by Pentapharm A. G. (Basel). The phosphate buffer used in these experiments had the following composition: 0.012 M KH_2PO_4, 0.003 M K_2HPO_4 and 0.075 M NaCl.

Clotting Experiments at pH 6.2.—Freshly purified fibrinogen precipitated with ammonium sulfate was dissolved to a concentration of 10 mg/ml in 0.3 M KCl and dialyzed exhaustively at 4°. The solution was then diluted to three times its volume with phosphate buffer (giving a final *p*H of 6.2) and divided into 20-ml portions in several 50-ml beakers. Reaction was started in all but two beakers by adding, with stirring, one ml of a freshly prepared thrombin solution (in phosphate buffer) or a chosen aliquot of the Hemostase solution. The enzyme concentrations were in a range to produce the first small particles of clotted protein in about ½ hour.

Reaction was stopped at various times by addition (with vigorous stirring) of 50–100 μl of 50% formic acid, which served to lower the *p*H to about 3.2. Nine ml of the solution was added to 3 ml of 20% trichloroacetic acid and saved at 4° for nitrogen analysis. The remaining 12 ml was started dialyzing at 4° against a 20-fold volume of precooled 0.01 M acetic acid (*p*H 3.5). After 1 hour, the dialysis bath was changed to a lower concentration ($0.5–2.0 \times 10^{-3}$ M) of acetic acid, and thereafter the bath was changed every hour for 5 or 6 hours. The conductance of the final solution was very low, as required for electric birefringence

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† Procter and Gamble Fellow in Chemistry, 1959–1960. Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge 39, Massachusetts.

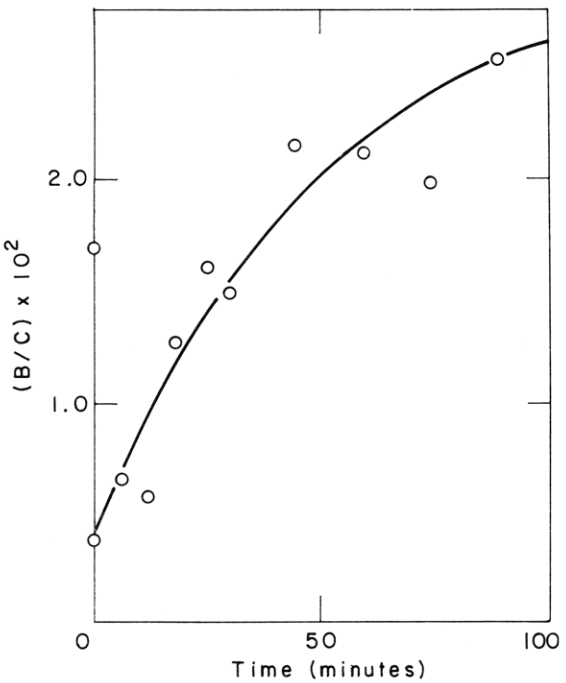

FIG. 1.—Birefringence of reaction mixture as measured at *p*H 4.0–4.1 vs. time of incubation with thrombin at *p*H 6.2, ionic strength 0.17; fibrinogen concentration = 3.2 mg/ml; thrombin concentration = 0.01 unit/ml.

measurements; the final *p*H varied, depending on the time of dialysis. After dialysis, solutions containing the polar intermediate were stable in appearance and birefringence properties during several weeks of storage in the cold. A zero-time sample, in which the formic acid was added before the enzyme, and a control with no enzyme added were prepared and dialyzed in the same manner. Results for the controls were consistently reproducible and have been reported in an earlier publication (Haschemeyer and Tinoco, 1962).

In the preparation of fibrin an excess of enzyme (about 1 unit/ml of thrombin or 300 μl of Hemostase) was used in order to achieve rapid and complete clotting. The clots were collected on a muslin cloth, washed with phosphate buffer, then dissolved in *p*H 3.0–3.5 acetic acid (or formic acid) and dialyzed under the same conditions as the other samples. Aliquots of the clot supernatants were added to 20% trichloroacetic acid in preparation for nitrogen analysis.

Peptide Nitrogen Analysis.—Each trichloroacetic acid–treated sample, while still cold, was shaken and filtered under nitrogen gas pressure through an ultrafine glass frit. The filtrate, containing the soluble peptides, was then analyzed for nitrogen by the micro-Nessler method developed by Schaffer and Sprecher (1957) with slight modifications (Haschemeyer, 1961). The completeness of the protein precipitation and the subsequent filtration were verified by nitrogen analyses on controls.

Clotting Experiments at pH 7–8.—Fibrinogen solutions were prepared by dialysis against 0.1 M glycine supplemented with ammonium hydroxide. After measurement of the birefringence at a convenient voltage (usually 1000 v), a drop of very dilute thrombin in water was added to the solution and further measurements were made periodically for several hours.

