[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

# The Conversion of Fibrinogen to Fibrin. XVII. Further Studies of Electrical Birefringence of Fibrinogen

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The electrical birefringence of fibrinogen and activated fibrinogen has been studied in salt-free solutions (containing glycerol) near *p*H 4.5 and 8.5, using the single rectangular pulse method introduced by Benoit and modified by Tinoco. The rise and decay of birefringence were followed by oscilloscopic measurements of the response of a photomultiplier tube to light transmitted through crossed Nicol prisms. At a fibrinogen concentration of 7 g./l., near *p*H 8.5, the rotary diffusion constant ($\theta$) reduced to water at 20° was $2.5 \times 10^4$ sec.$^{-1}$; addition of urea up to 2 *M* increased this value progressively, approaching $4.0 \times 10^4$. Near *p*H 4.5, $\theta$ was $4.0 \times 10^4$ and independent of urea concentration. The specific Kerr coefficients ($B/c$) were sensibly independent of urea concentration. At *p*H 8.5, $B/c$ was about $0.8 \times 10^{-2}$ cm.$^4$/volt$^2$ g., comparable with corresponding values measured by Tinoco in 3 *M* urea; at *p*H 4.5 it was about $0.5 \times 10^{-2}$. It was concluded that urea up to 3 *M* does not seriously affect $B/c$, and affects $\theta$ only through modifying the protein interactions. Activation by thrombin did not change $\theta$ but increased $B/c$ by about $0.1 \times 10^{-2}$ (at *p*H 4.5). At *p*H 4.5 the birefringence rise curves were symmetrical to the decay curves, indicating no dipole moment parallel to the long axis of the molecule. At *p*H 8.5, however, the shape of the rise curves indicated that there was a component of the dipole moment in this direction.

## Introduction

In a recent study by Tinoco[2] of the electrical birefringence of solutions of bovine fibrinogen before and after its activation by thrombin, values of Kerr coefficients and rotary diffusion coefficients were obtained over a *p*H range from 6.0 to 9.6; some conclusions were drawn concerning the magnitudes of the dipole moment and its change upon activation, and the location of the site of attack by thrombin on fibrinogen. In these experiments, 2 or 3 *M* urea was present to keep the fibrinogen in solution in the absence of salt. Although denaturation did not occur at such levels of urea concentration, higher concentrations can cause profound changes,[3,4] and it was therefore desirable to obtain some similar data with no urea present to determine whether this component had any influence on the results. The observation that fibrinogen is soluble in very dilute ammonia[5] and acetic acid without salt has now made it possible to prepare solutions with sufficiently low conductivities for this purpose at *p*H 4.3–4.6 and 8.2–8.8, respectively. The electrical birefringence of these solutions has been studied both with and without added urea.

## Materials

The sources of the fibrinogen and thrombin were the same as in the previous study,[2] and the fibrinogen was refractionated and assayed in the manner previously described. The clottability ranged from 88 to 95%.

Stock solutions of fibrinogen in dilute ammonia were prepared by dialyzing solutions in salt (0.15–0.45 *M* sodium chloride and/or buffer, 20–25 g. fibrinogen per l.) against repeated changes of $10^{-3}$ *M* ammonium hydroxide. When buffer was present, the protein would precipitate and redissolve in the course of dialysis. The final protein concentration was determined both spectrophotometrically and by dry weight.

Stock solutions of activated fibrinogen (which does not polymerize below *p*H 5) in dilute acetic acid were prepared by clotting a similar solution in sodium chloride and buffer at *p*H 6.2 with thrombin, allowing it to stand for at least ten times the clotting time to ensure complete conversion to fibrin, and then dialyzing against repeated changes of $10^{-3}$ *M* acetic acid, which completely dissolved the clot. Stock solutions of unactivated fibrinogen in acetic acid were prepared in the same manner (from an identical aliquot of the original salt solution in each experiment) except that the thrombin was of course omitted. The final protein concentration was in every case determined by dry weight.

Sedimentation experiments[6] on the fibrinogen solutions in acetic acid and ammonia (at a fibrinogen concentration of 7 g./l.) revealed in both solvents a single extremely sharp peak. The sedimentation constants reduced to water at 20° were 3.6 *S* in acetic acid and 5.6 *S* in ammonia; the Donnan effect in the absence of salt naturally depresses these values considerably below the normal figure[7] of about 8 *S*.

