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Charge Distribution of Fibrinogen as Determined by Transient Electric Birefringence Studies*

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This paper reports the results of a study of the transient electric birefringence of bovine fibrinogen in the *p*H regions of 4.0 to 5.0 and 7.0 to 9.0. A transverse permanent dipole moment leading to negative birefringence in the low *p*H region has been found; anomalous titration behavior associated with it suggests an interpretation which involves an all-or-none titration of several dissociable groups. The presence of more than one fibrinogen species in solution at the same time has been established from analysis of the birefringence transients. The presence of a longitudinal permanent dipole moment in the high *p*H region, as found by previous workers, has been verified. The magnitude and *p*H dependence are consistent with the titration of two α-amino groups, one at each end of the molecule, a distance of about 250 A from the center. From the results and interpretations given the conclusion is reached that the distribution of groups titrating in the *p*H regions studied is symmetrical about the center of the protein molecule.

A recent study by Hartley and Waugh (1960) of the solubility behavior of bovine fibrinogen has indicated that native clottable fibrinogen may exist in a variety of isomeric structures. Because of differences in folding of the polypeptide chains such isomers may differ in number and location of available dissociable groups. A valuable technique for the study of charge distribution in an anisometric molecule like fibrinogen is the method of transient electric birefringence, which is highly sensitive to changes in electrical symmetry. This technique has the additional advantage of providing a continuous check on the gross structure of the molecule through measurement of the rotational diffusion coefficient.

Although previous investigations of the transient electric birefringence of bovine fibrinogen have been reported (Tinoco, 1955a; Billick and Ferry, 1956) use of the more sensitive high-speed apparatus developed by O'Konski and Haltner (1956, 1957) has permitted a more critical study

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to be made and has revealed some unusual behavior at low *p*H.

Materials

The fibrinogen, obtained in the form of the Cohn Fraction I from Pentex, Inc. (Kankakee, Ill.) was purified by ammonium sulfate fractionation, according to the method of Laki (1951). The precipitate formed at 23% saturation of ammonium sulfate was dissolved in 0.3 M potassium chloride or in 3 M urea and was extensively dialyzed. All of the fibrinogen used in this research showed a clottability of 95% ± 3% when assayed by the method of Morrison (1947) within a day or two after purification. Total protein determinations were made by optical density measurement. The solutions for birefringence measurement were prepared from the purified fibrinogen by a variety of methods; not until much later in the work was it realized that the experimental results depended to some extent on the history of the samples.

For most of the experiments at low *p*H, samples in the salt or urea solvent were dialyzed directly against the desired final solvent, although direct addition of formic acid to a *p*H of about 3.2 was frequently made to permit fast passage through the isoelectric *p*H of the protein. Methods used to change the *p*H of a sample after it had already been dialyzed free of salt included direct addition of acetic acid or ammonium hydroxide, or treatment with Amberlite MB-1 acid-base ion exchange resin. The latter was effective in lowering the conductivity of the solution. The final solvent for most samples was a solution of approximately 10^{-3} M acetic acid. Other substances such as ethylene bromohydrin, formamide, etc., which were used in an attempt to solubilize the protein at a *p*H closer to its isoelectric point in the absence of salt, were generally ineffectual. All samples were dialyzed to achieve as low a conductivity as possible.

The solvents used for high *p*H measurements were 3 M urea, 0.1 M glycine, or water, the last two being supplemented with ammonium hydroxide to achieve the desired *p*H. At these *p*H values the protein showed a slight tendency to aggregate under the low-salt conditions required for the birefringence experiments. In order to reduce the interference from aggregated species, many of the solutions were clarified just before the birefringence run by centrifugation for 20 minutes at 30,000 × *g* in a Spinco Model L Preparative Centrifuge.

Methods

Apparatus.—The apparatus built by O'Konski and Haltner (1956, 1957) for the measurement of transient electric birefringence has been described in detail in their publications. A low voltage power supply was used to obtain fields up to 10,000 v/cm; a high voltage supply designed by Krause and O'Konski (1959) was used for birefringence measurements at high fields (up to 50,000 v/cm). A rectangular electric pulse of 50 microseconds duration was applied to the solution in the Kerr cell by means of the electrodes described by Pytkowicz and O'Konski (1959). A silica Beckman spectrophotometer cell with a path length of 1.0 cm was used as the Kerr cell; the separation of the electrodes was 0.200 ± 0.005 cm. The cell assembly was thermostatted at 25°.

The optical system designed by O'Konski and Haltner (1956, 1957), including a fast cathode follower stage at the output of the photomultiplier, has the advantage of providing a polarized output with a high signal-to-noise ratio. Analysis of the birefringence decay of 0.1 M glycine solutions indicated that under optimum conditions for measurement the response time of the photomultiplier circuit was less than 0.03 microsecond. The traces of the electric pulse and the birefringence transient produced on a Hewlett-Packard Model 150A Oscilloscope (with Model 152B Dual Trace Amplifier) were recorded on Kodak Linagraph film. In measurements to determine the Kerr coefficient the chopped presentation of the oscilloscope, which permitted both pulse and birefringence transient to be displayed simultaneously, was usually employed. Measurements were made at five or six values of the applied electric field.