Birefringence at Low Fields.—The methods used in the measurement of transient electric birefringence at low fields and in analysis of the data have been discussed elsewhere (Haschemeyer, 1961; Haschemeyer and Tinoco, 1962). The optical retardation δ resulting from

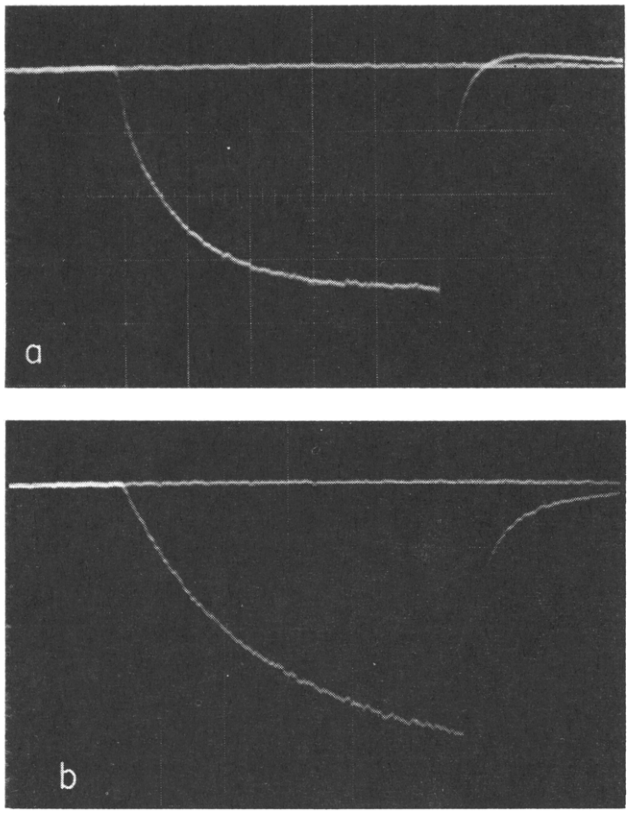

FIG. 2.—Birefringence transients showing strong permanent dipole orientation observed after a short incubation of fibrinogen with thrombin. The presence of an aggregate with negative birefringence (a) or with positive birefringence (b) is indicated by the decay curves. The distance between vertical lines corresponds to a time interval of 10 μsec.

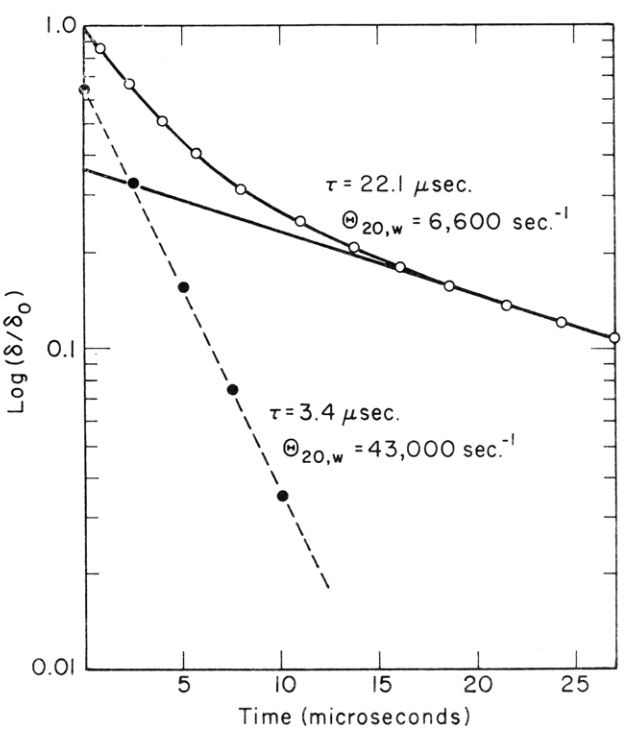

FIG. 3.—Log δ vs. time in the birefringence decay of a sample of thrombin-activated fibrinogen, $(B/C) = 2.6 \times 10^{-2}$, showing presence of the normal monomer ($\theta_{20,w} = 43 \times 10^3$ sec^{-1}) and an end-to-end dimer ($\theta_{20,w} = 6{,}600$ sec^{-1}).

the orientation of the molecules was determined as a function of the applied electric field strength and plotted to obtain the Kerr coefficient B from the slope, $(\delta/E^2)_{E\to 0}$. The time curves of the rise and decay of the birefringence were analyzed to obtain values of the rotational diffusion coefficient and the orientation parameter α. The latter represents the ratio of slow permanent dipole orientation to induced polarization effects.

Birefringence at Saturating Fields.—General equations for the magnitude of the birefringence at saturating fields and the shape of the birefringence transients have been derived by O'Konski, Yoshioka, and Orttung (1959). The steady-state birefringence (Δn_s) at infinite field strength is given by

$$\Delta n_s = (2\pi V_m/n)(g) \qquad (1)$$

where V_m = volume fraction of particles, n = index of refraction of the solution, and (g) = optical anisotropy of the particles in the solution. Δn represents the difference in refractive indices in the two directions (parallel and perpendicular to the field), and is related to the observed retardation by the equation, $\Delta n = (\lambda/2\pi l)\delta$, where λ is the wavelength of light and l is the light path. The rise of the birefringence at saturating fields for pure permanent dipole orientation was shown to be

$$\Delta n/\Delta n_s = 1 + \frac{12e^x}{(e^x-1)^2} - \frac{6xe^x(e^x+1)}{(e^x-1)^3} \qquad (2)$$

$$x = 2\beta\theta t \qquad \beta = \mu E/kT$$

E is the applied field strength; μ is the effective longitudinal dipole moment. The birefringence decay is the same as that at low fields, given by $\Delta n/\Delta n_s = e^{-6\theta t}$, where θ is the rotational diffusion coefficient.

