ELECTRIC BIREFRINGENCE IN SOLUTIONS OF HIGH MOLECULAR WEIGHT RIBONUCLEIC ACID

E. I. GOLUB *and* V. G. NAZARENKO

From the Institute of Biophysics, Academy of Sciences of the USSR, Moscow

ABSTRACT The electric birefringence of low ionic strength solutions of high molecular weight ribonucleic acids from various sources was studied. RNA preparations with a high helical content were found to have negative electric birefringence as a result of the segment anisotropy of the helical portions of the RNA molecule. For completely unfolded molecules of RNA, the electric birefringence is positive and results from the orientation of the macromolecular coil. In intermediate cases, the observed electric birefringence is the sum of negative and positive birefringence. The negative birefringence is caused by the segment orientation of helical sections, and the positive birefringence is caused by the orientation of the macromolecular coil as a whole. Different relaxation times for the positive and negative birefringence permit the pulsed electric birefringence method to analyze these separate phenomena.

INTRODUCTION

Recent studies of the birefringence of macromolecular solutions in pulsed electric fields give valuable information on the geometric, electric, and optical parameters of the molecules. For rigid particles this method may provide data on the values of the rotary diffusion coefficients and dipole moments and on the anisotropy of electric and optical polarizability (Benoit, 1951; Tolstoi and Feofilov, 1949; Tinoko, 1955; Tinoko and Yamaoka, 1959; O'Konski, Yoshioka and Orttung, 1959).

The complicated character of the phenomena occurring during flexible macromolecular chain orientation in an electric field makes these phenomena difficult to interpret. Nevertheless, observation of birefringence as a method for revealing the structure and properties of chain macromolecules seems to be very promising. Indeed, it has been shown that in DNA solutions, electric birefringence and the character of the relaxation phenomena in an electric field are determined by the rigidity of the macromolecular chains and can be used as a sensitive criterion of structural change (Golub, Dvorkin, and Nazarenko, 1963; Golub, 1964).

This paper examines some experimental studies of electric birefringence of high molecular weight RNA solutions in pulsed electric fields. Our method permits us to separate the effects of orienting helical sections of a macromolecule and of orient-

ing the entire macromolecular coil. Consequently, it allows us to draw some conclusions on the character of changes in macromolecular structure of the nucleic acid molecules as the result of various treatments.

EXPERIMENTAL PROCEDURE

The solutions used were: Tobacco mosaic virus RNA (I, II),[1] ribosomal RNA from *Escherichia coli* (III, IV), and RNA from extractions of ascites hepatoma (V, VI), rat liver (VII, VIII), and calf thymus (IX). All RNA preparations were isolated by the phenol extraction method (Kirby, 1956, 1957) with the use of 0.01 M EDTA. The *E. coli* RNA was isolated from ribosomes that had previously been separated by centrifugation. The original properties of preparations I–IV were established by measuring sedimentation constants and the temperature dependence of reduced viscosity (Spirin, 1963). For preparation V, only the sedimentation constants were measured. All the samples analyzed were composed of native high-molecular weight RNA.

Electric birefringence was measured in the apparatus described by Dvorkin and Golub (1963). Single rectangular pulses 50–2000 μsec long and with field strengths up to 8.5 kv/cm were used for orientation. The rise and decay times of the pulses were no more than 5 μsec. The time constant of the detecting system varied between 5 and 500 μsec. Hollow electrodes of stainless steel, 25 mm long, were used. The solution was thermostated by passing constant-temperature water through the electrodes, or in some cases was measured at room temperature without thermostating. Optical birefringence measurements were made in the visible region of the spectrum (effective wavelength in vacuum $\lambda^{\circ}_{eff} = 450$ mμ) by measuring the phase difference (δ) of the light wave vector component polarized along the field and normal to it that arises upon the application of an orienting electric field. The birefringence, Δn ($= \delta\lambda^{\circ}/2\pi l$), and the electric field strength, E, were measured to an accuracy of no less than 5%.

The RNA concentrations were between 0.01 and 0.04% by weight. Distilled or deionized water, or a 0.001M solution of NaCl was used as the solvent. RNA concentration was determined by measuring optical density of the solutions in the ultraviolet (wavelengths 270 and 290 mμ) (Spirin, 1958).