The retardation of light produced by the orientation of the protein molecules in the electric field is readily obtained from the voltage deflection observed on the oscilloscope screen. Standard curves calculated for a rotation of the analyzer of three degrees from the crossed position[1] were used to obtain retardation values by means of the exact equation of the optical system, as given by Krause and O'Konski (1959).

The AC resistance of the solution in the Kerr cell was determined with an EMI Model 250-DA Impedance Bridge. The resistance of a 0.0100 M potassium chloride solution was used to obtain the cell constant necessary for the calculation of conductivities. The *p*H was determined with a Beckman Laboratory Model G *p*H meter.

Calculations.—When anisometric molecules with an index of refraction different from that of their solvent become oriented in an electric field, the refractive index of the solution in the direction of the field will differ from that perpendicular to the field. Under these circumstances plane polarized light passing through the solution will emerge elliptically polarized. The retardation δ (measured in radians) of one component of the light with respect to the other is related to Δn, the difference in refractive indices in the two directions, by the equation $\delta = (2\pi l/\lambda)\Delta n$, where l =

[1] The analyzing prism was ordinarily turned in a direction such that positive birefringence produced an increase in light reaching the photomultiplier. Negative birefringence also could be observed under these conditions; however, in order to avoid loss of sensitivity, the direction of rotation of the analyzer was usually changed.

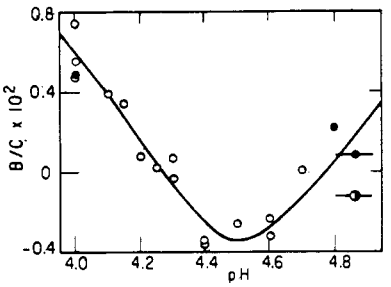

FIG. 1.—The *p*H dependence of the specific birefringence of fibrinogen. The *p*H was adjusted by dialysis. The Kerr coefficient B (cm/statvolt²) divided by the concentration in g/cm³ is plotted *vs.* *p*H. The solvents were aqueous solutions containing: open circles, acetic acid; filled circles, 2-bromoethanol; half-filled circle, 0.1 M glycine, 2 M formamide, or 0.1 M formamide.

path length of light in the cell and λ = wave length of light. Values of the Kerr coefficient, B, may be obtained from a plot of δ *vs.* E^2, the square of the applied field strength, by the relation:

$$B = (1/2\pi l)(\delta/E^2)_{E\to 0} \qquad (1)$$

The specific Kerr coefficient, (B/C), has been shown by Benoit (1951) to depend upon the presence of a longitudinal permanent dipole moment in a macromolecule in addition to the difference in polarizability between the molecule and the solvent. In Tinoco's (1955b) extension of the theory an additional term has been added to take into account the presence of a transverse dipole moment. With the notation of Tinoco and Yamaoka (1959) the equation may be conveniently expressed as

$$B/C = (B/C)_{\text{ind}} + (2\pi V/15\lambda n)(g)(p_3 - p_1) \qquad (2)$$

where the subscript 3 refers to the principal axis of an ellipsoid of revolution and the subscript 1 to a transverse axis. The units of (B/C) are cm⁴/g-statvolt². The other symbols are as follows: C = concentration of the birefringent solute in g/ml; V = partial specific volume of solute (0.71 ml/g for fibrinogen); λ = wave length of light used in the birefringence measurement (5100 A); n = index of refraction of the solvent (1.333 for water); g = the optical anisotropy given by $(\alpha_{0,33}-\alpha_{0,11})/v$ where v is the volume of the protein molecule (3.9×10^{-19} ml for fibrinogen) and $\alpha_{0,33}$ and $\alpha_{0,11}$ are components of optical polarizability; $p_3 = \langle\mu^2\rangle/(kT)^2$ where $\langle\mu^2\rangle$ refers to the effect mean square permanent dipole moment in the direction of the symmetry axis of the molecule, k is the Boltzman constant, and T is the absolute temperature (298°K); $p_1 = \langle\mu_t^2\rangle/(kT)^2$ where $\langle\mu_t^2\rangle$ is the effective mean square transverse permanent dipole moment. The quantity $(B/C)_{\text{ind}}$, which refers to the contribution of effects induced by the presence of the field, includes a term for electronic and atomic polarizability given by $(2\pi V/15\lambda n)$ (g) (q_e) where q_e is $(\alpha_{E,33}-\alpha_{E,11})/kT$. Other possible contributions to $(B/C)_{\text{ind}}$ have not yet been treated quantitatively.

