Anisotropy Effects in Temperature-Jump Relaxation Studies on Solutions Containing Linear Polymers

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Abstract: The strong transient electric field applied in Joule heating temperature-jump instruments can produce partial orientation of linear macromolecules. In the case of absorption detection, the dichroic effects resulting from this transient anisotropy may complicate the study of the chemical relaxation processes. However, the use of plane polarized light simplifies the analysis of the data, as shown by a simplified theoretical approach. This theory predicts that the orientation dichroism vanishes as the polarization plane is oriented at 55° from the direction of the applied field, thus making it possible to observe pure chemical relaxation signals. The experimental data obtained with proflavine–poly A mixtures are in satisfactory agreement with the theory, provided that corrections are made for the possible depolarization and rotation of light through the entrance window of the temperature-jump cell. The existence of temperature independent isosbestic wavelengths allows one to study the electric dichroism of the oriented solution independently of the chemical relaxation processes. In the case of poly A–proflavine mixtures, the results obtained at these wavelengths provide direct experimental evidence for the existence of at least two structurally different complexes between the dye and the single-stranded neutral form of the polynucleotide. At low polymer to dye ratio ($P/D \sim 1$) most of the bound dye molecules are cooperatively stacked along the linear macromolecule and the solution exhibits positive (parallel) dichroism unlike pure poly A. Moreover the aggregation of the dye molecules along the single-stranded poly A molecule seems to increase the stiffness of the backbone to an extent similar to that of double helix formation in acidic medium. At very large polymer-to-dye ratio ($P/D \sim 130$), most of the dye molecules are bound as monomers and the solution exhibits negative (perpendicular) dichroism as do adenine bases, in agreement with dye–base stacking interactions. These results show that Joule heating temperature-jump instruments can be used to obtain qualitative informations on the structure of macromolecules, and of their complexes with small molecules under ionic strength conditions which are generally too high to allow the use of a conventional electric dichroism apparatus.

The experimental study of fast chemical reactions in solutions requires the use of relaxation techniques[1] whose principle rests upon analyzing the response of the chemical system to a small perturbation of the equilibrium conditions. The perturbation is produced by a transient or a periodic change of one of the equilibrium factors: pressure, temperature, electric field, ... Among the various possible relaxation techniques, the temperature-jump method[2] has been widely used. In this case the transient perturbation is a rapid heating from the initial equilibrium temperature T_i to a new one T_f ($T_f = T_i + \Delta T_0$). Information about the reaction mechanism is obtained from the study of the time dependence of the relaxation phase (*i.e.*, the evolution of the reacting species toward the equilibrium concentrations defined by the final temperature T_f).

In most temperature-jump equipments the fast rise of temperature (ΔT_0) results from Joule heating due to the discharge of a high voltage capacitor between two electrodes immersed into the solution. Although the lifetime of the discharge is generally between 1 and 100 microseconds (depending on the conductivity of the solution), the torque exerted on the molecules by the transient electric field may be large enough to produce partial orientation of those species which have a large permanent or induced dipole moment. This condition is fulfilled by linear polyelectrolytes and, especially, by nucleic acids.[3–5]

The relaxation phase is conveniently studied by monitoring the variations of one of the optical properties of the solution. Most often this property is the transmission of a monochromatic wave whose wavelength belongs to the absorption spectrum of the medium. Linearization of the relaxation equations requires that the changes of the concentrations of the reacting species between the initial and final equilibrium states be small. Accordingly, one has to use very sensitive differential detectors whose resolution can reach 10^{-4}–10^{-3} absorbance units.[6] Since the molecular orientation of polymer solutions can lead to very large dichroic effects,[7] it may be expected that even a small transient anisotropy can yield a transmission change which is comparable to that resulting from the chemical relaxation process.

The superposition of a physical relaxation process (*i.e.*, the orientation decay toward isotropic distribution) to a chemical one may lead to incorrect evaluation of the chemical relaxation times if the contribution of the orientation process is not taken into account. It is therefore important to be able to discriminate between the physical and the chemical parts of the observed signals. The aim of the present paper is to provide an elementary study of this problem. Although most of the considerations will be restricted to a few simple typical cases, it is expected that some conclusions are of general applicability. In particular, it will be suggested that "pure" chemical relaxation signals can be obtained with the use of appropriately plane polarized light.