RESULTS

Fig. 1 is a typical photograph of a photoelectric current pulse that appears when a rectangular orienting pulse is applied to an RNA solution. The observed phase difference, δ, is seen to follow the equation:

$$\delta = \delta_p[1 - \exp(-t/\tau_p)] - \delta_n[1 - \exp(-t/\tau_n)] \qquad (1a)$$

after the initial rising part of the rectangular pulse and

$$\delta = \delta_p \cdot \exp(-t/\tau_p) - \delta_n \cdot \exp(-t/\tau_n) \qquad (1b)$$

after the final decreasing part of the pulse. There often appear to be several values for τ_p and for τ_n when these curves are carefully analyzed (with a series of terms of the type used in equation (1*a*) and (1*b*)). The phase differences are functions of the

[1] The various preparations are numbered with Roman numerals.

square of the field voltage E (Fig. 2). Here δ_p and τ_p represent the phase difference and relaxation time of a positive effect ($\Delta n > 0$), and δ_n and τ_n represent these parameters for a negative effect ($\Delta n < 0$). The relaxation time of the positive signal, τ_p, usually has a magnitude of the order of hundreds of microseconds, while τ_n usually has a smaller magnitude. The observed signal accordingly usually shows a short initial phase of negative magnitude, followed by a rapid decay on the rising edge of the orienting pulse and a short initial phase of positive magnitude on the declining edge of the orienting pulse.

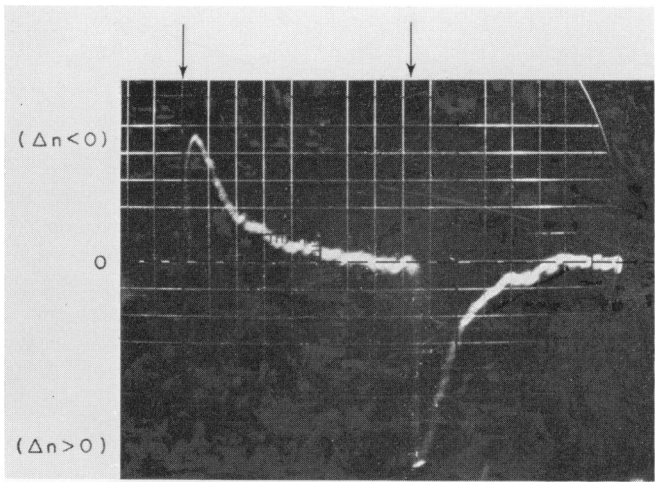

FIGURE 1 Photoelectric pulse resulting from the application of a rectangular orienting pulse to a solution of RNA from *E. coli* (preparation IV) in water. RNA concentration, 0.04%; electric field strength, 2.5 kv/cm; pulse duration, 250 μsec. The analyzer is displaced from the crossed position by 1°, and a quarter-wave plate is inserted between the cell and the analyzer (Dvorkin and Golub, 1963). The signal above the dashed line corresponds to negative birefringence and the one below to positive birefringence. The beginning and the end of the orienting pulse are indicated by arrows. One horizontal scale division is 30 μsec.

The magnitudes of the positive and the negative phase difference, δ_p and δ_n, depend upon the temperature, the ionic strength, and the concentration of bivalent cations. These are the conditions that specify the helical content and the configuration of a nucleic acid macromolecule. The factors that favor the nonhelical form of RNA molecules (increase of temperature, removal of Mg^{++}) tend to decrease δ_n and τ_n and to increase δ_p. Conversely, factors promoting the helical form (decrease of temperature, increase of Mg^{++}) cause an increase of δ_n and a decrease of δ_p. Thus, preparations III, IV, and VII gave only negative signals at room temperature (Figs. 3 and 4), and these preparations were thought to be primarily in the helical form.

