

THE ELECTRIC BIREFRINGENCE BUILDUP CURVE AS APPLIED TO THE DETERMINATION OF THE DIPOLE MOMENT OF SOLUBLE COLLAGEN

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SUMMARY

Electric birefringence patterns of calfskin corium collagen dissolved in citrate buffer in the acid pH range were made. At high concentrations of collagen, these showed an anomalous electric birefringence pattern which was indicative of time-dependent variations in permanent and induced dipole moments. Measurements of dipole moments showed that when the square wave pulsed electric field is created, permanent and induced dipole moments are in the same direction. This is followed by a progressive decrease in permanent dipole moment with pulse duration, and at high field strength the electric field eventually brings about a reversal in direction between permanent and induced dipole moments. The effect of pH on this phenomenon was studied.

INTRODUCTION

Pulsed electric birefringence presents a means of measuring dipole moments, electric polarizability and optical anisotropy of macromolecular particles in suspension. These parameters are usually difficult to measure by more direct methods, especially in macromolecular suspensions where the presence of water and small buffer ions introduce so much loss that capacitance measurements are not feasible. Electric birefringence is especially useful in observing time dependent changes in the permanent and induced dipole moments of suspended particles in a pulsed electric field. The data obtained from this technique can lead to an understanding of electric charge pattern, aggregation behavior and molecular conformation.

The aim of this paper is to show the application of pulsed electric birefringence to the study of a polyelectrolyte suspension using collagen as a model. Measurements were made on calfskin corium collagen dissolved in citrate buffer over the pH range of 3.25-4.85, and the effects of collagen concentration, electric field strength and pH were investigated. To date, only two papers on the electric birefringence of dissolved collagen have appeared in the literature^{1,2}.

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APPARATUS

A schematic diagram of the apparatus used has already been published². It consists of a transparent optical cell holding the collagen sample being observed which is located between crossed Rochon prisms. Two flat parallel electrodes are immersed in the solution so that the light beam passes between them. A square wave pulse generator and amplifier create an electric field between the electrodes and its trace is displayed on an oscilloscope screen. A photomultiplier at the exit of the optical path senses the light output of the system and displays it on the oscilloscope screen simultaneously with the square wave pulse.

The optical train was obtained by modifying a Rudolph Model 80 Polarimeter so that the polarimeter tube holder and housing were replaced by a cell and holder similar to the one described by O'KONSKI AND HALTNER³. The electrodes were 1.0 cm² and the gap between them was 0.273 cm with the electric field set at an angle of 45° to the axes of the Rochon prisms. The light source was a General Electric CPG projection lamp. No optical filter was used and the average wavelength of the white light was found to be 535 nm. The fidelity of the photoelectric portion of the system was tested with square wave light pulses emitted by an avalanche diode. The oscilloscope used was a Tektronix Model 549 equipped with a 1A1 front end unit which displays a dual trace by electronic switching, and which has a storage system so that a single pulse can be held on the screen for long periods of time. The apparatus was calibrated to give electric birefringence in terms of degrees of angle by a modification⁴ of the method described by BENOIT⁵.

EXPERIMENTAL

The details of making the dissolved collagen preparations used in this work have been given elsewhere⁶. The calfskin corium was placed in contact with citrate buffer at pH 3.5 and ionic strength 0.40, and was kept well below the gelatinization temperature of 35° during preparation. The resulting collagen solutions were verified by electron microscopic examination of fibers reconstituted by dialysis to exhaustion of the electrolyte, and by the presence of a single hypersharp peak in the sedimentation pattern which had a sedimentation coefficient between 2.8 and 3.0 S for different preparations. Collagen assay was *via* Kjeldahl nitrogen determination on the basis that collagen contains 17.5% nitrogen.