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Material and Methods

The temperature-jump apparatus and cell are standard equipments purchased from Messanlagen Studiengesellschaft (Göttingen). The nominal capacitance of the high-voltage discharge capacitor is 50 nF. The distance between the cell electrodes is 9.5 ± 0.5 mm. Assuming that the electric field is homogeneous during the discharge, the linear relationship between the capacitor voltage U and the field intensity E is then

$$E\,(\text{kV/cm}) = U\,(\text{kV})/0.95 \simeq U\,(\text{kV})$$

The equipment is used without any modification except for the addition of a linear polarizer (Polaroid Corporation, Type HNP'B, unsupported) mounted in front of the entrance window (quartz Suprasil) of the temperature-jump cell. This window produces a depolarization of the incident light. In order to compare the experimental results with the theoretical predictions (see next section), a quantitative study of the depolarizing effect has been performed on the empty cell. Taking the direction of the electric field as the reference axis ($0z$), the parallel and perpendicular transmitted light intensities have been measured as a function of the angle ψ between the polarizer axis and the reference axis. The corresponding results are shown in Figure 1.

The quality of the polarizer is good between 290 and 500 nm. Below 285 nm, absorption and partial depolarization occur. These defects become worse as the wavelength decreases, so that quantitative studies are no longer possible at the maximum absorption wavelength of polyadenylic acid (poly A) whose orientation properties were investigated in the experimental part of this study. Because a conformational helix–coil equilibrium exists at the temperature of the experiments, the measurements relative to the orientation of the single-stranded poly A molecule have been performed at 280 nm, which is the isosbestic wavelength between the helical and the coiled conformations.

Poly A was purchased as potassium salt from Miles Research Products Division and used without further purification. Proflavine base purified by column chromatography was a gift from Dr. M. Charlier. Unless otherwise stated the experimental conditions are T_i = 11 ± 1°; solvent, 10 mM NaCl, 1 mM Na cacodylate in bidistilled water; pH 6.8. The sedimentation coefficient of the single-stranded (ss) form of poly A was $(s_{20,w})_{ss}$ = 7 S in this solvent. In 10 mM acetate buffer (pH 4.6), the sedimentation coefficient of the double-stranded (ds) form was $(s_{20,w})_{ds}$ = 13 S.

Elementary Theoretical Approach

(1) Light Absorption in the Presence of a Static Electric Field. The orientation of polyelectrolyte macromolecules causes anisotropy of their optical properties. Electric linear dichroism refers to the fact that the extinction coefficient for plane polarized light depends on the angle between the direction of polarization and that of an applied electric field. The complete theoretical analysis of this phenomenon is a complex problem because of the number of factors which must be taken into account:[5] shape and flexibility of the molecules, permanent dipole moment, polarizability, ionic environment, local field effects, polydispersity, ... Several advanced treatments have been reported.[7-10] However, in order to stress the basic phenomenon, a possible simplified approach consists in neglecting the last three factors in the above enumeration. Futher simplification occurs if one considers that the macromolecules behave as cylindrical rigid rods whose dipole moment is parallel to their long axis.