The absence of positive or negative signals means that the absolute magnitude of

δ is less than 0.01°/cm at a field E equal to 8.5 kv/cm. A field E of 8.5 kv/cm is the maximum strength of the orienting fields used in this work. Negative phase differences decreased when solutions were heated and positive phase differences appeared (Table I). At sufficiently high temperatures the preparations studied gave only positive phase differences. Preparations that gave finite values for both

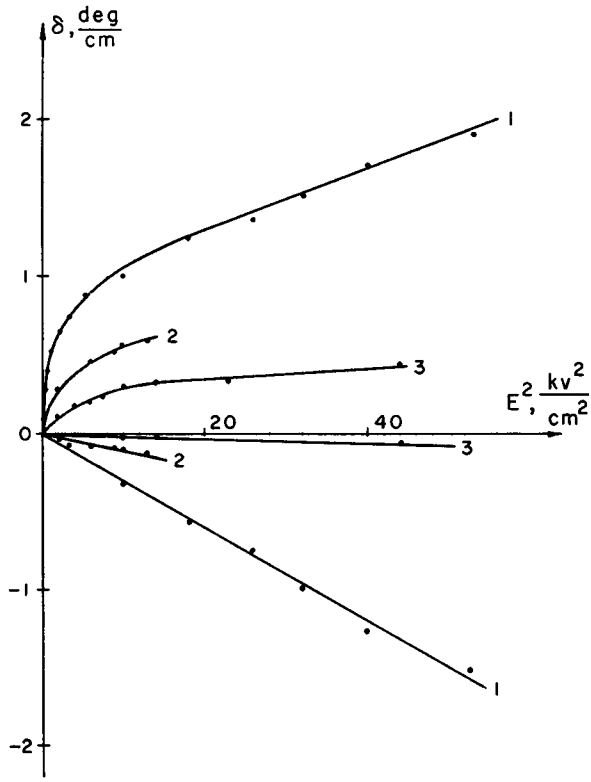

FIGURE 2 Plot of the phase difference δ vs. the square of the field strength E^2 for RNA solutions in water. Concentration, 0.01%. Curve 1 for rat liver RNA (preparation VIII). Curve 2 for calf thymus RNA (preparation IX). Curve 3 for TMV-RNA (preparation I) with the RNA precipitated from 0.01 M EDTA solution.

δ_p and δ_n at room temperature show a decrease of δ_n and an increase of δ_p upon heating of the solutions. The δ_n values usually become nearly zero between 40° and 50°C-(preparations I and V in Table I).

When RNA is reprecipitated from 0.01 to 0.005 M solutions of EDTA (for removal of bivalent cations), a purely positive effect ($\delta_n < 0.01$°/cm) is observed at temperatures considerably lower than those for preparations not so precipitated (Table I). In some cases the negative effect is absent even at room temperature (Figs. 5 and 6), but even these solutions show an increase of δ_p with heating (II*a*

and II*b* in Table I). In all cases, both δ_p and δ_n become vanishingly small after incubation of the preparations of RNA and RNAse.

The change of sign of the electric birefringence when RNA molecules are converted to the nonhelical form is similar to the temperature induced changes of sign of the electric birefringence in DNA solutions. The solutions of native DNA have negative birefringence (Golub, 1964). In a temperature range corresponding to "melting" of the molecular helix of DNA (70–80°C), the negative birefringence falls abruptly. At the same time relaxation times τ decrease to values that the experimental apparatus cannot register (τ less than 5 μsec). This is followed by positive birefringence and relaxation times of the order of milliseconds. When the solutions are then cooled to room temperature, the birefringence becomes negative again. If

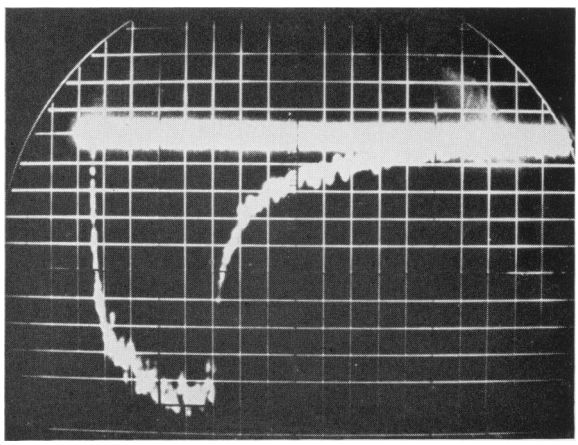

FIGURE 3 Photoelectric pulse resulting from the application of a rectangular orienting pulse to an aqueous solution of RNA from *E. coli* (preparation III). Concentration, 0.04%; electric field strength, 0.8 kv/cm. Prisms are crossed. One horizontal scale division is 160 μsec.