The solutions for electrical birefringence measurements were made up in glycerol, usually 64% by weight, to provide a medium of high viscosity; and in some cases contained added urea as well. Each was prepared by mixing the appropriate stock fibrinogen solution with 96% glycerol, together with a stock solution of 3 *M* urea in 96% glycerol if required, to give the desired concentrations of all components. The final fibrinogen concentration was usually 7 g./l. The conductance was estimated by measuring the resistance of the Kerr cell filled with solution. The viscosity of the solvent was determined in each case by making up a similar mixture with $10^{-3}$ *M* acid or ammonia substituted for the stock protein solution, and measuring its flow time in an Ostwald viscosimeter.

## Method

The apparatus of Tinoco,[2] which had been patterned after that of Benoit,[8] was modified slightly. For a light source, a 6 volt, 18 amp. projection bulb was used, and a lens was inserted to focus it on the polarizing Nicol, providing about three times the previous intensity. The voltage for the photomultiplier tube was supplied by a Furst[9] Power Supply, Model 810-P, and that for the Kerr cell itself from a Sorenson[10] B-Nobatron, Model 560 BB. A new and improved rectangular pulse generator was constructed.[11]

All measurements were made at room temperature, 25 ± 1°. The procedure and calculations were as described by Tinoco,[2] except that a more elaborate calculation was used to obtain the rotary diffusion coefficient. The response time of the photomultiplier circuit was first determined by measuring the apparent rate of decay of birefringence of fibrinogen in an aqueous solvent containing no glycerol. In this case the rotary diffusion is so rapid that the decay reflects only the response time, $\tau$, which was found to be 14 microseconds. In the presence of glycerol, the decay is determined by both $\tau$ and the rotary diffusion coefficient of fibrinogen, $\theta$. Network analysis of the photomultiplier circuit[11] shows that the relative height of the oscilloscope

(1) Union Carbide and Carbon Fellow in Physical Chemistry, 1954–1955.

(2) I. Tinoco, Jr., THIS JOURNAL, **77**, 3476 (1955).

(3) E. Mihályi, *Acta Chem. Scand.*, **4**, 317, 334 (1950).

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

(5) F. R. Bettelheim, personal communication.

(6) The Svedberg oil turbine ultracentrifuge, operated by Mr. Edwin M. Hanson, was made available through the kindness of Professor J. W. Williams.

(7) S. Shulman, THIS JOURNAL, **75**, 584b (1953).

(8) H. Benoit, *Ann. Phys.*, **6**, 561 (1951).

(9) Furst Electronics, Chicago, Ill.

(10) Sorensen and Co., Stamford, Conn.

(11) Further details are given in the Ph.D. Thesis of I. H. Billick, University of Wisconsin, 1955.

signal, $y$, as a function of time, $t$, should follow the equation

$$y = [1/(1 - 12\theta\tau)]e^{-12\theta t} - [12\theta\tau/(1 - 12\theta\tau)]e^{-t/\tau}$$

The usual plots[2] of log $y$ against $t$ were somewhat curved, but afforded a first approximation calculation of $\theta$. Then the logarithm of $y + [12\ \theta\tau/(1 - 12\ \theta\tau)]e^{-t/\tau}$ was plotted against $t$, giving a much better straight line from the slope of which the final calculation of $\theta$ was made. The values of $\theta$ were reduced to water at 20° using the measured viscosities of the respective solvents.

## Results and Discussion

**Rotary Diffusion Coefficients.**—The rotary diffusion coefficients at $p$H 8.2–8.9, in ammonia solutions, are given in Table I and plotted against the urea concentration in Fig. 1. In the absence of urea, $\theta$ is about $2.5 \times 10^4$ sec.$^{-1}$, perceptibly smaller than the values found by Tinoco in 3 $M$ urea, which averaged $3.6 \times 10^4$ and were recognized to be somewhat too low because of the neglect of $\tau$ in their calculation. But with increasing urea concentration $\theta$ rises and appears to approach a value of about $4 \times 10^4$, in reasonable agreement with the previous results.

TABLE I

ROTARY DIFFUSION COEFFICIENTS AT HIGH $p$H

Fibrinogen 7 g./l., glycerol 64% by weight

| Expt. | Urea concn., $M$ | $p$H | $\theta_{20,w} \times 10^{-4}$, sec.$^{-1}$ |
|---|---|---|---|
| B-8-2 | 0 | 8.50 | 2.6 |
| B-12-1 | 0 | 8.60 | 2.6 |
| B-12-2[a] | 0 | 8.32 | 2.4 |
| B-12-6[a] | 0.25 | 8.20 | 2.5 |
| B-8-4[b] | 0.50 | 8.78 | 3.2 |
| B-12-5[a] | 0.50 | 8.34 | 3.3 |
| B-8-5[c] | 1.0 | 8.90 | 3.5 |
| B-12-4[a] | 1.0 | 8.20 | 3.5 |
| B-12-3[a] | 2.0 | 8.30 | 3.8 |

[a] Fibrinogen concentration 5.47 g./l. [b] Glycerol concentration 64.3% by weight. [c] Glycerol concentration 61.6% by weight.

