

ELECTRIC BIREFRINGENCE OF COLLAGEN*

LEO D. KAHN AND LEE P. WITNAUER

2991

*Eastern Utilization Research and Development Division
Agricultural Research Service, U. S. Department of Agriculture
Philadelphia, Pennsylvania 19118*

ABSTRACT

Electric birefringence presents a means of determining molecular parameters by measuring the alignment of electrically charged solubilized particles oriented by an electric field. The apparatus consists of a cell holding the solubilized macromolecular sample between two electrodes and in the light path of an optical system capable of measuring birefringence. When a square wave pulse is applied to the electrodes there is a build-up of birefringence followed by its decay. From the resulting curves of birefringence versus time, it is possible to determine whether a suspension is monodisperse, as well as measure dimensional and electric parameters of the solubilized macromolecules. Application of this technique to the study of solubilized collagen is discussed.



INTRODUCTION

Solubilized collagen preparations made by conventional techniques appear to be monodisperse by some criteria, but when examined by light scattering, free boundary electrophoresis, or dynamic osmometry, they seem to be paucidisperse systems of interacting components. To look for the presence of small amounts of gelatins and lower polymers which might account for this, a species-selective technique is needed. Electric birefringence fills this need by exploiting either the frequency dependence of dispersed particles in an electric field, or the relaxation rates of particles that have been aligned momentarily in an electric field and then allowed to return to a random orientation spontaneously.

APPARATUS

A schematic representation of the apparatus used is shown in Figure 1. A light source and monochromatic filter are aligned along an optical bench with two Rochon prisms and a photomultiplier. A transparent optical cell holding the collagen solution under study is located between the Rochon prisms, and two flat parallel platinum electrodes are immersed in the solution so that the light

*Presented at the ALCA Meeting, Mackinac Island, Michigan, June 1968.

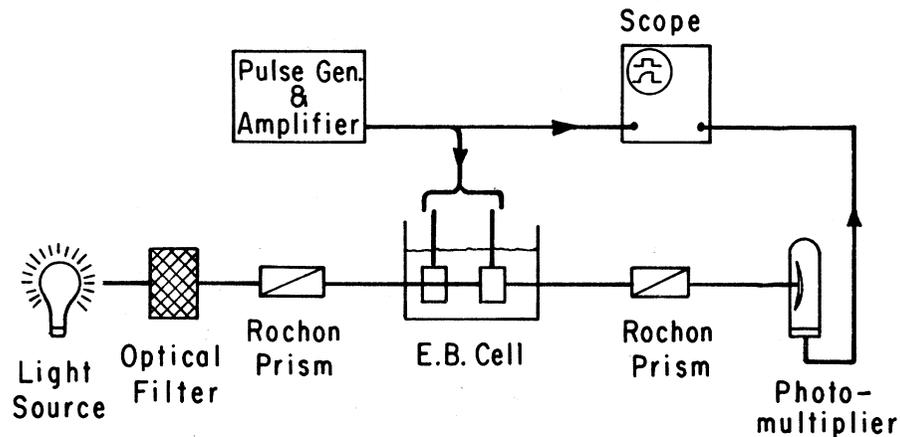


FIGURE 1.—Electric birefringence apparatus.

beam passes between them. A square wave pulse generator and amplifier are connected to the electrodes, and a dual trace oscilloscope is used to display both the applied pulse and the optical response of the system. The oscilloscope has a data storage system so that when a single pulse is applied the displayed data can be retained on the screen.

The Rochon prisms are in the crossed position and in the quiescent state no light passes to the photomultiplier. When a pulse is applied to the electrodes this condition no longer holds. Since each suspended collagen particle possesses an overall dipole moment, an electric field in the birefringence cell tends to align the particles at rates which are dependent on their geometry, dipole moment, field strength, ion atmosphere, and frictional effects. The aligned, or even partially aligned, particles form a uniaxial crystal which is birefringent. This allows light to pass along the optical axis, and its intensity at the photomultiplier follows the mode of rotation of the collagen molecules or aggregates. When the pulse is terminated there is a spontaneous return to random orientation and a corresponding loss of birefringence. A single pulse, rather than a train of pulses, is generally used so as to minimize heating, electrolysis and electrophoresis effects.