DNA is heated in a 1% solution of formaldehyde, which is known to prevent renaturation, and then cooled, the positive birefringence is preserved.

It is natural to suppose that RNA preparations yielding pure negative effects contain a large number of Mg^{++}, so that even at very low ionic strength the number of helical molecules is high. Indeed, preparation III*a* in water, when heated from 25–80°C, showed a hyperchromic effect of 17% at $\lambda = 260$ mμ. According to Doty et al. (1959), this is characteristic of ribosomal RNA preparations with the highest content of helical molecules. The same preparation at the same wavelength showed no decrease in absorption on increasing the NaCl concentration to 0.1 M. Further, when preparation VI was precipitated from 0.01 M EDTA, the ratio of positive to negative birefringence increased, and when it was precipitated from 0.01% solution of $MgCl_2$, it showed pure negative birefringence at room temperature.

Thus it appears that the negative part of the electric birefringence of the RNA solutions depends on the presence of helical sections in the RNA molecules. On the other hand, the completely unfolded polynucleotide chain has positive electric birefringence. The negative birefringence of RNA solutions appears to be caused by segment anisotropy of helical sections of the molecule, as had been shown for DNA by Dvorkin and Golub (1963).

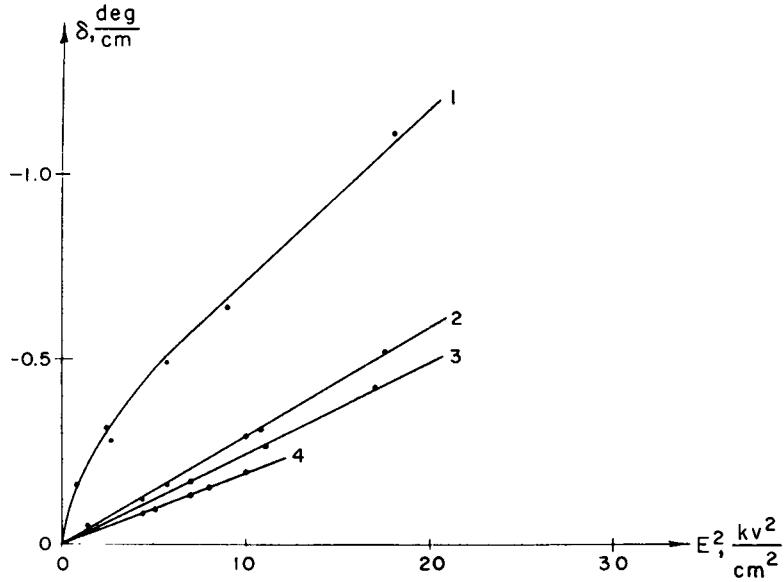

FIGURE 4 Plot of the phase difference δ vs. the square of the field strength E^2 for RNA solutions. Concentration, 0.01%. Curve 1 for *E. coli* RNA in water (preparation III). Curve 2 for rat liver RNA in water (preparation VII). Curve 3 for *E. coli* RNA in water (preparation III) with the RNA precipitated from 0.005 M EDTA solution. Curve 4 is for rat liver RNA in 0.0014 M NaCl (preparation VII).

From the theory of dielectric relaxation of chain macromolecules in solution (Kuhn, 1950; van Beek and Hermans, 1957), it follows that the relaxation time of the macromolecules in an electric field is determined by the rates of coil orientation and segment orientation.

1. Coil orientation is the orientation of the entire macromolecule as a rigid unit. This would occur only for a rigid coil of nucleic acid. The rate of coil orientation is determined by the rotary diffusion coefficient of the coil, which in turn is determined by the anisotropy of optical polarizability and by the shape of the coil.

2. Segment orientation is the deformation of the macromolecule, i.e., the orientation of its separate sections. Its rate is determined by the local properties of the macromolecules, e.g., subchain parameters. Segment orientation values may be calculated approximately (Stuart and Peterlin, 1950) from the concept of a "gas of segments," where the word *segments* refers to Kuhn's statistical chain elements (Kuhn, 1934; Kuhn and Grün, 1942).