Results

Kinetics of Thrombin-Catalyzed Reactions.—Figure 1 illustrates the increase of birefringence observed upon incubation of fibrinogen with thrombin. The shape of the birefringence transients (Fig. 2) established that the increased birefringence was due to a permanent dipole orientation. The data are collected in Table I. In addition to the polar species responsible for most of the birefringence, which had $\theta_{20,w}$ like that of fibrinogen, a more slowly rotating component also appeared in these solutions. At *p*H 4.0–4.3 this species gave negative birefringence seen as a negative tail on the decay curve (Fig. 2a) with $\theta_{20,w}$ about 7×10^3 sec^{-1}. At higher *p*H a component of similar size but with strong permanent dipole orientation appeared (Fig. 2b). Such curves were analyzed as a sum of two exponential terms, as shown in Figure 3.

Under the conditions given in Figure 1 the release of peptide nitrogen in the reaction catalyzed by thrombin was linear with a yield of 0.0080%/minute of total protein nitrogen during the first 90 minutes. The yield from completely clotted mixtures averaged about 2.3%. This may be compared with the value of 2.6% predicted from the stoichiometry of the reaction using molecular weights of 340×10^3 for fibrinogen, 1890 for peptide A, and 2460 for peptide B (Gladner *et al.*, 1959).

In several experiments 5% formic acid was substituted for the 50% formic acid normally used for stopping the reaction, and this was followed by dialysis against *p*H 4.0 acetic acid. Under these conditions where the *p*H of the samples never fell below 4.0 no permanent dipole was observed in the enzyme-treated samples, whereas the results obtained for fibrinogen controls were not affected. Dialysis of the reaction mixture during the incubation did not affect the results.

Table I

Transient Electric Birefringence of Fibrinogen Solutions after Incubation with Thrombin
The specific birefringence and rotational diffusion coefficients are presented.

Sample	T_0[a]	Incubation Time (min)	*p*H of Birefringence Measurement	$(B/C) \times 10^2$ (cm^4/statvolt^2-g)	$\theta_{20,w}$ of Principal Component
1-1	0.01	0	4.0	0.37	48,000
1-2	0.01	6	4.0	0.65	(55,000)
1-3	0.01	12	4.0	0.57	(59,000)
1-4	0.01	18	4.0	1.26	43,000
1-5	0.01	25	4.0	1.61	38,000
2-B	0.01	0	4.0	1.70	42,000
2-1	0.01	30	4.0	1.50	42,000
2-2	0.01	60	—	2.14	39,000
2-3	0.01	90	—	2.55	43,000
3-1	0.02	75	4.0	2.00	46,000
4-4	0.03	30	4.2	0.90	42,000
5-1	0.01	55	4.5	2.60	36,000
			4.2	2.10	40,000
			4.1	1.70	40,000
			4.6	3.20	40,000
8-1	0.01	45	4.2	1.50	36,000
			4.0	2.20	38,000
			4.4	1.90	42,000
			9.5	4.00	38,000
9-1	0.01	65	4.7	3.20	40,000
			4.5	2.00	46,000
			9.5	4.50	46,000
			10.5	3.40	46,000
10-4	0.01	60	4.3	1.50	40,000
11-1	0.006	40	4.3	0.40	44,000
11-2	0.006	80	4.3	1.10	45,000
13-1	0.005	40	4.3	0.21	41,000
13-2	0.005	80	4.3	0.64	41,000

[a] T_0 = thrombin concentration (in units/ml) in reaction mixture.

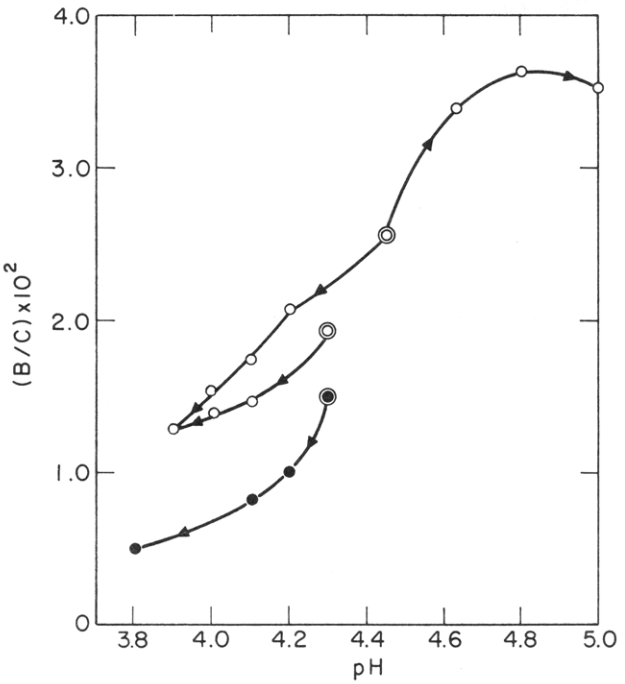

FIG. 4.—Birefringence vs. *p*H in the titration of two thrombin-activated fibrinogen samples: CET 5-1 (open circles) at a concentration of 3.1 mg/ml and CET 10-4 (closed circles) at a concentration of 3.2 mg/ml. The samples were prepared by dialysis at the *p*H values indicated by the double circles. Arrows show the direction of titration carried out by additions of 0.1 M acetic acid or 0.1 M ammonium hydroxide. The time lapse between measurements was 10-15 minutes for CET 5-1 and 1–2 hours for CET 10-4.