In all electric birefringence measurements, the ionic strength of the collagen preparations had to be reduced so that specific conductivities were less than 400 $\mu\Omega^{-1}\cdot\text{cm}^{-3}$, so as not to overload the pulse amplifier. This was done by dialyzing the original preparation against water to a very low value of electrolyte concentration and then adding microliter volumes of solutions of citric acid and sodium citrate to achieve the desired pH and ionic strength. Under these conditions, it is very difficult to determine exact values of ionic strength; therefore, the electrolytic state of the collagen preparations used in this study will be specified by pH and specific electrical conductivity. From these data it is estimated that ionic strength varied from 0.005 to 0.05, and over this limited range, variations in ionic strength seemed to have no effect on the experimental results. Electric birefringence measurements were made at a temperature of 24–27°.

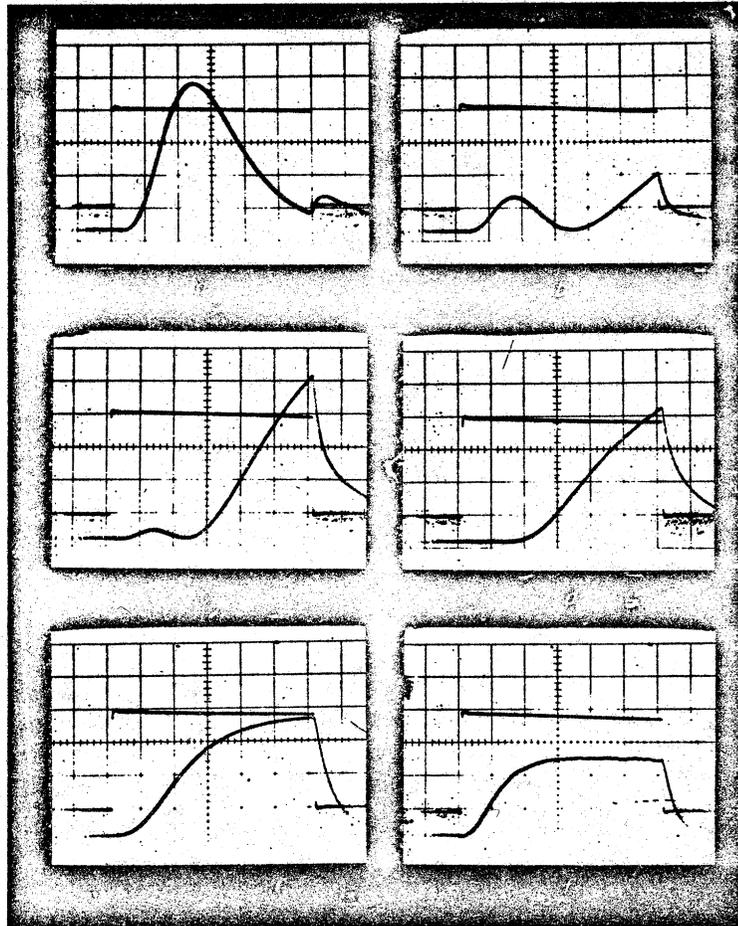


Fig. 1. Electric birefringence pattern of dissolved collagen at decreasing values of concentration at pH 4.13 and at a specific electric conductivity of $185 \mu\Omega^{-1}$. The oscilloscope patterns show magnitude of birefringence *versus* time at a horizontal sweep of 0.5 msec/cm. The collagen concentration of each pattern is: a, 1.05%; b, 0.90%; c, 0.80%; d, 0.70%; e, 0.27%; f, 0.05%.

RESULTS

The relation between collagen concentration and shape of the electric birefringence pattern is shown in the six oscillograms of Fig. 1, which depict the square wave pulse applied to the collagen sample and its birefringent response. These were taken at progressively decreasing values of collagen concentration. The horizontal scale in each of these oscillograms is 0.5 msec/cm and the applied pulse amplitude is always 600 V, which corresponds to an electric field of 2200 V/cm. The vertical scale of the electric birefringence trace was adjusted as necessary from oscillogram to oscillogram so that the pattern would fill the screen. It must be borne in mind that the electric birefringence trace is actually a representation of the potential drop across the load resistance of the photomultiplier, and to convert it to displacement

of light waves in either linear or angular terms, it must be modified according to a sine squared law, which was done via the calibration technique already mentioned.