A plot of log δ *vs.* time for the birefringence decay gives the birefringence relaxation time τ related to the rotational diffusion coefficient by the equation

$$\theta = 1/6\tau \qquad (3)$$

The results are expressed in terms of standard conditions as $\theta_{20,w}$ by use of the relation

$$\theta_{20,w} = \frac{(293)\ \eta_{25,s}}{(298)\ \eta_{20,w}}\,\theta_{25,s} \qquad (4)$$

where $\eta_{25,s}$ = viscosity of solvent at 25° and $\eta_{20,w}$ = viscosity of water at 20° = 0.01005 poise.

The rotational diffusion coefficient, denoted $\theta_{20,w}$ in this report, refers to rotation about a transverse axis. In the case of fibrinogen the relaxation time for rotation about the long axis of the molecule is much too small to be measured.

Perrin (1934) has shown for a prolate ellipsoid of revolution with semi-axes a and b and axial ratio $(a/b) > 5$ that the rotational diffusion coefficient for rotation about a transverse axis is given by

$$\theta = \frac{3kT}{16\pi\eta a^3}\,[2\log_e(2a/b) - 1] \qquad (5)$$

where η is the viscosity of the solvent. Thus, a length for the molecule (length = $2a$) may be calculated from knowledge of the rotational diffusion coefficient and the axial ratio.

The shape of the birefringence rise curve provides a value of the orientation parameter α, which corresponds to the ratio of longitudinal permanent dipole (or slowly induced dipole) contributions to those of rapidly induced dipoles and transverse permanent moments. In simple cases it may be represented theoretically by the ratio $p_3/(q - p_1)$; however, in the presence of ion atmosphere polarization, for example, the equation must be modified (Tinoco and Yamaoka, 1959).

RESULTS

Low Field Birefringence, pH 4.0–5.0.—Figure 1 shows a plot of the specific birefringence *vs.* *p*H for fibrinogen solutions at concentrations from 0.30 to 9.0 mg/ml. A complete set of the data, including the values of $\theta_{20,w}$ determined from the birefringence decay curves, is collected in Table I. A curious feature of these results is the occurrence of negative birefringence in part of the *p*H region; this was not observed by Billick and Ferry (1956), because their apparatus only measured the absolute magnitude of the birefringence.

The change in the appearance of the birefringence transients through the *p*H region of 4.0

TABLE I

TRANSIENT ELECTRIC BIREFRINGENCE OF FIBRINOGEN, *p*H 4–6; THE SPECIFIC CONDUCTANCE, SPECIFIC BIREFRINGENCE, AND ROTATIONAL DIFFUSION COEFFICIENTS ARE PRESENTED

Sample	Solvent	*p*H	Conc. (mg/ml)	$\kappa \times 10^4$ (mho/cm)	$B/C \times 10^2$	$\theta_{20,w}$ (sec.$^{-1}$)
Q-2	HOAc	4.0	0.7	0.65	0.75	44,000
W-1	HOAc	4.0	3.2	0.84	0.55	41,500
F-1	BrEtOH	4.0	6.0	1.27	0.49	48,500
F-3	HOAc	4.0	6.0	1.31	0.48	50,000
V-1	HOAc	4.1	8.1	1.42	0.39	41,000
V-2	HOAc	4.15	7.9	0.90	0.35	41,500
S-2a	HOAc	4.2	2.8	0.89	0.08	—
R-1a	HOAc	4.25	3.0	0.61	0.02	—
M-1	HOAc	4.3	2.5	0.65	0.07	—
S-1	HOAc	4.3	3.0	0.61	−0.03	—
J-1	HOAc	4.4	7.6	0.81	−0.37	41,000
I-2	HOAc	4.4	5.7	0.59	−0.36	43,000
S-2b	HOAc	4.5	2.8	0.90	−0.26	39,000
V-4	HOAc	4.6	0.3	0.20	−0.23	40,500
R-1b	HOAc	4.6	3.0	1.70	−0.32	39,500
F-5	0.1 M gly.	[a]	7.5	1.05	−0.13	36,500
C-1	2 M form.	[a]	6.4	1.42	−0.12	31,000
B-2	0.1 M form.	[a]	9.0	0.96	−0.10	25,000
R-1c	HOAc	4.7	3.0	1.90	0.01	—
F-4	BrEtOH	4.8	6.2	0.61	0.22	250,000
E-1	BrEtOH	[a]	7.9	0.81	0.08	250,000
C-3	0.1 M acet.	[b]	6.0	0.48	0.12	250,000

[a] *p*H 4.7–5.0 [b] *p*H 5–6.

Abbreviations: HOAc = acetic acid; BrEtOH = ethylene bromohydrin; gly. = glycine; form. = formamide; acet. = acetamide. The units of B/C are cm ml/statvolt^2-g.