The positive birefringence observed in solutions of completely nonhelical RNA (and DNA) molecules is ascribable to coil orientation. The relaxation times found in such solutions exceed by some orders of magnitude those due to the segment orientation observed in solutions of flexible-chain macromolecules (de Brouskère

TABLE I

EFFECT OF HEATING ON THE ELECTRIC BIREFRINGENCE OF RNA SOLUTIONS IN WATER*

Preparation No.		I	II*a*	II*b*	III*a*‡	III*c*‡	V
Source		TMV§	TMV§	TMV§	*E. coli*	*E. coli*	Ascites hepatoma
RNA concn., (%)		0.010	0.017	0.027	0.014	0.020	0.010
Electric field strength (kv/cm)		7.2	4.4	3.9	3.4	3.8	2.6
Phase difference, δ, (degrees/cm) = $f(T)$							
Temperature, T =	25°C	−0.13‖ 0.41	0.77	0.55	−2.4	−0.52	−0.3‖ 1.0
	30°					−0.35	
	33°			0.59		−0.29	
	35°	−0.04‖ 0.45				(−)¶	
	38°		0.79	0.65			
	43°			0.67	−2.2		
	45°	0.47	0.92			0.29	
	50°	0.48		0.69	−2.0	0.52	
	55°		0.96		−1.5	0.62	1.4
	62°				(−)¶		
	65°					0.62	
	80°				0.8		
(After cooling)	25°		0.74	0.50		−0.50	

* Because the positive and negative birefringence depend on electric field strength in different ways (Fig. 2), the ratio of these signals depends on the field strength at which the comparison is made. This fact should be taken into account when the data of this table are analyzed.

‡ Experiments were repeated three times with similar results.

§ RNA was precipitated from 0.005–0.01 M EDTA solution.

‖ In these experiments the observed phase difference was resolved into two components, δ_n and δ_p. Other points represent the total phase difference.

¶ The pulse was negative but too small to be measured.

et al., 1955, 1956, 1957). On the other hand, the relaxation times of positive birefringence have the same order of magnitude as those calculated by the Riseman-Kirkwood formulas (1949) for the rotary diffusion coefficient of a macromolecular coil. Also, the shapes of the curves of $\delta = F(E^2)$ (Fig. 6; see also Fig. 2) are contrary to the assumption that the positive signal is the result of segment anisotropy. In fact, deviations from the Kerr law occur in fields smaller than might be explained by a high degree of orientation of segments of a single-stranded chain.

When molecules are made nonhelical by heating, a subsequent decrease in temperature (as noted above) first causes δ_p to decrease and then causes δ_n to increase from its zero value at the high temperature. A simultaneous decrease in the optical density at 260 mμ is evidence that helix formation causes the birefringence changes, i.e., the decrease in the value of the positive birefringence may be explained mainly

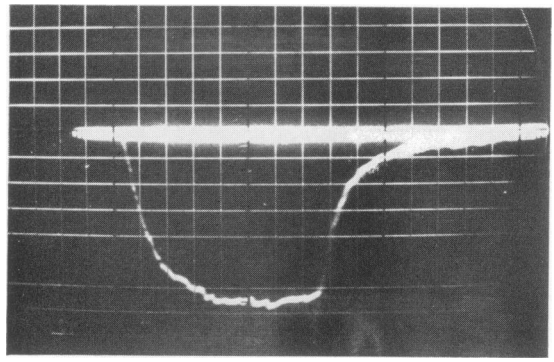

FIGURE 5 Photoelectric pulse resulting from the application of a rectangular orienting pulse to a solution of RNA from TMV (preparation II precipitated from 0.01 M EDTA) in water. Concentration, 0.027%; electric field strength, 8.4 kv/cm. Prisms are crossed. One horizontal scale division is 30 μsec.

by the appearance of helical portions (segments with strong negative anisotropy of optical polarization) in the coil. The relaxation times of the negative birefringence are at least two orders of magnitude smaller than those of the positive signal arising from the orientation process of the macromolecular coil. Therefore it may be supposed that the negative signal is caused by the segment orientation of the helical sections of an RNA molecule and, according to Stuart and Peterlin (1950), the specific Kerr constant for the negative birefringence, K_{sp}, may be expressed as:

$$K_{sp} = (1/C_v n)(\Delta n/E^2)_{E\to 0} = A(g_1 - g_2)(\alpha_1 - \alpha_2) \qquad (2)$$

where C_v is the volume fraction of macromolecules, n is the refractive index of the solution, Δn is the electric birefringence under steady-state conditions, A is a constant independent of macromolecular properties, $(g_1 - g_2)$ is the mean value of the optical anisotropy of the segments per unit of segment volume, and $(\alpha_1 - \alpha_2)$ is

the mean value of electric anisotropy of the segments. Values of K_{sp} are recorded in Table II.