RESULTS AND DISCUSSION

A typical oscilloscope pattern of the applied square wave (upper trace) and the birefringent response of a solubilized collagen preparation is shown in Figure 2. The build-up of birefringence and its subsequent decay after the pulse is terminated are shown. Both build-up and decay curves yield information on the

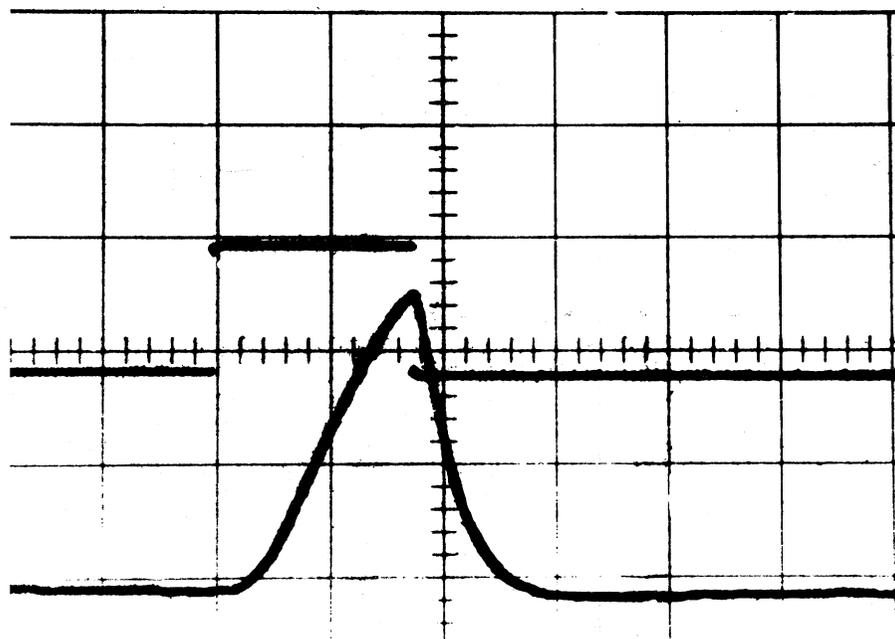


FIGURE 2.—Oscilloscope pattern of electric birefringence trace for solubilized calfskin collagen. Top trace is exciting pulse and bottom trace is optical response of the system.

characteristics of the suspended particles and are usually displayed separately. The mathematics of this process have been developed by Benoit (1), Tinoco (2), and O'Konski, Yoshioka, and Orttung (3).

If a collagen solution is monodisperse, its birefringence decay curve after square wave excitation will follow the law (1) $\eta = \eta_0 e^{-6Dt}$ where η is birefringence, η_0 is birefringence at the instant the electric field is removed, D is the rotatory diffusion constant of the collagen particles and t is decay time. This means that a plot of log birefringence versus decay time will be a straight line of negative slope $6Dt$, and D will give the axial ratio of one cylindrical particle via Burger's formula (4). If a solubilized collagen preparation includes aggregates or subunits, each will have its own rotatory diffusion constant and a plot of log birefringence versus decay time will be the sum of a number of straight lines of different slopes, and will appear on the graph as a curved line.

Yoshioka and O'Konski (5) have investigated the electric birefringence of collagen. Working at collagen concentrations ranging from 0.011 to 0.042 percent they observed a rotatory diffusion constant close to $1.1 \times 10^{-3} \text{ sec}^{-1}$. The aim of the present investigation is to extend these data to higher concentrations.

Figure 3 shows the relation between the relative birefringence and decay time for a sample of calfskin collagen solubilized in citrate buffer at a number of concentrations. The method of solubilization has been described elsewhere (6),