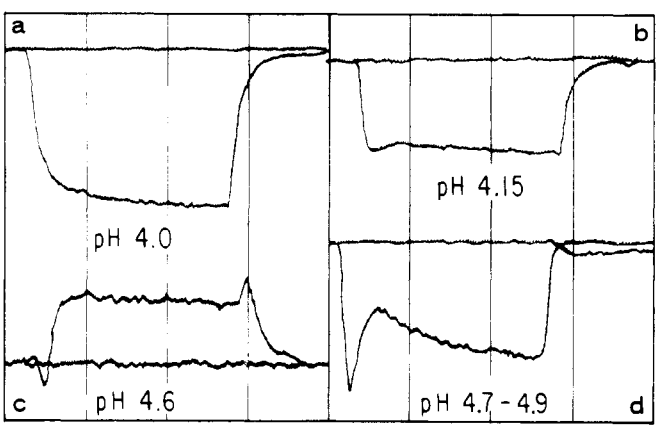

FIG. 2.—The transient electric birefringence as photographed on the oscilloscope screen. A signal proportional to the birefringence is plotted *vs.* time. The vertical lines are 20 μsec. apart. *a*, A large positive birefringence at *p*H 4.0; *b*, a small positive birefringence at *p*H 4.15; *c*, a negative birefringence at *p*H 4.6; *d*, a positive birefringence at *p*H 4.7–4.9.

to 5.0 was quite marked, as indicated by the oscilloscope tracings shown in Figures 2a-2d. The horizontal sweep time is 10 μsec/cm; a positive birefringence signal goes below the base line. In a typical transient observed at *p*H 4.0 (Fig. 2a) the birefringence is large and positive; the rise follows the same exponential as the decay. This signal corresponds to a single fibrinogen species orienting parallel to the electric field by an induced dipole mechanism. At *p*H 4.15 (Fig. 2b) both the rise and decay curves indicate some inhomogeneity, which implies a second species orienting to give birefringence of opposite sign. Critical analysis of the decay curves shows that the two species rotate at approximately the same velocity, although the positive species is slightly (less than 15%) faster.

An example of the large negative birefringence observed for fibrinogen at *p*H 4.4–4.6 is shown in Figure 2c. Since the values of $\theta_{20,w}$ determined in this *p*H region are still characteristic of fibrinogen, there is no reason to believe that a radical change in molecular structure has taken place. Presumably, then, the negatively birefringent species,

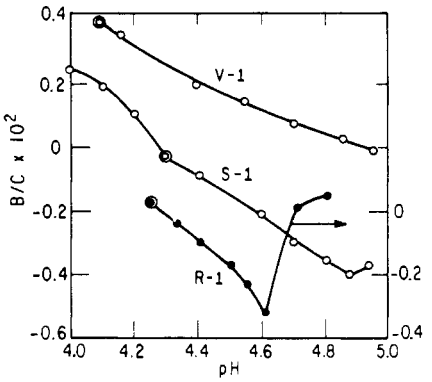

FIG. 3.—The *p*H dependence of the specific birefringence of three samples of fibrinogen. The *p*H was adjusted by addition of acetic acid or ammonium hydroxide to the dialyzed solution identified by the double circle in the figure. The open circles refer to the scale on the left; the filled circles refer to the scale on the right.

which orients perpendicular to the electric field, consists of fibrinogen molecules that have acquired a permanent dipole moment along a transverse axis, such that the contribution of the term involving p_1 in equation (2) exceeds $(B/C)_{\text{and}}$. The fact that the orientation parameter α is zero throughout this *p*H region indicates that p_3 is negligible. In addition to the principal negatively birefringent species the shape of the transients suggested a small contribution from a very rapidly orienting species with positive birefringence.

At higher *p*H values the birefringence became positive again; unfortunately only a few measurements could be made because of precipitation of the protein on the electrodes. The rise of the birefringence in a typical case (Fig. 2d) shows several competing mechanisms of orientation with a possible p_3 contribution. The fast decay of the resultant birefringence again suggests that a rapidly orienting species is present in the mixture. The appearance of this component in the decay may mean that a change in structure has occurred in some molecules.

Titration Experiments.—In order to further investigate the *p*H dependence of the specific birefringence of fibrinogen, titration experiments were carried out with several solutions. The results are shown in Figure 3. The double circle in each plot corresponds to the *p*H at which the samples were prepared by dialysis; changes in *p*H were made by addition of 1 N ammonium hydroxide or 1 N acetic acid. The birefringence measurements were taken within 15 minutes after the *p*H change.

These results clearly show the trends observed with changing *p*H. A *p*H-dependent conversion between components with positive and negative birefringence is strongly indicated. The rate of change of birefringence with *p*H is similar in samples V-1 and S-1, although the absolute values of (B/C) are clearly a function of the original state of the samples. Sample R-1 showed a somewhat faster change of birefringence with *p*H; this may have been related to its slightly greater ionic strength. However, it is doubtful that the small changes of ionic strength due to the addition of acid and base in these titrations had an appreciable effect on the results. It was observed, for example, at *p*H 4.2–4.3, where the birefringence changes sign, that the birefringence followed roughly the same path when the *p*H was varied up and down by successive additions of acid and base.