Assuming that the segment length exceeds its diameter considerably, we can write the expression for $(g_1 - g_2)$ in the form

$$g_1 - g_2 = g_o l/(\pi r^2 l) = g_o/(\pi r^2)$$

where g_o is the optical anisotropy per unit of segment length, l is the mean length of the segments, and r is the radius of a segment (helix).

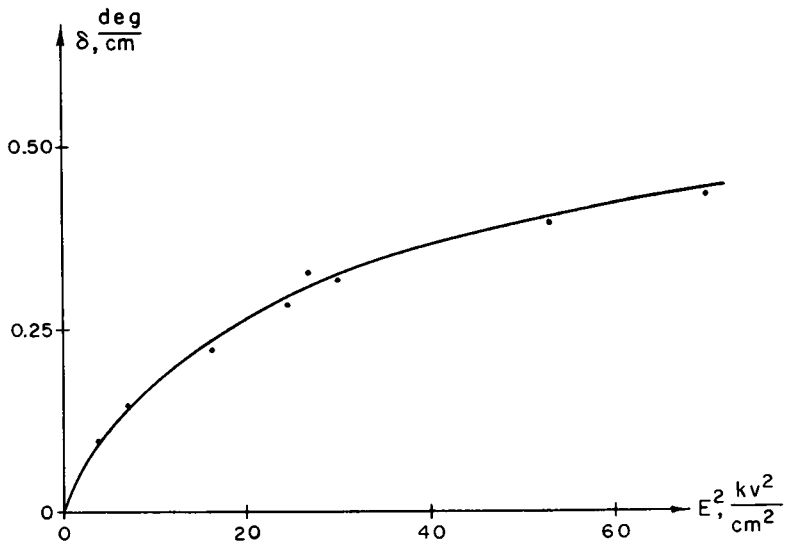

FIGURE 6 Plot of the phase difference δ vs. the square of the field strength E^2 for RNA solutions in water. The RNA is from TMV (preparation II precipitated from 0.01 M EDTA). Concentration, 0.01%.

If we assume that the polarizability of the surrounding ions is the chief factor in the electric polarizability of a segment (O'Konski and Haltner, 1957; O'Konski, 1960; Mandel and Jenard, 1963; Eigen and Schwartz, 1962), then according to Schwarz (1959) and Mandel (1961), the anisotropy of the segment polarizability is proportional to the third power of its length.

In Golub et al. (1963) and Golub (1964), where the birefringence of DNA solutions in an electric field was reported, it was shown that under those experimental conditions segment orientation was the main mechanism of orientation. Consequently, equation (2) may be used to calculate K_{sp} for the electric birefringence of both DNA and RNA solutions. Thus, if we consider that the segments of DNA molecules and helical sections of RNA molecules differ only in size,

$$K_{sp}(\text{DNA})/K^{h}_{sp}(\text{RNA}) = [l(\text{DNA})/l(\text{RNA})]^3 \cdot [r(\text{RNA})/r(\text{DNA})]^2 \qquad (3)$$

where $K^{h}_{sp}(RNA)$ is the specific Kerr constant for RNA molecules with respect to their helical sections, i.e., $K^{h}_{sp}(RNA) = K_{sp}(RNA)/P$, where P is the ratio of nucleotides in helices to the total number.

For the solutions of DNA (three different preparations) in NaCl of 0.001 ionic strength studied in this work,[2] $K_{sp}(DNA) = -0.0007$ to -0.0019 e.s.u. Using the value $K_{sp}(RNA) = -0.0000030$ e.s.u. (in 0.001 M NaCl) (preparation VIII*a* in Table II) and assuming P to be 0.5, we obtain $K_{sp}(DNA)/K^{h}_{sp}(RNA) = 120$–$320$.