A conventional electric birefringence pattern is shown in Oscillogram f of Fig. 1, which was made at a collagen concentration of 0.05%. There is an exponential buildup of birefringence to a saturation point which persists until the termination of the applied pulse, at which point decay of birefringence begins. Oscillograms a, b, and c of the same figure, made at the high end of the concentration scale show a very different picture. These three oscillograms were made at the same value of oscilloscope gain and corresponding magnitudes of birefringence can therefore be compared. In Oscillogram a of Fig. 1, made at a collagen concentration of 1.05%, buildup of birefringence is very rapid, and instead of reaching a steady saturation point, as it did in Oscillogram f, it passes through a maximum and decreases in magnitude, even though the applied pulse is still active. At the termination of the pulse, there is a brief secondary increase in electric birefringence. Decreasing the collagen concentration of the sample to 0.90%, as shown in Oscillogram b, produces further changes in the pattern. Here the initial buildup is greatly attenuated, and after the birefringence has dropped to zero (after the pulse has been active for about 1.75 msec), what appears to be a normal buildup ensues. Decreasing the collagen concentration to 0.80%, as shown in Oscillogram c, results in further diminution of the initial maximum of the electric birefringence curve, a return to zero birefringence at an earlier point (about 1.25 msec after the pulse was initiated), and again a normal buildup. Further reduction of collagen concentration to 0.70% results in Oscillogram d, which appears conventional except for the rather lengthy induction period (about 0.75 msec) before buildup of birefringence begins. Oscillogram e where the collagen concentration is 0.47% shows a birefringence trace that is intermediate to d and f. When pulse amplitude was varied over a range of 300-800 V, the general shape of the birefringence curve for a given collagen concentration was preserved and only its amplitude changed.

It is seen from Oscillograms d, e, and f of Fig. 1 that the lower the collagen concentration, the sooner is the electric birefringence saturation point reached.

Furthermore, comparison of Oscillograms d and e (both taken at the same vertical gain) shows the unexpected result that from the time the pulse is initiated and during a time interval of about 2.5 msec, the more dilute solution has a higher birefringence. It has also been observed that birefringence saturation is reached sooner if the pulse amplitude is raised.

A graph of electric birefringence at saturation *versus* pulse amplitude for several concentrations of collagen covering the range from 0.30 to 0.60% is shown in Fig. 2. Electric birefringence increases with collagen concentration and also with pulse amplitude. When these data are plotted as electric birefringence *versus* the square of the electric field, each shows a linear relation below a definite value of field, thus establishing a definite Kerr region whose upper limit increases with collagen concentration⁷.

The effect of pH on electric birefringence at saturation is shown in Fig. 3 which shows a rapid increase in electric birefringence as the pH is raised from 3.5 to 4.4. No measurements above pH 4.85 were possible because at this point collagen precipitates.

The relation of permanent-to-induced dipole moments was investigated by

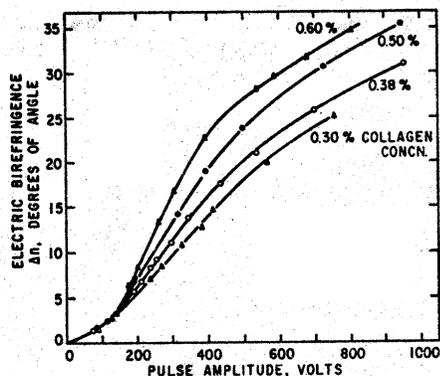


Fig. 2. Electric birefringence of dissolved collagen at saturation *versus* pulse amplitude and concentration at pH 4.33 at a specific electric conductivity of $140 \mu\Omega^{-1}$.