It must be concluded from these titration data that charge isomers of fibrinogen exist which are not in rapid equilibrium under the conditions of these experiments. Their relative concentrations, which are dependent on the previous history of the sample, determine the magnitude and sign of the observed birefringence. Since anomalies in titration behavior have not previously been reported for fibrinogen, it is possible that these effects occur only at the very low ionic strengths of the birefringence measurements.

Birefringence at High Fields.—Very dilute solutions of fibrinogen at *p*H 4.5 or higher have a sufficiently low conductivity to permit the establishment of very high fields across the electrodes, within the power limitations of the apparatus. At these *p*H levels the low field birefringence is negative; as the field is increased, the birefringence reaches a maximum negative value, then decreases and eventually changes sign. Although the applied fields were not large enough to achieve complete saturation of the birefringence, the observations indicate the beginning of saturation behavior for those molecules with a transverse dipole moment; *i.e.*, the birefringence of this species is falling off from its initial linear dependence on E^2. Thus, positive induced dipole birefringence produced by molecules that have no transverse moment becomes the more important factor. An example of the field dependence of the birefringence expressed as the retardation δ is given in Figure 4. The birefringence transients also clearly showed the effect of a competition between two different modes of orientation; at the highest field applied the transient appeared as shown in Figure 5.

A value of approximately 1150 Debye for the root mean square transverse dipole moment was found for the species which orients to give negative birefringence. Although complete saturation of the birefringence was not achieved, the determination was made possible by the use of empirical curves based on data for birefringence saturation of an enzymatically activated species of fibrinogen (Haschemeyer, 1961). These data have shown that in the region between Kerr Law behavior and complete saturation the shape of a curve of observed retardation *vs.* the square of applied field strength is characteristic of the magnitude of the permanent dipole contribution to the orienting force. It is necessary, however,

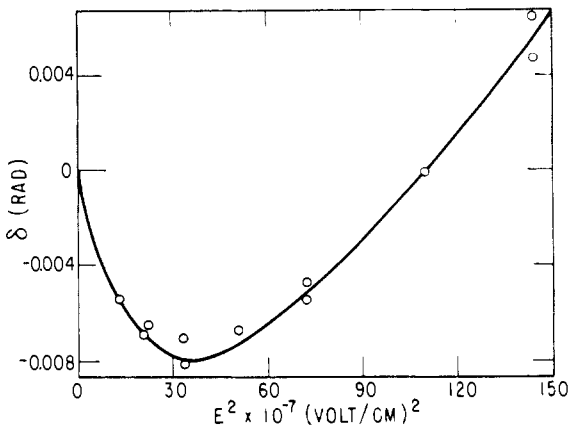

FIG. 4.—The electric field dependence of the birefringence of fibrinogen at *p*H 4.6. The retardation δ in radians is plotted *vs.* electric field strength squared $(v/cm)^2$.

to know the magnitude of the birefringence due to induced dipole orientation and other fast interactions (*e.g.*, ion atmosphere polarization) leading to positive birefringence. For the purposes of this calculation a value of 0.8×10^{-2} was taken for $(B/C)_{ind}$ based on the data at *p*H 4.0 where the orientation is entirely of this type, in addition to the values obtained at high *p*H from measurements of (B/C) and α.

Low Field Birefringence, pH 7.0–9.0.—The electric birefringence of fibrinogen at alkaline *p*H both in the presence and in the absence of urea has been reported by other workers. Using aqueous glycerol–3 M urea solvents, Tinoco (1955) found definite evidence for a permanent dipole type of orientation with values of the specific Kerr coefficient up to 1.3×10^{-2} and values of the orientation parameter α from 0 to more than 5. Billick and Ferry (1956) were able to confirm Tinoco's results at *p*H 8.2–8.8 in the absence of urea, although they still found it necessary to use a high concentration (64%) of glycerol to decrease the speed of orientation and permit determination of the rotational diffusion coefficient. Values of $\theta_{20,w}$ from 24,000 to 38,000 sec.$^{-1}$ were reported.

The high speed electric birefringence apparatus of O'Konski and Haltner (1956, 1957) has made it possible for the first time to observe the transient birefringence of fibrinogen without the requirement for glycerol or any other viscous substance to slow the rotational diffusion. The results obtained in the *p*H region of 7.0 to 9.0 are collected in Table II. The specific birefringence is plotted against *p*H in Figure 6. The amount of variation in the values of $\theta_{20,w}$, determined from analysis of several birefringence transients for each sample, does not actually reflect experimental error. In all but two cases the presence of slow-moving species, probably aggregated protein, was evidenced by deviation from simple exponential decay of the birefringence. For two samples which showed complete monodispersity throughout their decay, $\theta_{20,w}$ was found to be 43,000 sec.$^{-1}$. This is consistent with values obtained at low *p*H (Table I).