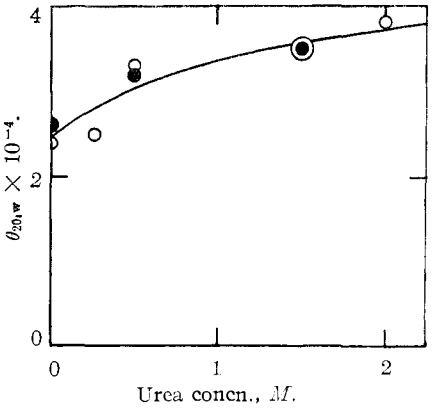


Fig. 1.—Rotary diffusion constant of fibrinogen, reduced to water at 20°, plotted against urea concentration ($p$H 8.2–8.9): black circles, expt. 8; open circles, expt. 12.

At a fibrinogen concentration of 7 g./l., $\theta$ might be expected to be somewhat smaller than the value extrapolated to infinite dilution from flow birefringence measurements in more dilute solutions. The latest estimate for the latter[4] is $4.4 \times 10^4$ sec.$^{-1}$, in 0.3 $M$ salt solutions in the $p$H range 6.0–8.2, as compared with $2.3 \times 10^4$ at a concentration of 11 g./l. in the same type of solvent. Our value of $2.5 \times 10^4$ with no urea at an intermediate concentration fits in well with these data, reflecting a moderate slowing of rotary Brownian motion by protein–protein interaction.

The addition of urea appears to diminish the interaction and allow $\theta$ to approach the infinite dilution value. It is true that urea, even at these moderate concentrations, might be expected to have other effects due to swelling or binding, but such changes should decrease rather than increase $\theta$ and it seems likely that the primary manifestation is a reduction of molecular interactions although it is not clear just what these interactions are.

The rotary diffusion coefficients at $p$H 4.2–4.6, in acetic acid solutions, are given in Table II. Although the values scatter rather badly, there seems to be no effect of urea concentration, and the average is $4 \times 10^4$ sec.$^{-1}$, close to the infinite dilution value at higher $p$H. Apparently in this $p$H range the effects of interaction are much less, and there is nothing to be counteracted by addition of urea.

TABLE II

ROTARY DIFFUSION COEFFICIENTS AT LOW $p$H

Fibrinogen or activated fibrinogen 7 g./l., glycerol 64% by weight; A denotes activated fibrinogen.

| Expt. | Urea concn., $M$ | $p$H | $\theta_{20,w} \times 10^{-4}$, sec.$^{-1}$ |
|---|---|---|---|
| B-9-1 | 0 | 4.28 | 4.5 |
| B-9-2 | 0 | 4.30 | 4.8 |
| B-9A-1 | 0 | 4.31 | 5.1 |
| B-9A-2 | 0 | 4.21 | 2.9 |
| B-10-1 | 0 | 4.50 | 3.9 |
| B-10A-1 | 0 | 4.30 | 3.6 |
| B-11-1 | 0 | 4.40 | 5.0 |
| B-11-2 | 1 | 4.61 | 3.8 |
| B-11A-1[a] | 0 | 4.25 | 3.8 |
| B-11A-2[b] | 0.5 | 4.35 | 3.9 |
| B-11A-3 | 0 | 4.25 | 3.1 |
| | | Av. | 4.0 |

[a] Fibrinogen concentration 9.33 g./l. [b] Fibrinogen concentration 8.92 g./l.

Table II also shows that the rotary diffusion coefficients of fibrinogen and activated fibrinogen are practically identical, as already concluded from Tinoco's measurements[2] under the quite different conditions of 3 $M$ urea and $p$H 9.0–9.7. The present conditions might be considered as somewhat closer to the physiological environment.

**Kerr Coefficients.**—The specific Kerr coefficients ($B/c$, where $B = \Delta n/\lambda E^2$ and $c$ is protein concentration in g./cc.; $\Delta n$ is the birefringence, $\lambda$ the wave length of light, and $E$ the electric field strength) at $p$H 8.2–8.9 are given in Table III, together with the estimated conductivities ($\kappa$). It is evident that increasing the urea concentration has no effect on the Kerr coefficient perceptible within experimental scatter. Moreover, the values are similar in magnitude to those obtained by Tinoco in 3 $M$ urea, although the conditions of $p$H and conductivity are not exactly comparable.