E. B. DECAY CURVES

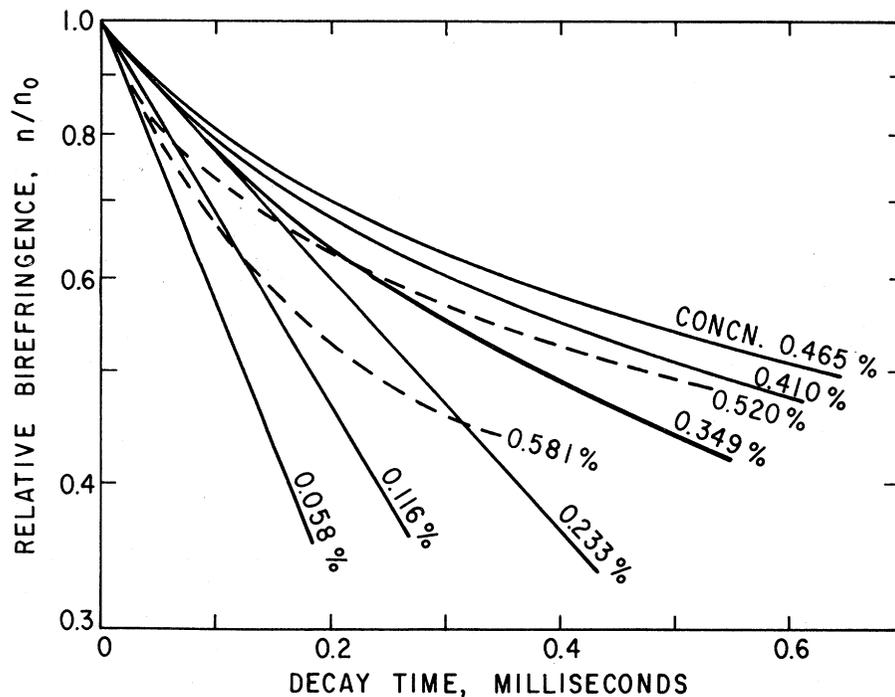


FIGURE 3.—Graph of relative birefringence versus decay time for solubilized collagen at indicated concentrations.

and the preparation was dialyzed against citrate buffer of pH 3.4 and 0.04 ionic strength. At collagen concentrations ranging from 0.058 percent to 0.233 percent the plot shows straight lines of decreasing negative slope. At a concentration of 0.349 percent the plotted line becomes curved, and as collagen concentration is increased this curvature becomes greater and the initial slope less until a peak is reached at a concentration of 0.465 percent. Increasing the collagen concentration beyond this point and up to a maximum of 0.581 percent gives a reversal in that the initial slope of each line increases with increasing collagen concentration and the curvature keeps increasing.

Figure 4 shows the relation between rotatory diffusion constant and collagen concentration. The data include the values obtained from the curves at the three lowest concentrations in Figure 3 plus values from other collagen preparations at the same pH and ionic strength.

The curvature in the plotted lines at higher concentrations shown in Figure 3 can be due to either aggregation, interaction of collagen molecules, or viscosity effects, and experiments to resolve this question are currently under way. At any

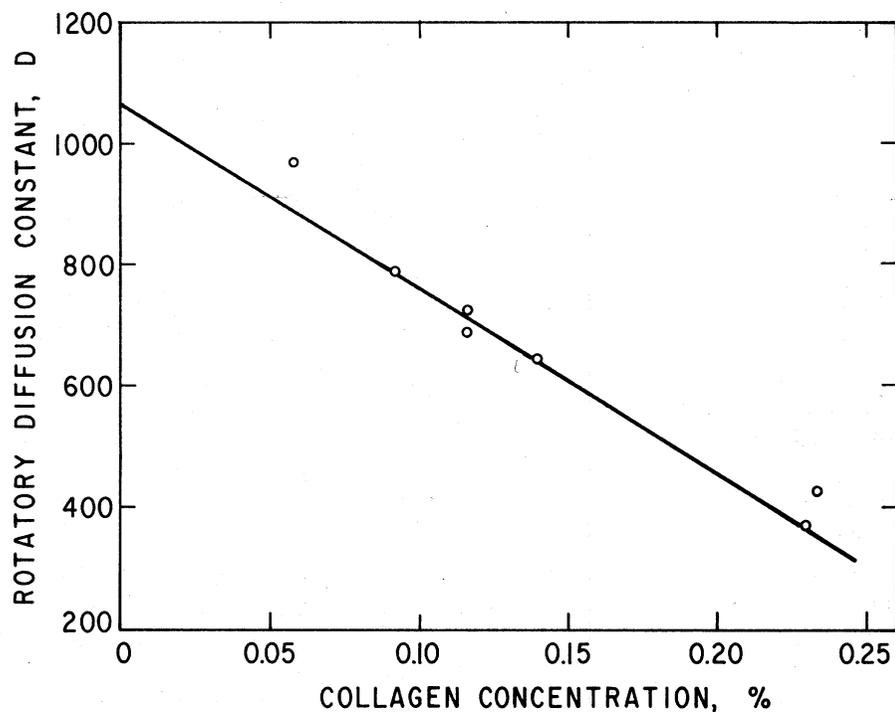


FIGURE 4.—Graph of rotatory diffusion constant versus collagen concentration for solubilized collagen in region where the Relative Birefringence vs. Decay Time plot is a straight line.

rate, at concentrations of 0.233 percent and less there is now added evidence that solubilized collagen preparations of this type are monodisperse. Future studies will apply electric birefringence to the investigation of electrical and optical properties of collagen.