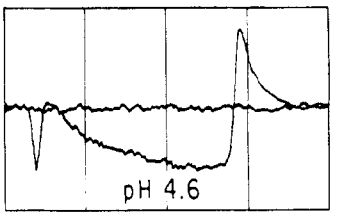

FIG. 5.—The transient electric birefringence of fibrinogen at *p*H 4.6 under a field strength of 37,500 v/cm.

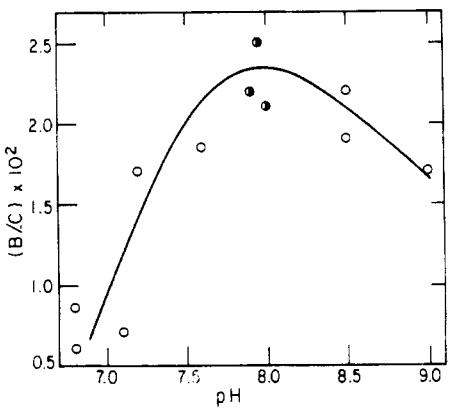

FIG. 6.—The *p*H dependence of the specific birefringence of fibrinogen. The *p*H was adjusted by dialysis.

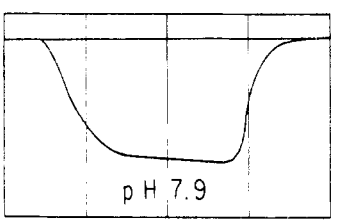

FIG. 7.—The transient electric birefringence of fibrinogen at *p*H 7.9. The birefringence is positive and shows a permanent dipole orientation mechanism.

In the last column of Table II are listed the values of the orientation parameter α obtained by comparing the birefringence rise curves with standard curves based on the theoretical equation (Benoit, 1951). An example of a birefringence transient at *p*H 7.95 is given in Figure 7. The shape of the curves was quite reproducible among the various samples and was independent of the applied voltage in the range normally used for low field birefringence measurements; however, the curves did not actually fit any of the standards. The initial slope of the rise indicated $2 \leq \alpha \leq 5$; however, the shape above $\theta t = 0.3$ corresponds more closely to the standard curves

TABLE II

TRANSIENT ELECTRIC BIREFRINGENCE OF FIBRINOGEN, *p*H 6.8-9; THE SPECIFIC CONDUCTANCE, SPECIFIC BIREFRINGENCE, ROTATIONAL DIFFUSION COEFFICIENTS, AND ORIENTATION PARAMETERS ARE PRESENTED

Sample	Solvent	*p*H	Conc. (mg/ml)	$\kappa \times 10^4$ (mho/cm)	$B/C \times 10^2$ (cm^4/v^2-g)	$\theta_{20,w}$ ($sec.^{-1}$)	α
B-1a	3 M urea	6.8	10.0	1.14	0.85	43,000	1
B-1b	3 M urea	6.8	10.0	1.12	0.60	36,000	
C-2	3 M urea	7.1	5.9	1.36	0.70	33,000	1/2
H-1	3 M urea	7.0	—	—	—	39,500	1
G-6a	NH_4OH	7.1	3.6	0.76	0.80	—	
G-5	NH_4OH	7.2	4.7	0.65	1.70	36,000	2
G-2	NH_4OH	7.6	1.6	0.17	1.85	32,000	2
G-3	NH_4OH	8.5	3.1	1.48	1.90	31,500	1-2
G-7	NH_4OH	8.5	3.5	0.88	2.20	33,500	1-2
F-2	NH_4OH	9.0	6.2	1.00	1.70	43,000	2-3
G-2	0.1 M gly.	7.9	2.8	0.70	2.20	33,000	
G-2b	0.1 M gly.	7.95	2.8	0.40	2.50	40,000	1
G-2a	0.1 M gly.	8.0	1.3	0.28	2.10	37,000	1

for $\alpha = 1$ or $\alpha = 2$. The latter are probably the better values; a slow rise at early times may be attributable to the rise time of the electrical pulse.

DISCUSSION

Birefringence at Low pH.—One of the most distinctive features of the data in Figure 1 is the symmetry of the birefringence values about the *p*H of greatest negative birefringence. If it is assumed that only one type of group is titrating in this region (*e.g.* carboxyl), then the possibility of an asymmetric distribution of groups is completely excluded by these data. However, the fact that a transverse dipole moment occurs means that charge asymmetry must exist in some molecules in the solution. It will be shown that the observed data can be interpreted in terms of a symmetrical distribution of groups.