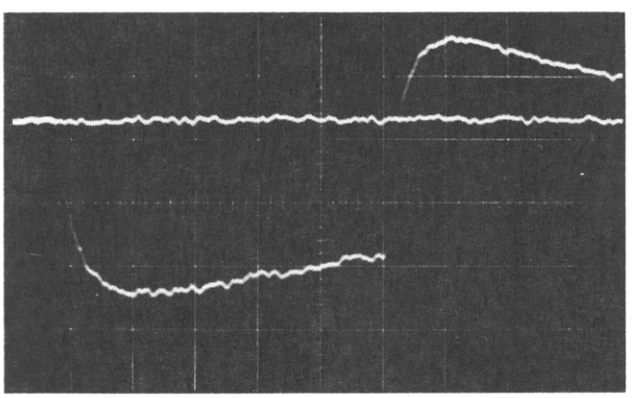

FIG. 5.—Birefringence transient of thrombin activated fibrinogen at *p*H 3.8. Orientation is due entirely to induced effects with $(B/C) = 0.5 \times 10^{-2}$. A negatively birefringent aggregate is present.

pH Dependence of the Birefringence of the Polar Intermediate.—Figure 4 shows the results obtained by direct titration of several highly birefringent samples. Although the (B/C) values obtained at a given measured *p*H varied with the protein concentration and the time elapsed after the *p*H change, the trends were reproducible. The decrease of birefringence with decreasing *p*H was accompanied by a loss of permanent dipole character in the rise curves. In the case of sample 10-4, a pure induced dipole type of rise was observed at *p*H 3.8 (Fig. 5). Measurements were also made at high *p*H's immediately following addition of concentrated ammonium hydroxide. The birefringence at *p*H 9.5 was greater than at the *p*H 4.8 value by an amount consistent with the greater birefringence of native fibrinogen at this *p*H. Aggregation prevented further study in this *p*H region.

Saturation Birefringence of the Polar Intermediate.—The extremely large birefringence observed in many samples of thrombin-activated fibrinogen made it possible to obtain measurable signals even in very dilute solutions. Reduction of the solution conductivity by dilution with distilled water permitted the establishment of fields as high as 50×10^3 v/cm across the solution in the Kerr cell. Under these conditions saturation of the birefringence of the polar intermediate was readily achieved.

The data from the saturation experiments are collected in Table II. The root mean square dipole moment was determined from the ratio of the low field birefringence $(\delta/E^2)_{E\to 0}$ to the retardation at saturation, δ_s. This is based upon a combination of the equation for permanent dipole birefringence at low fields (Benoit, 1951) and equation (1):

$$\langle \mu^2 \rangle = \frac{15(kT)^2(\delta/E^2)_{E\to 0}}{\delta_s} \qquad (3)$$

The error incurred by the assumption of infinite field strength in these measurements is approximately cancelled by the 10–15% contribution of fibrinogen to the measured birefringence. The random error in the determination of $\langle \mu^2 \rangle$ does not exceed ± 10%.

A plot of δ vs. the square of the field strength is shown in Figure 6 for two samples measured at fields up to saturation. The shapes of these curves suggested an empirical relation for the birefringence at fields intermediate between the Kerr law region and saturation. When a quantity representing the deviation of the birefringence at a high field from the Kerr law was plotted against $\langle \mu^2 \rangle$, a straight line was obtained. Plots for three values of E^2 are shown in Figure 7. An unknown permanent dipole moment therefore can be estimated from knowledge of the birefringence behavior at fields slightly above the Kerr law region. The theoretical birefringence equations have not been solved for these field strengths.

The birefringence rise curves at saturating fields for samples with $\alpha = \infty$ were compared with a standard curve based on equation (2). The observed (δ/δ_s) for the rise was plotted against $2\beta\theta t$ where θ was determined from the decay of the birefringence, and values of $\beta = (\mu E/kT)$ were chosen. The initial slope of the rise generally fit the standard curve fairly well when a value of β calculated from the measured field strength and the experimental dipole moment was used. For most samples this value was about 10 at the highest