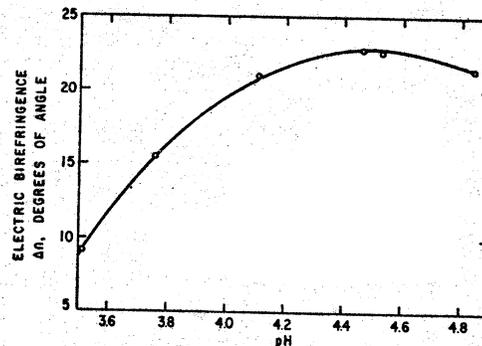


Fig. 3. pH dependence of the electric birefringence of dissolved collagen at saturation. The specific electric conductivity of the preparations varied from 120 to $140 \mu\Omega^{-1}$. The pulse amplitude was 720 V.

applying BENOIT'S⁵ equation for the buildup phase of the electric birefringence curve at low field strength.

$$\frac{\Delta n}{\Delta n_{\infty}} = 1 - \frac{3r}{2(r+1)} e^{-2Dt} + \frac{r-2}{2(r+1)} e^{-8Dt} \quad (1)$$

where Δn is the birefringence at any point on the buildup curve, Δn_{∞} is the birefringence at saturation and

$$r = \frac{P}{Q}$$

with

$$P = \frac{\mu^2}{k^2 T^2}$$

and

$$Q = \frac{\alpha_1 - \alpha_2}{kT}$$

Here μ is the permanent dipole moment along the principal hydrodynamic axis, k is the Boltzmann constant, T is the temperature, and $(\alpha_1 - \alpha_2)$ the difference in the excess polarizabilities of the suspended particles over the solvent in directions parallel to and perpendicular to their principle hydrodynamic axis.

Since the induced dipole moment, ψ , is given by

$$\psi = (\alpha_1 - \alpha_2) E$$

E is the electric field strength, r is proportional to the ratio of permanent-to-induced dipole moments. Curves of r *versus* buildup time are shown in Figs. 4 and 5 for a number of values of pulse amplitude. At low values of pulse amplitude, these are monotonic curves in the first quadrant that show a decrease in r with buildup time. At pulse amplitudes greater than 600 V, these curves reach a point in time where

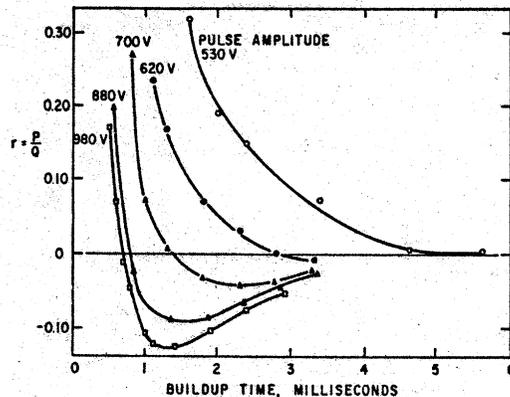
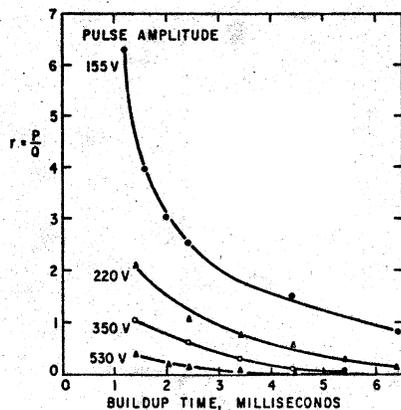


Fig. 4. Graph of r versus buildup time of electric birefringence of dissolved collagen at several values of pulse amplitude. The preparation has a concentration of 0.20%, pH 4.22, and specific electric conductivity of $140 \mu\Omega^{-1}$.

Fig. 5. Graph of r versus buildup time of electric birefringence of dissolved collagen at several values of pulse amplitude. The preparation has a concentration of 0.20%, pH 4.22, and specific electric conductivity of $140 \mu\Omega^{-1}$.

there is a reversal in the algebraic sign of r , and this is followed by a reversal in the direction of the curve. While these curves appear to approach the zero level asymptotically, this is actually not the case as is shown by subsequent measurements of dipole moment at saturation.