Consider a distribution of groups at either end of a transverse axis through the center of rotation of the molecule. If they are symmetrically arranged, no transverse moment will exist in any direction when all groups are charged, *e.g.* in carboxylate form. Obviously, there will be no dipole moment when all groups are uncharged, *e.g.* as undissociated carboxyls. However, at *p*H levels within the titration region of these groups, some molecules will possess a transverse dipole moment simply because more groups have been titrated on one side of the molecule than on the other. These species will orient with their transverse moments in the direction of the applied electric field and produce negative birefringence. Thus, as the *p*H is raised from the point where all groups are uncharged, negative birefringence will be produced, reaching a maximum at the *pK* of the titrating groups, where the concentration of partially titrated species will be at a maximum. As titration is continued, the number of molecules with a transverse dipole moment will decrease and the negative birefringence will decrease. When all groups have been titrated the observed birefringence should be the same as it was before the titration was begun.

For quantitative treatment of the data a simple model will be chosen. Let there be a total of ν dissociable groups with charge zero or e (the electronic charge) which are placed symmetrically at either end of a transverse axis a distance of $\pm r$ from the center of rotation. It will be assumed that these groups titrate at random and that there are no interactions of any kind between them. From a simple consideration of the statistical distribution of species it can be shown that the apparent mean square dipole moment at the *pK* of the titrating groups is given by

$$\langle \mu_t^2 \rangle_{app} = (\nu/4)(er)^2 \qquad (6)$$

This result may also be obtained as a special case from the general equation for dipole moment fluctuations derived by Kirkwood and Shumaker (1952).

The value of $\langle \mu_t^2 \rangle_{app}$ may be calculated from the birefringence maximum at *p*H 4.5 with the aid of equation (2), which reduces to

$$(B/C) = (B/C)_{ind} - K(g)\langle \mu_t^2 \rangle_{app} \qquad (7)$$

in the absence of a permanent dipole moment along the major axis of the molecule, *i.e.*, $p_3 = 0$. The constant K equals 2.6×10^{-6} for (B/C) in the usual units of $cm^4/g\text{-statvolt}^2$. Using the value of 0.8×10^{-2} for $(B/C)_{ind}$ as before and a value for the optical anisotropy factor (g) of 6.5×10^{-3} based on flow birefringence data (Hocking *et al.*, 1952) yields a result of $0.7 \times 10^6 D^2$ for $\langle \mu_t^2 \rangle_{app}$. From equation (6) one then finds that the total number of groups, ν, responsible for the negative birefringence is approximately 50. In this calculation a value of 50 A was used for r, the length of a transverse semi-axis, based on reasonable values for the length (500 A) and axial ratio (5) of fibrinogen (Scheraga and Laskowski, 1957).

The result above based on random titration is clearly too high to be meaningful and cannot be considered a good explanation of the data. Some

kind of cooperation must be assumed to exist among the groups because, in this way, the number of groups required to account for the negative birefringence could be greatly reduced. Such a possibility also is consistent with the apparent resistance of the protein to titration. There are many possible models involving interactions between the titrating groups which may be constructed; only the simplest will be considered here. If there are two clusters of $(\nu/2)$ dissociable groups, each of which titrates as a unit in a type of unmasking or all-or-none reaction, a transverse dipole moment of $(\nu er/2)$ will exist in those molecules which have one cluster charged and the other uncharged. At the pK of the groups, where half of the molecules in solution are in the partially titrated state,

$$<\mu_t^2>_{app} = \frac{1}{2}(\nu er/2)^2 \qquad (8)$$

With this equation the experimental data then predict a value of $\nu = 10$ for $r = 50$ A, *i.e.*, five groups on either side of the molecule. This is a much more reasonable result. For $\nu = 10$, the transverse dipole moment $(\nu er/2)$ is 1200 Debye. This agrees well with the experimental value estimated from the high field birefringence measurements.

Thus, a reasonable interpretation of the pH dependence of the negative birefringence of fibrinogen at low pH has been possible in terms of an all-or-none titration of groups composing two identical and independent clusters on either side of the molecule. The model predicts the existence of three charge isomers of fibrinogen: two will have no transverse dipole moment while the other has a large one. The presence of at least two isomers was established directly from analysis of the birefringence decay curves. In addition, the interpretation is consistent with the high degree of structural symmetry which probably exists if the molecule is a dimer, as indicated by clotting studies (Blombäck, 1958). The idea of a cooperative phenomenon is supported by the time dependence of the titration.

It would be most interesting in the future to investigate the nature of the interactions, perhaps hydrogen-bonding, that lead to the anomalous birefringence behavior of the protein and the postulated cooperative unmasking reaction. Unfortunately, it is not possible at this time to ascertain what significance these interactions may have with regard to the structure of the protein, particularly that which occurs under physiological conditions. However, they may play an important role in maintaining the tertiary structure of the molecule under the conditions of these experiments. This may be inferred from the changes in the decay of the birefringence which occurred on the high pH side of the titration region.