Using equation (3) with the above value of the Kerr constant ratio and assuming that $r(RNA) = 14$ A (Rich, 1959) and that $r(DNA) = 10$ A (Wilkins, 1961), we find that the mean segment length of the RNA molecules is 3.9–5.4 times smaller

TABLE II

SPECIFIC KERR CONSTANTS FOR NEGATIVE BIREFRINGENCE OF RNA SOLUTIONS

Temperature, 25°C

Arranged in order of decreasing Kerr constant

Preparation No.	Source	Solvent	K_{sp}* (e.s.u.)
III*b*	*E. coli*	Water	-3.5×10^{-5}
III*a*	*E. coli*	Water	-2.6×10^{-5}
IV*a*	*E. coli*	Water	-9.5×10^{-6}
IV*b*	*E. coli*	Water	-6.1×10^{-6}
III*b*	*E. coli*	0.001 M NaCl	-5.6×10^{-6}
VIII	Rat liver	Water	-4.7×10^{-6}
VII*a*	Rat liver	Water	-4.6×10^{-6}
III*c*	*E. coli*‡	Water	-3.9×10^{-6}
VIII*a*	Rat liver	0.001 M NaCl	-3.0×10^{-6}
IX	Calf thymus	Water	-1.6×10^{-6}
I	TMV§	Water	-1.8×10^{-7}

* The specific Kerr constant, K_{sp}, is estimated to have an accuracy of approximately ±15%.

‡ Preparation precipitated from 0.005 M EDTA solution.

§ Preparation precipitated from 0.01 M EDTA solution.

than the mean segment length of the DNA molecules. Therefore, assuming the length of the statistical segment of a DNA molecule in 0.001 M NaCl to be equal to about 600 A (Ptizin and Fedorov, 1963a; Ptizin and Eizner, 1961; Hearst and Stockmayer, 1962), we find that $l(RNA) = 120$–150 A.

These calculations presuppose the above mentioned third-power relation of segment polarizability to length as the chief means of orientation of double-chain polynucleotide helices in an electric field. Although this third-power relationship is merely supposition and cannot be considered as proved, it seems highly probable.

[2] The properties of bacteriophage T2 and calf thymus DNA used here, and the measuring techniques of electric birefringence in DNA solutions are given in Golub et al. (1963) and in Dvorkin and Golub (1963).

It is interesting to point out the agreement between this result for the statistical segment length of the RNA molecule and the results of a study of 1–4% RNA gels by the small-angle X-ray scattering method (Timasheff et al., 1961). The data obtained by them show that RNA molecules contain rigid rods approximately 120 A long (Ptizin and Fedorov, 1963b) with the electron density per unit length prescribed by the model of a double-stranded helix of the Crick-Watson type.

The analysis of the decay of electric birefringence upon removing the electric field for preparations which produce only a negative phase difference (preparations III and IV) showed that in this case the birefringence decay cannot be described as a relaxation process with a single relaxation time. As previously observed in such experiments (Golub, 1964), the observed relaxation times depended greatly on the electric field and shortened with an increase of field. Thus, for preparation III, the maximum relaxation time τ_{max} (defined from the slope of the long-time end of the curve of $\log \delta = f(t)$) in water was 500 ± 100 μsec at $E = 1.1$ kv/cm and 200 ± 100 μsec at $E = 3.5$ kv/cm.

With increasing ionic strength, the relaxation times shorten sharply as a result of the decrease in rigidity of the macromolecular structure. This decrease occurs when the added cations screen the negative charges of phosphate groups of the main macromolecular chain (Golub, 1964). In 0.001 M NaCl, $\tau_{max} = 30 \pm 10$ μsec ($E = 1.1$ kv/cm, preparation III). Under similar conditions the maximum relaxation time of DNA solutions was 1000 ± 200 μsec (Golub, 1964).

CONCLUSION

The data obtained in the study reported here prove that an RNA molecule contains helical sections that can be independently oriented in an electric field, irrespective of the orientation of the macromolecular coil (that is, the sections are not bound rigidly into the total macromolecular structure). The pulse technique gives us a means of evaluating the role played by the orientation of these sections. Further study is required to increase our knowledge of the length and relative positions of the helical portions in an RNA molecule.

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