The specific Kerr coefficients at $p$H 4.2–4.6 for both fibrinogen and activated fibrinogen are given in Table IV. Again there is no indication of change with addition of urea. The values are similar in

TABLE III

KERR COEFFICIENTS AT HIGH *p*H

Glycerol 64% by weight.

| Expt. | Fibrinogen concn., g./l. | Urea concn., *M* | *p*H | $\kappa \times 10^5$, mho cm.$^{-1}$ | $B/c \times 10^2$, cm.$^4$/volt$^2$ g. |
|---|---|---|---|---|---|
| B8-2 | 7 | 0 | 8.50 | 3.0 | 0.82 |
| B8-6[a] | 7 | 0.25 | 8.68 | 3.2 | .81 |
| B8-4 | 7 | 0.5 | 8.78 | 3.5 | .74 |
| B8-5[b] | 7 | 1 | 8.90 | 4.6 | .99 |
| B12-1 | 7 | 0 | 8.60 | 2.2 | 0.91 |
| B12-2 | 5.5 | 0 | 8.32 | 2.2 | .76 |
| B12-6 | 5.5 | 0.25 | 8.20 | 2.2 | .75 |
| B12-5 | 5.5 | 0.5 | 8.34 | 2.1 | .91 |
| B12-4 | 5.5 | 1 | 8.20 | 1.7 | .84 |
| B12-3 | 5.5 | 2 | 8.30 | 1.2 | .92 |

[a] 61.6% glycerol. [b] 65.5% glycerol.

magnitude to those obtained by Tinoco between *p*H 6 and 7; his range did not extend, of course, so far to the acid side. There appears to be a small but definite increase in $B/c$ with activation, of the order of $0.1 \times 10^{-2}$. Larger increases were observed by Tinoco at higher *p*H.

TABLE IV

KERR COEFFICIENTS AT LOW *p*H

Glycerol 64% by weight.

| Expt. | Fibrinogen concn., g./l. | Urea concn., *M* | *p*H | $\kappa \times 10^5$, mho cm.$^{-1}$ | $B/c \times 10^2$, cm.$^4$/volt$^2$ g. |
|---|---|---|---|---|---|
| | | Unactivated fibrinogen | | | |
| B9-1 | 7 | 0 | 4.28 | 2.0 | 0.55 |
| B11-1 | 7 | 0 | 4.40 | 1.8 | 0.41 |
| B11-2 | 7 | 1 | 4.61 | 1.8 | .35 |
| | | | | Av. | .38 |
| | | Activated fibrinogen | | | |
| B9-A1 | 7 | 0 | 4.30 | 1.9 | 0.64 |
| B9-A2 | 7 | 0 | 4.21 | 2.0 | .62 |
| | | | | Av. | .63 |
| B11-A1 | 9.3 | 0 | 4.25 | 2.1 | 0.46 |
| B11-A3 | 7 | 0 | 4.25 | 1.3 | .55 |
| B11-A2 | 8.9 | 0.5 | 4.35 | 2.2 | .51 |
| | | | | Av. | .51 |

**Interpretation of Polarization.**—The curves for rise of birefringence at *p*H 4.2–4.6 were symmetrical with the decay curves. From this behavior, which was observed also by Tinoco at *p*H 6–7, the theory of Benoit[8] would lead to the conclusion that the permanent dipole moment parallel to the long molecular axis is zero. Tinoco's extension[12] of this theory shows, however, that a component of dipole moment parallel to the *short* axis cannot be excluded. The fact that $B$ increases with activation indicates that such a component does exist and that it changes when the negatively charged polypeptide is split off by thrombin. This would indeed be expected if the site of attack is near the center of one side of the fibrinogen molecule as previously deduced.[2] Unfortunately, the magnitude of the moment and its change on activation cannot be calculated without more precise knowledge of the optical anisotropy of the molecule than is available at present.

At *p*H 8.2–8.9 the rise of birefringence was not symmetrical with the decay, but resembled the time dependence calculated by Benoit[8] for a ratio $\alpha$ of the order of 1 to 5. This behavior indicates a component of permanent dipole moment parallel to the long axis of the molecule. Since both the shape of the rise curve and the magnitude of the Kerr coefficient were similar to those obtained by Tinoco at comparable *p*H in 3 *M* urea, his analysis in terms of this moment would be qualitatively applicable to the new data as well. It may be concluded that the presence of urea in his experiments introduced no serious complications in the interpretation of the data. However, since it is no longer certain[12] that the orientation at low *p*H is due solely to induced polarization, quantitative calculations of the dipole moments must await knowledge of the molecular optical anisotropy in this case also.

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