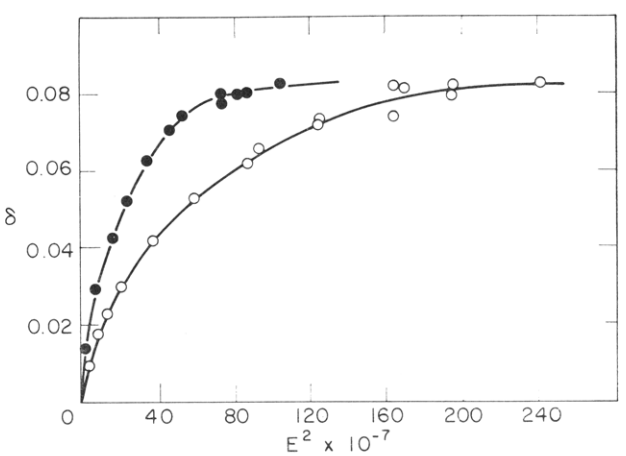

FIG. 6.—δ vs. the square of the applied electric field (volt²/cm²) for two samples of thrombin-activated fibrinogen, showing saturation of the birefringence of the polar intermediate: CET 9 (solid circles), *p*H 4.7, μ = 4300 Debye; CET 11-2 (open circles), *p*H 4.5, μ = 2400 Debye

TABLE II

ELECTRIC BIREFRINGENCE DATA FOR THROMBIN-ACTIVATED FIBRINOGEN SOLUTIONS

The specific conductance and the specific birefringence determined from measurement at low field strengths are given. Values for the permanent dipole moment and the fraction of polar molecules were obtained from saturation of the birefringence. The amount of trichloroacetic acid–soluble peptide material released in the reaction is expressed as the per cent of the total protein nitrogen.

Sample	Conc. (mg/ml)	$\kappa \times 10^4$ (mho/cm)	pH	$B/C \times 10^2$	μ	χ	% Total Protein N
CET 5	0.15	0.12	4.8	3.9	3800	0.17	0.50
	0.30	0.27	4.6	3.2	3400	0.18	
	3.0	0.57	4.5	2.5	3100	0.18	
CET 8	0.32	0.34	4.4	1.9	2400	0.23	0.40
CET 9	0.30	0.17	4.7	3.2	4300	0.11	0.43
CET 10-1						0.056	0.13
-2						0.051	0.21
-3						0.034	0.31
-4	0.32	0.29	4.5	1.1	2500	0.15	0.38
-5						0.070	0.63
CET 11-1						0.060	0.24
-2	0.35	0.21	4.5	0.9	2400	0.13	0.48
CET 13-1						0.037	0.13
-2	0.30	0.34	4.4	1.0	3400	0.07	0.30

field where measurements were made. At later times ($\theta t > 0.2$) the rise corresponded better to $\beta = 5$–7. This deviation may have been due to a contribution from the relatively nonpolar fibrinogen and fibrin molecules also present in the solutions.

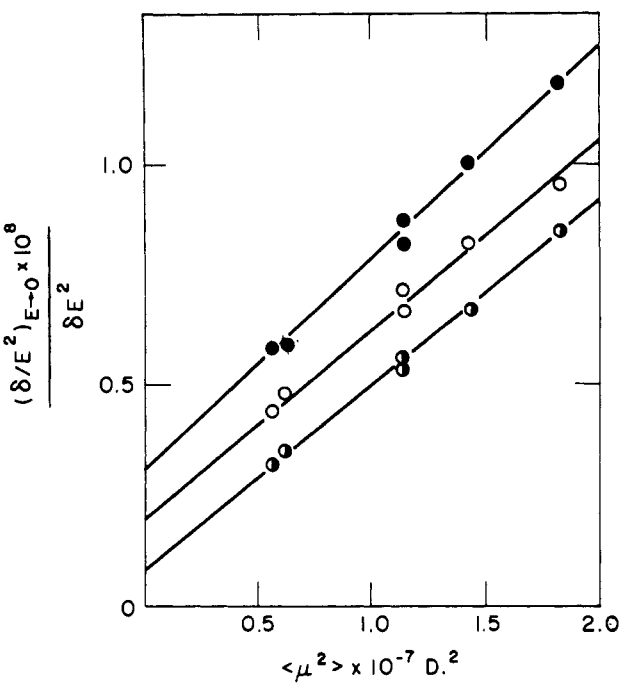

FIG. 7.—Deviations of the birefringence from Kerr law behavior at high fields obtained from the data of six saturation experiments. The ratio of the birefringence in the Kerr law region to the observed retardation at a given applied field is plotted against the square of the applied field for three values (in volts^2/cm^2): $E^2 \times 10^{-7} = 30$ (solid circles), 50 (open circles), and 100 (half-solid circles). The hysteresis observed is typical of these titrations.

The quantity χ in Table II represents the fraction of molecules which possess the permanent dipole moment. These values were calculated from the low field birefringence data (corrected for the negative birefringence of fibrinogen) and the experimental μ with the aid of the equation

$$(B/C) = 2.6 \times 10^{-6} \chi(g)\langle\mu^2\rangle \qquad (4)$$

which is readily derived from the equations given previously (Haschemeyer and Tinoco, 1962). The optical anisotropy factor (g) was taken as 6.5×10^{-3}. Values of χ were also determined from ratios of low-field birefringence for other samples measured at the same pH (and with the same pH history). The results of nitrogen analyses for peptide release are also given in Table II. The data show an approximate correlation at early reaction times between the concentration of activated molecules and the amount of peptide material released.