Birefringence at High pH.—From the best experimental value of the rotational diffusion coefficient ($\theta_{20,w} = 43{,}000$ sec.$^{-1}$) a calculation may be made from equation (5) to obtain the length of the fibrinogen molecule, as represented by a prolate ellipsoid of revolution. Using a value of 0.01005 poise as the viscosity of the solvent (water at 20°), one finds lengths of 550 A and 610 A for assumed axial ratios of 5 and 10, respectively. This agrees well with values obtained by other methods (Scheraga and Laskowski, 1957).

A knowledge of the orientation parameter α and the magnitude of the total birefringence permits a calculation of the induced dipole contribution to the birefringence, $(B/C)_{ind}$, a quantity which includes all fast interactions which lead to positive birefringence. It will be assumed that there are no transverse permanent dipole contributions to the birefringence. Then equation (2) reduces to

$$(B/C) = (B/C)_{ind} + K(g)<\mu^2> = (1 + \alpha)(B/C)_{ind} \qquad (9)$$

where K is a constant and (g) is the optical anisotropy. From the experimental values of approximately 2.3×10^{-2} for (B/C) and 1.5 for α at a pH of about 8.1, one obtains $(B/C)_{ind} = 0.9 \times 10^{-2}$, in good agreement with the low pH value. This calculation is valid, however, only if the system is monodisperse.

The value of $(B/C)_{ind}$ calculated as above from the birefringence data at pH 8.1 may be compared with the theoretical contribution of induced polarization due to differences in dielectric constants between solute and solvent. This is given by $(2\pi V/15\lambda n)(g)(q_e)$ (Benoit, 1951). With the aid of Maxwell's (1873) equation for the polarization of a dielectric ellipsoid in an electric field it may be readily shown that the term $(\alpha_{E,33}-\alpha_{E,11})$ in the quantity (q_e) reduces to $0.0795\ v\epsilon_0$ if the assumption is made that the principal dielectric constants of the protein are small compared to the dielectric constant of the solvent. The volume of the fibrinogen molecule, v, is equal to 3.9×10^{19} ml, based on a molecular weight of 330,000 and a partial specific volume of 0.71 ml/g; the dielectric constant ϵ_0 of the solvent is approximately 80 for water. Using values of $(g) = 6.5 \times 10^{-3}$ and $T = 298°$, one finds the theoretical value for $(B/C)_{ind}$ due only to induced polarization to be 0.17×10^{-2}. Since this accounts for only a fraction of the value obtained from the experimental data, it would appear that other types of interactions are more important in producing the fast orientation of the molecules in the electric field. Theoretical considerations have shown the importance of ion atmosphere polarization as a contribution to the orienting force in electric birefringence (O'Konski, 1960). Estimation of times for ion redistribution about an ellipsoid of revolution indicates these effects are fast compared to the orientation of the molecule (Passchemeyer, 1961), and therefore would be included in $(B/C)_{ind}$.

If, as in the previous calculation, the system of protein molecules is assumed to be monodisperse

(*i.e.*, all molecules possess the same polarizabilities and dipole moments), equation (9) may also be used to obtain a value for the permanent dipole moment μ along the symmetry axis. The result is 840 Debye, which could represent, for example, an asymmetrical charge distribution with one excess electronic charge at a distance of 175 A from the center of rotation of the molecule.

Just as was observed at low *p*H, the dependence of the birefringence on *p*H is suggestive of titration behavior. Therefore, an interpretation based on the titration of symmetrically placed groups is conceivable. One type of group which has its *pK* near the observed birefringence maximum at *p*H 8.0–8.2 is the α-amino group of an N-terminal amino acid. Evidence will be presented in a later publication to show that the two N-terminal glutamyl residues of bovine fibrinogen, which are part of Peptide A (one of the peptides released in clotting), are located at opposite ends of the molecule. At the *pK* of these groups the contribution of the partially titrated species to the observed electric birefringence may be calculated with a relation like that given in equation (6) letting $\nu = 2$ and $r = 250$ A. Using the same values for the constants and for $(B/C)_{ind}$ as before, one finds that this effect can account completely for the difference between the maximum experimental birefringence (2.1×10^{-2}) and $(B/C)_{ind}$. Thus, the titration of two groups satisfies the requirements of the experimental data both in the magnitude of the birefringence as its maximum and in the *p*H dependence of the birefringence.

It must be pointed out that the significance of the above calculation depends upon the assumption that the partially titrated molecules retain their state throughout the 50 microsecond time of measurement (in order to account for the slow permanent dipole character of the birefringence rise). In any case, this calculation, together with the calculations on the low *p*H effect, does present the interesting possibility that despite the occurrence of a transverse dipole moment in the low *p*H region (4.0–5.0) and a longitudinal dipole moment at high *p*H (7.0–9.0), there is actually no asymmetry in the distribution of groups titrating in these regions.

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