The plotted values of r were obtained by solving Eqn. 1 after each value of D

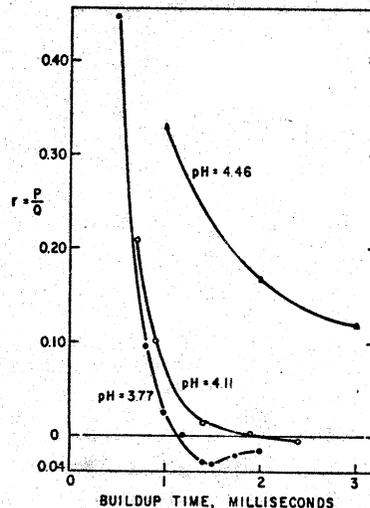
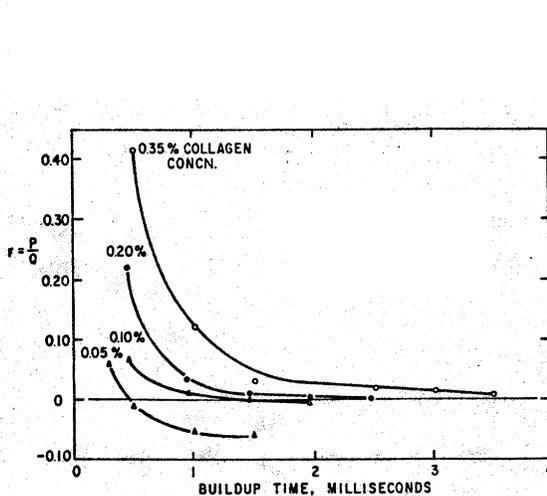


Fig. 6. Graph of r versus buildup time of electric birefringence of dissolved collagen at several values of concentration. The pulse amplitude is 800 V; pH of the preparation is 4.34 and its specific electric conductivity is $115 \mu\Omega^{-1}$.

Fig. 7. Graph of r versus buildup time of electric birefringence of dissolved collagen at several values of pH. The pulse amplitude is 720 V; concentration of the sample is 0.20%.

was obtained from the slope of the corresponding decay curve. These experiments were limited to values of collagen concentration which yielded a straight line when values of the logarithm of the relative birefringence were plotted *versus* decay time.

The effect of collagen concentration on the relation between τ and buildup time is shown in Fig. 6. Decrease in collagen concentration at a fixed pulse amplitude of 800 V is shown to favor reversal of the algebraic sign of the birefringence. Reduction in pH also increases the tendency toward sign reversal as shown in Fig. 7, where τ is plotted *versus* buildup time for three different values of pH.

The permanent dipole moment and the polarizability of dissolved collagen at saturation of electric birefringence were determined using a method proposed by YOSHIOKA AND WATANABE⁸ and by YAMAOKA⁹, based on work of O'KONSKI *et al.*¹⁰. Collagen preparations at pH 4.2 and having a specific electric conductivity of $85 \mu\Omega^{-1} \cdot \text{cm}^{-3}$, showed at saturation a permanent dipole moment of 31 000 debyes regardless of collagen concentration. The polarizability varied with collagen concentration as shown in the graph of Fig. 8. The dependence of permanent dipole moment