Clotting Experiments Catalyzed by Hemostase.—Hemostase was found to be equally effective in producing a large permanent dipole moment in fibrinogen after short incubation times; however, the time dependence was erratic. Apparently, the reaction was not stopped by the addition of acid, as indicated by large values of (B/C) in zero-time samples. The birefringence transients were generally like those observed in the thrombin reactions except that, in some cases, very high values of $\theta_{90,w}$ (60×10^3–80×10^3 sec^{-1}) were found, indicating a possible alteration in structure. Negatively and positively birefringent aggregates occurred, as in the thrombin-activated samples. The release of soluble peptides was linear at a rate of 0.0020%/minute of the total protein nitrogen during the early stages, with Hemostase at 10 μl/ml. The yield at 100% reaction was found to be 1.45% of the total protein nitrogen, slightly greater than the 1.1% expected if only peptide A were released by this enzyme.

Birefringence Study of Two Types of Fibrin.—Experiments on nine preparations of fibrin prepared both with thrombin and with Hemostase confirmed previous work (Tinoco, 1955; Billick and Ferry, 1956) showing the similarity of fibrin monomer to fibrinogen. However, although the charge symmetry is identical in the pH range of 4.2 to 4.8, the birefringence of fibrin monomer is negative below pH 4.2, as shown in Figure 8, whereas that of fibrinogen is positive. An example of the birefringence transient in this region is given in Figure 9.

The principal birefringence results are presented in Table III. Almost all samples showed the presence of a negatively birefringent aggregate in addition to the monomeric component. Where possible, the decay curves were analyzed in terms of two relaxation times; however, because of the low birefringence, the error in

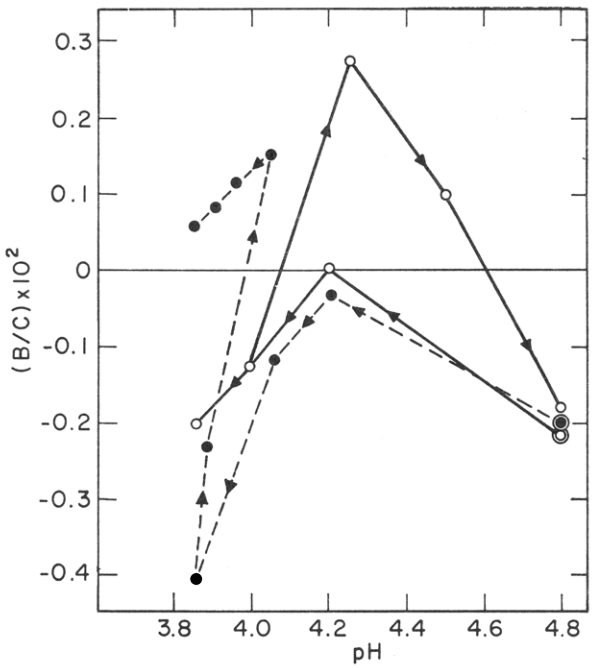

FIG. 8.—Birefringence vs. *p*H in the titration of fibrin monomer sample W-f1 at a concentration of 3.2 mg/ml (solid circles) and at a concentration of 0.80 mg/ml (open circles). Arrows show direction of titration. The hysteresis observed is typical of these titrations.

$(\theta_{20,w})_2$ may have been as much as 30% in some cases. The orientation for both species was entirely of the "fast" types ($\alpha = 0$). In two cases where the clots were not carefully washed, some permanent dipole orientation was observed.

Birefringence Measurements of Clotting Solutions at pH 7–8.—A study was made of the birefringence behavior of fibrinogen-thrombin mixtures in 0.1 M glycine where birefringence measurements could be made directly during the course of reaction. The early stages of the polymerization were followed by analysis of the decay curves. As shown in Table IV, the first aggregate to appear has $\theta_{20,w}$ about 6000 sec^{-1}. The subsequent decrease of this value is due to the contribution of larger aggregates. After 15 minutes' incubation

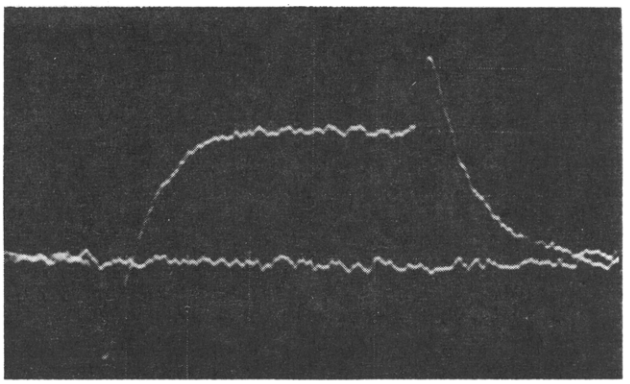

FIG. 9.—Birefringence transient of fibrin monomer at *p*H 4.0 showing transverse dipole orientation. $(B/C) = -0.13 \times 10^{-2}$.

a third relaxation time was determined from the decay at times greater than 25 μsec after removal of the electric field. After subtraction of this component the resultant curve gave a good fit with a 2-term equation. Little change occurred in the magnitude of the birefringence in these experiments. Apparently, then, no polar intermediates are formed under these conditions, or perhaps their aggregation is too rapid to permit their detection over the high background of fibrinogen birefringence.