REFERENCES

1. Benoit, H. *Ann. Phys.*, **6**, 561 (1951).
2. Tinoco, I., Jr. *JACS*, **77**, 4486 (1955).
3. O'Konski, C. T., Yoshioka, K., and Orttung, W. H. *J. Phys. Chem.*, **63**, 1558 (1959).
4. Burgers, J. M. *Verhandel Koninkl. Ned. Akad. Wetenschap., Afdel Natuurk., Sect. I*, **16**, No. 4, 113 (1938).
5. Yoshioka, K., and O'Konski, C. T. *Biopolymers*, **4**, 499 (1966).
6. Kahn, L. D., and Witnauer, L. P. *J. Appl. Polymer Sci.*, in press.

DISCUSSION

MR. MEO: The discussion leader for this paper will be James Cassel of the National Bureau of Standards.

DR. CASSEL: This is a tremendous presentation of a most difficult problem. It's the type of presentation and the type of material which one expects to hear if he's fortunate enough to go to Gordon research conferences given on proteins. I want to personally congratulate Dr. Kahn and Dr. Witnauer on a real fine bit of work.

Dr. Kahn, you can also get at this same sort of problem with flow birefringence measurements. You mentioned dielectric measurements. There, the problem is that you've got so much water in the system that you're swamped out. But from what I know, I think you could get somewhat the same information, but would you care to comment on that?

DR. KAHN: Yes, but you cannot get some information by flow birefringence that you can get by electric birefringence, and that is the electric susceptibility of the collagen molecule. Electric susceptibility looks very promising for studying things like denaturation and ion-binding, because a very slight change in a molecule, or the binding of a very small number of small ions, would have a tremendous effect on its electric susceptibility.

According to Benoit's mathematics, or O'Konsky's mathematics, the shape of the build-up curve is very much dependent upon electric susceptibility, and since this would help us to study things like ion-binding and denaturation it does go beyond flow birefringence. It would also make it possible for us to study some other things such as traces of gelatins and low polymers in the presence of a large concentration of monomer.

DR. CASSEL: I do gather from this, though, that you're a firm believer in the fact that one is able to obtain a more disperse collagen system without going through an enzyme treatment to do so. Is that correct?

DR. KAHN: No, I'm not so sure about that. In fact, I feel that all these mono-disperse (quotes around the "mono-disperse") systems are anything but that. Now, talking off the cuff with no evidence to back it up, I strongly feel that in all these "mono-disperse" systems we do have traces of gelatins: the alphas, the betas, and the gammas. Now, of course, collagen gelatinizes at a definite temperature, but I feel that even below that temperature we still have traces of gelatin, and we also have traces of low order polymers, such as dimer, trimer, tetramer and so on, and this suspicion is one of the things that led me into this particular problem.

DR. CASSEL: There has been a long controversy over the fact that at the ends of this unique molecule that is the basis of leather making, there may be certain telopeptide groups or portions which are not in this helical structure. You gave us a rotational diffusion constant which was similar to what O'Konsky derived, and I looked up O'Konsky's paper, and using the Burgers-Brersma equation for cylindrical particles he calculated a length for the rod-like monomer of

around 2700 or 2800 angstroms. Does this mean that the telopeptide ends or the non-helical ends, therefore, don't really have any influence on the behavior of this rod-like molecule, or might it indicate that they weren't there in the first place?

DR. KAHN: Well, the telopeptides probably would not show up as modifying the dimensions of the collagen molecule, but they might carry electric charges, in which case, if they were present, evidence of their existence could be shown through the build-up portion of the electric birefringence curve. One of the beauties of electric birefringence is that there's so much information that you can get from it. If there's any slight modification of the electrical condition of the molecule, it's going to have a very profound effect on the build-up curve.

DR. CASSEL: Are there any comments or questions from the audience?

DR. ROSS DONOVAN (Canada Packers Limited): I believe you can calculate axial ratios from these measurements, can you not? I'm just wondering if there's any evidence whether the polymerization is end-to-end or side-to-side?

DR. KAHN: That is something that I haven't gotten around to as yet. I've only been at this about a year now. It took me about nine months just to get my apparatus together.

DR. DONOVAN: I think that's very good.

DR. CASSEL: With the tremendous advantage of this equipment and so forth, I just want to caution everybody not to rush out and pick up a pulse generator and amplifier which can be used in this system, because Dr. Kahn built his own, and he did this because he couldn't get the one which it was necessary to use.

Are there any other comments or questions from the audience? I'd like to thank Dr. Kahn for a very fine presentation and thank his co-author as well. Thank you, gentlemen.