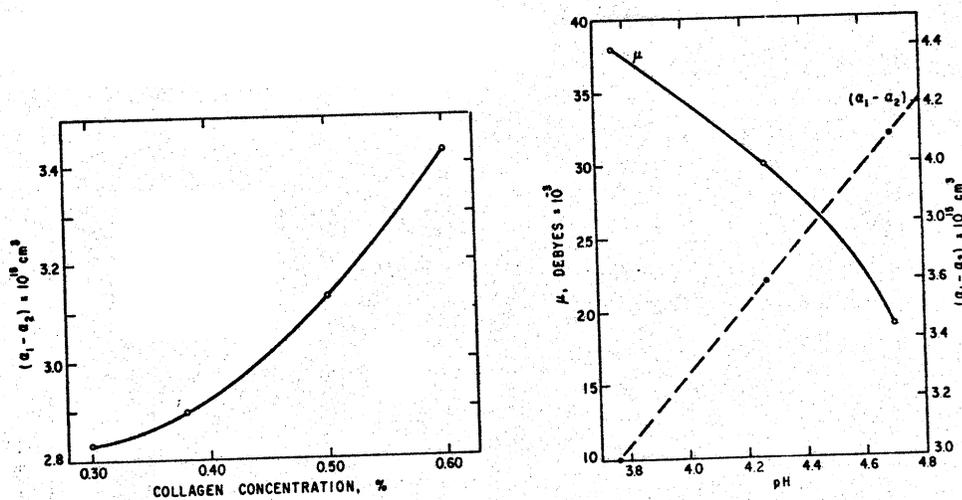


Fig. 8. Graph of polarizability of dissolved collagen *versus* collagen concentration at pH 4.32.

Fig. 9. Graph of permanent dipole moment and polarizability of dissolved collagen *versus* pH. The collagen concentration is 0.35%.

and polarizability on pH are shown in Fig. 9, where it is observed that permanent dipole moment decreases and induced dipole moment increases as pH is raised. Further details of the application of this type of calculation to dissolved collagen will be published elsewhere⁷. Working with solutions of rat tail tendon collagen in dilute acetic acid, YOSHIOKA AND O'KONSKI¹ observed a permanent dipole moment of 15 000 debyes and a polarizability of $2.7 \cdot 10^{-15} \text{ cm}^3$.

The amount of heat generated in the cell by the applied pulse was calculated and found to produce a temperature rise of less than 0.5° . Because of this and the very short duration of each single pulse, it is believed that the effects of heating, electrophoresis, and electrolysis have not influenced the results.

DISCUSSION

To explain electric birefringence behavior which deviates from that expected of a monodisperse dissolved collagen preparation, several possibilities must be considered. The most obvious is bulk interference between adjacent particles as they are caused to rotate in an electric field. Because of its 200:1 axial ratio, a monomeric collagen particle in suspension cannot rotate very far without colliding with one of its neighbors. Polydispersity may be a complicating factor, especially if aggregation or disaggregation is promoted by the presence of the electric field. Electrostatic interaction is very possible because of the complicated electric charge pattern running the length of the rod-like collagen molecule, and may possibly reach a point where adjacent particles so strongly influence each other in this manner that they move as an entity even though they do not form an aggregate in the usual sense by being held together by either covalent or by hydrogen bonds. Conformational changes may be brought about by the solution environment and/or the electric field, and in this way make a change in the permanent dipole moment. Finally, change in charge pattern of the collagen molecule may be brought about through ion binding effects which would alter the induced dipole moment.

In studying the electric birefringence patterns of dissolved collagen at low concentrations, the decrease in rate of buildup of birefringence, and the longer time necessary to reach saturation as collagen concentration is increased, as seen in Fig. 1, Oscillograms d, e and f, is a consequence that is expected because of electrostatic interaction. Consideration should also be given to the fact that as the collagen rods move into alignment with the electric field, they set up a counter potential whose polarity is opposed to the field; also, the ion atmosphere within the solvent is shifted by the field so as to oppose it. The induction period seems to vanish at a collagen concentration of about 0.5%. It has already been shown² that the initial slope of decay curves plotted to a semi-logarithmic scale have their minimum slope at this concentration. This coincidence may indicate that this is a critical concentration and above it a definite energy of activation must be accumulated by the system before orientation changes in the suspended particles can begin to take place.

The decrease in electric birefringence after a maximum has been reached in the case of high collagen concentration as shown in Fig. 1a is in keeping with Eqn. 1. When this equation is applied to a macromolecular species where $P = -Q$, the birefringence passes through a maximum, as has been discussed by BENOIT⁵. If the value of P were very close to $-Q$, the curves shown in Oscillogram b and c would be obtained. The buildup of birefringence after the electric field is quenched, shown in Oscillogram a, is an unexplained hysteresis effect.