DISCUSSION

The results of the clotting experiments clearly show that a highly polar protein species occurs as an intermediate in the conversion of fibrinogen to fibrin monomer, both by thrombin and by Hemostase. Because of its large electric birefringence with permanent dipole orientation in the *p*H region of 4–5, this protein species may be readily distinguished from fibrinogen and fibrin monomer which have relatively little birefringence at these *p*H values. No change occurs in the rotational diffusion coefficient, a quantity that is sensitive to changes in length of a molecule, in the conversion to the polar species. The fact that the polar intermediate appears very early in the thrombin-catalyzed reaction, during A peptide release, and that it is also produced by Hemostase, suggests the following representation of the reaction:

TABLE III

TRANSIENT ELECTRIC BIREFRINGENCE OF FIBRIN MONOMER PREPARED WITH THROMBIN AND HEMOSTASE

The specific birefringence and rotational diffusion coefficients are presented. Enzymes: T = thrombin (Parke-Davis); T_S = Seegers' thrombin; H = Hemostase. For samples L-fl and M-f, $\alpha = 5$; for all others $\alpha = 0$. In cases where the birefringence of the two components was of different sign, only a rough estimate of $\theta_{20,w}$ (given in parentheses) could be made.

Sample	Enzyme	*p*H	Conc. (mg/ml)	$\kappa \times 10^4$ (mho/cm)	$(B/C) \times 10^2$	$[\theta_{20,w}]_1$	$[\theta_{20,w}]_2$
K-f	T	4.0	10.0	1.30	−0.19	—	7,000
L-f1	H	4.4	2.4	0.49	1.60	75,000	
L-f2	H	4.3	2.5	0.70	−0.10	—	
M-f	T	4.3	3.0	—	1.00	—	
CET-2f	T	4.1	2.9	—	0.60	(85,000)	(4,500)
CET-7f	T	4.2	2.6	—	0.14	(85,000)	(4,500)
W-f1	T	4.8	3.2	0.40	−0.21	36,000	6,300
		4.8	0.80	0.29	−0.22	32,000	
		4.8	0.70	1.90	−0.19	36,000	11,500
		4.25	0.75	1.00	0.27	47,000	
		3.9	3.2	1.35	−0.40	38,000	7,400
W-f2	T_S	4.5	1.46	0.37	−0.25	35,000	6,400
		4.2	1.44	0.61	−0.20	34,000	5,000
		4.0	1.42	1.04	−0.50	42,000	10,000
		3.9	1.39	1.31	−0.63	38,000	9,000
W-f3	H	4.2	1.20	0.41	0.11	—	(12,000)
		3.9	1.10	1.20	−0.44	49,000	7,200

TABLE IV

ROTATIONAL DIFFUSION COEFFICIENTS MEASURED DURING A TYPICAL INCUBATION OF FIBRINOGEN WITH THROMBIN IN 0.1 M GLYCINE, *p*H 8.0

Concentration = 2.8 mg/ml, $\kappa = 0.65 \times 10^{-4}$ mho/cm.

Incubation Time (min)	$[\theta_{20,w}]_1$	$[\theta_{20,w}]_2$	$[\theta_{20,w}]_3$	% Contribution of Aggregates
0	41,000	—	—	0
1	41,000	5,900	—	10–15
4	40,000	5,000	—	25
8	40,000	4,600	—	35
15	41,000	5,900	3,000	50

$$F_{AA} \xrightarrow{k} F_A + A \qquad (5)$$
$$\downarrow k'$$
$$f + A$$

where F_{AA} is fibrinogen, $F_A$is the polar intermediate and f is fibrin monomer (neglecting the release of the B peptides). The rate constants k and k' include the enzyme concentration.

The distance r from the center of the molecule to the site of charge alteration may be calculated from the measured dipole moment. If the observed asymmetry is due to an excess of charge at one site only, the dipole moment may be expressed by $\mu = ver$, where v is the number of charges and e is the electronic charge. From the amino acid composition of peptide A (Gladner *et al.*, 1959; Sjöquist, 1959) the charge of the peptide should be $-4e$ when all groups are charged. The maximum μ observed (4300 D at *p*H 4.7) then leads to $r = 220$ A. Comparison of this value with the half-length of fibrinogen (250–300 A) indicates that the peptide is located very near the end of the molecule. Since no longitudinal permanent dipole moment exists in fibrinogen at the *p*H of these measurements or in fibrin monomer after removal of both A peptides, the two peptide sites must be equidistant from the center of symmetry of the molecule.