The decrease in time to reach saturation with decreasing collagen concentration is perhaps due to decreased electrostatic interaction between suspended particles, and also to a decrease in tendency to aggregate. The lowering of the saturation time with increased pulse amplitude is expected on the basis of energy considerations.

Eqn. 1 is applicable to cases where the electric field strength is low and the macromolecular suspension is dilute, and it is possible that the experimental conditions of this research exceed its range of validity. For this reason, results stemming from the use of this equation must be considered an approximation. It is possible

that to explain the electric birefringence behavior of a complex molecule such as collagen an entirely new theoretical approach is needed.

When the algebraic sign of r is positive, the permanent and induced dipoles are in the same direction; and when it is negative, they are opposed. Applying this principle to the curves of r versus buildup time at pulse amplitudes of 700–980 V in Fig. 5, it appears that there must be a progressive decrease in permanent dipole moment as pulse amplitude is increased, rather than an increase in induced dipole moment, because the latter process would require the induced dipole moment to pass through an infinite value to effect the reversal of algebraic sign. The shift in permanent dipole moment would have to be the result of a conformational change involving the collagen molecule which moves a charged site from one end of the molecule toward the other. A speculative model which illustrates this process is shown in Fig. 10, where a single rod-like molecule is in suspension between two metal plates. This rod carries an overall dipole moment *plus* a flexible side-chain which has a positive charge. In Fig. 10a, where no electric field is active, the side chain takes up a position that brings its positively charged termination as close as possible to the negatively charged end of the rod. When an electric field is created between the plates, as shown in Fig. 10b, the rod-like particle moves to alignment with it and the positive charge of the

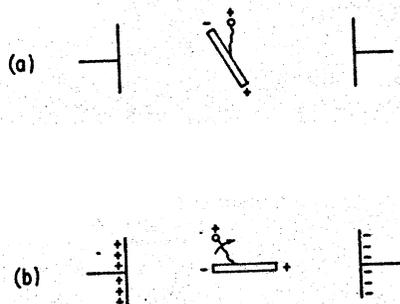


Fig. 10. Schematic representation of mechanism leading to change in the permanent dipole moment of a macromolecular particle in suspension: (a) particle between uncharged plates; (b) particle between charged plates.

side chain is repelled by the anode causing it to move toward the other end of the rod as shown by the arrow, possibly passing through one or more energy barriers. This point of view is favored by the fact that reduction of collagen concentration favors reversal of the algebraic sign of r as shown in Fig. 6, since at low concentration there would be less steric and electrostatic hindrance to such a change. Further confirmation of this explanation comes from the fact that lower pH also favors reversal of the sign of r . In this case, as pH is lowered, there is an increase in the net positive charge of that portion of the collagen molecule that is undergoing conformational change. At pulse amplitudes below 530 V shown in Fig. 4, there is insufficient energy supplied by the electric field to carry this conformational change to the point of sign reversal of r .

A comparison of Fig. 3 with Fig. 7 shows the striking relation that over the range of pH 3.5 to pH 4.1 there is a large change in electric birefringence, but a very

small change in r . Then, over the range of pH 4.1 to pH 4.8, birefringence is nearly constant, but values of r increase tremendously.

Comparison of Fig. 4 with Fig. 9 is also paradoxical in that it is seen that as pH is lowered, a decreasing electric birefringence is observed while the permanent dipole moment increases. This illustrates the fact that electric birefringence is the result of interplay between the permanent and induced dipole moments, rather than their vector sum. It is also important to note in this respect that optical anisotropy varies with induced dipole moment, since both are functions of electronic structure. The decrease in permanent dipole moment that takes place as pH is raised and approaches the isoelectric point at 5.8, as determined by free boundary electrophoresis¹¹, indicates that the negative charges added to a suspended collagen molecule are sandwiched between the positive charges already present to form a pattern of alternate charges.

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