The titration studies (Fig. 4) are consistent with the assumption that the charge asymmetry of the polar intermediate is directly associated with the carboxylate charges of the remaining A peptide. However, other possibilities must be considered in the light of probable hydrogen bonding between the peptides and the protein core. On the basis of thermodynamic measurements Laskowski *et al.* (1960) have proposed the existence of carboxyl · · · carboxyl hydrogen bonds. In our experiments the fact that acidification of the activated samples seemed to be required in order to observe a permanent dipole moment is consistent with the idea that hydrogen bonds are involved, which must be altered or broken. For example, the low *p*H treatment accompanied by dialysis may have served to release the peptide from a species such as $F_A \cdots A$, where A is held by hydrogen bonds. This species has been postulated to explain kinetic results obtained at *p*H 5.3 (Laskowski *et al.*, 1960). Another explanation might be that the A peptides that are still covalently linked are also involved in carboxyl · · · carboxylate hydrogen bonds stable in the low *p*H region. Strong acidification may then produce a conversion to the double carboxyl · · · carboxyl form which, after dialysis to low ionic strength, is stabilized. In this way the observed titration of μ could be due to the dissociation of those carboxyl groups that have been unmasked by the removal of the first A peptide. Further investigation in this area is required.

Kinetics of Formation of the Polar Intermediate and A Peptide Release.—For the reaction shown in equation (5) the release of peptide A will obey the rate law:

$$d(A)/dt = 2k(F_{AA}) + k'(F_A) \qquad (6)$$

while the formation of the polar intermediate F_A will be given by:

$$d(F_A)/dt = 2k(F_{AA}) - k'(F_A) \qquad (7)$$

Making use of the fact that $-d(F_{AA})/dt = 2k(F_{AA})$, one can integrate the above to obtain

$$A = 2C_0\left[1 - \frac{(k' - k)}{(k' - 2k)}e^{-2kt} + \frac{k}{(k' - 2k)}e^{-k't}\right]$$
$$F_A = \frac{2kC_0}{k' - 2k}[e^{-2kt} - e^{-k't}] \qquad (8)$$

for $k' \neq 2k$. C_0 is the total protein concentration in moles/liter. For the special case of $k' = 2k$ the solutions are

$$A = 2C_0[1 - e^{-k't}] - k'tC_0e^{-k't}$$
$$F_A = k'tC_0e^{-k't} \qquad (9)$$

When the experimental data of Table II (assuming all nitrogen released to be peptide A) are compared with theoretical curves based on the above equations, the following is noted: The early time dependence of the experimental χ corresponds most closely to $k'/k = 20$. However, the fact that values of χ as high as 0.2 were observed indicates that k'/k cannot be that large but may be as much as 5. The experimental maximum may be compared with the theoretical maximum value of $\chi = 0.5$ at half-reaction for $k'/k = 1$. The fact that the more reasonable result of $k'/k = 1$ is not obtained may be due to oversimplification of the reaction. For example, the existence of the nonpolar species $F_A \cdots A$ would reduce the apparent concentration of F_A without affecting the nitrogen analyses, and thus cause an apparent deviation from $k'/k = 1$.

Transverse Dipole Moment of Fibrin Monomer.—At *p*H's below 4.2 the birefringence of fibrinogen and fibrin monomer is quite different. Comparison of Figure 8 with a similar titration of fibrinogen (Haschemeyer and Tinoco, 1962) indicates a difference in (B/C) of about 0.8×10^{-2} at *p*H 3.9, corresponding to a transverse dipole moment of 700 Debye. Since fibrins prepared both with thrombin and with Hemostase (Table III) show this effect, it is probably associated with release of the A peptides. The result would suggest that the two peptide sites are not opposite each other as in the case of inversion symmetry, but may be on the same side of the molecule.

Release of B Peptides.—From a comparison of the results on fibrins prepared with both enzymes it would appear that no change in charge symmetry occurred with release of the B peptides in the thrombin-catalyzed reaction. Consequently, the two B peptides must be symmetrically placed with respect to the center of the molecule. Their exact position along the longitudinal axis, however, cannot be stated. It should be possible to determine this by studying the reaction in which only B peptides are released, e.g., in the action of thrombin upon Hemostase-produced fibrin.

End-to-End Dimerization.—In almost all samples of activated fibrinogen and of fibrin monomer in the *p*H range of 4–5, a slowly rotating species was observed that did not occur in fibrinogen solutions at similar concentrations. This species was also observed at very early times in the experiments at *p*H 7–8 where measurements were made during clotting, and it may be reasonably identified as an end-to-end dimer. Calcu-

lations were made with the aid of Perrin's (1934) equation for the rotational diffusion coefficient of a prolate ellipsoid of revolution of large axial ratio. For a monomer with $\theta_{20,w} = 43 \times 10^3$ sec^{-1} and axial ratio of 5 or 10, the equation predicts values of 7,500 sec^{-1} and 6,900 sec^{-1}, respectively, for an end-to-end dimer. The best experimental values were about 7000 sec^{-1}.

In some partially reacted samples (Fig. 3) dimers were observed which obviously possessed a longitudinal dipole moment of considerable magnitude, like the monomeric polar intermediate. This is consistent with a model of F_A joined at its peptide-free end to fibrin monomer. The resultant dimer would thus have an A peptide at one end only. At *p*H's below 4.3 the dimers showed the negative birefringence characteristic of fibrin monomer. These might include all possible combinations: F_A—F_A, f—f, and F_A—f, the species F_A—f having lost its polarity at low *p*H. The dimer f—f was also observed in the fibrin preparations. The fact that the apparent transverse dipole moment of fibrin monomer is preserved in the dimer suggests that the two monomer units have the same orientation in space.

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