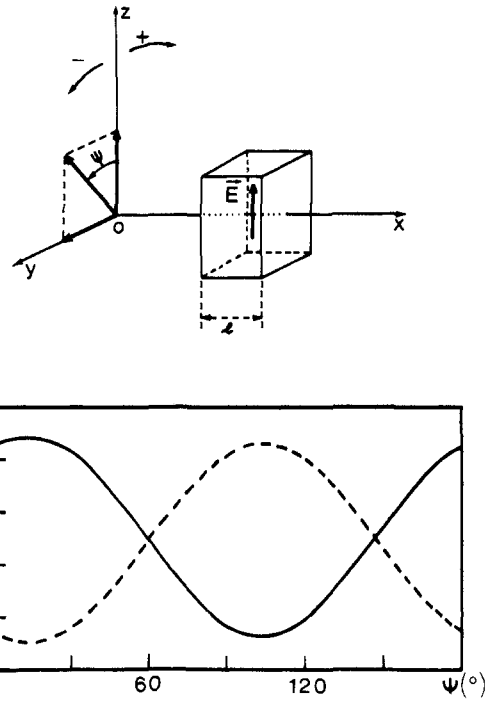

Figure 1. Top: schematic drawing showing how the parallel and perpendicular directions defined in the text are related to the direction of the field and to the polarization angle ψ used in the next sections. Bottom: transmission of a plane polarized wave (λ 436 nm) by the temperature-jump cell used in this work as a function of the polarization angle ψ: (—) $I_{\parallel}/(I_{\parallel} + I_{\perp})$; (- - -) $I_{\perp}/(I_{\parallel} + I_{\perp})$.

For each absorbing species two principal molar extinction coefficients are defined: $\epsilon_{\parallel}$ refers to light polarized parallel to the electric field E (Figure 1); $\epsilon_{\perp}$ refers to light polarized perpendicular to E. It can be shown[7] that these two coefficients are related to the isotropic molar extinction coefficient ϵ measured in the absence of applied field. The relationship between the three quantities is

$$\epsilon_{\parallel} + 2\epsilon_{\perp} = 3\epsilon \tag{1}$$

The reduced linear dichroism ρ is defined as

$$\rho = \frac{\Delta\epsilon}{\epsilon} = \frac{\epsilon_{\parallel} - \epsilon_{\perp}}{\epsilon} \tag{2}$$

and may vary between $-3/2$ and $+3$. The ρ value depends on the effective applied field, the electric properties of the macromolecules (permanent dipole moment μ_p, polarizability α), and the angle ϕ between the transition moment of the chromophore and the dipole moment of the polymer.

It is possible[7] to write ρ as the product

$$\rho = \frac{3}{4}(1 + 3\cos 2\phi)\Phi(E,\mu_p,\alpha) \tag{3}$$

(8) C. T. O'Konski, K. Yoshica, and W. H. Orttung, *J. Phys. Chem.*, **63**, 1558 (1959).
(9) J. Schweitzer and B. R. Jennings, *Biopolymers*, **11**, 1077 (1972).
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$\Phi(E,\mu_p,\alpha)$ is the orientation function defined by O'Konski, *et al.*[8] This quantity increases from zero (for $E = 0$) to unity at infinite field strength. The field dependence of the steady state dichroism at intermediate E values depend on μ_p and α. Experimental study of this dependence may yield useful information on the electric properties of the polymer.[7,11] At low field strength, the orientation function may be approximated by a quadratic law for rigid rods.[7] The occurrence of a quasilinear dependence at low field strength[12] has been assigned to a lack of rigidity of the polymer structure.[5] However, for rigid rods bearing only permanent moment ($\alpha = 0$), the deviation from the quadratic behavior occurs at moderate field strengths and pseudolinearity is observed in a rather extended range (see Figure 3 in ref 11).

The sign of the reduced dichroism ρ depends on the ϕ value (eq 3). Positive (or parallel) dichroism is observed for $\phi < 55°$, negative (or perpendicular) dichroism corresponds to $\phi > 55°$.

Using the following definition relations

$$\rho_{||} = -2\rho_{\perp} = {}^2/_3\rho \tag{4}$$

Eq 1 and 2 yield

$$\epsilon_{||} = \epsilon(1 + \rho_{||}) \tag{5}$$

$$\epsilon_{\perp} = \epsilon(1 + \rho_{\perp}) \tag{6}$$

If the oriented solution is irradiated by incoherent light, the intensity of the transmitted light is

$$I_t = I_{0,||}e^{-2.3\epsilon_{||}cl} + I_{0,\perp}e^{-2.3\epsilon_{\perp}cl} \tag{7}$$

In this equation $I_{0,||}$ and $I_{0,\perp}$ are the parallel and perpendicular intensities of the incident light, respectively, c is the concentration of the chromophore and l is the optical pathlength. Using eq 4, 5, and 6 and assuming small dichroism ($2.3\rho\epsilon cl \ll 1$), the above equation may be replaced by the first-order approximation

$$I_t = I_0e^{-2.3\epsilon cl}\left[1 - \frac{2.3\rho\epsilon cl}{3I_0}(2I_{0,||} - I_{0,\perp})\right] \tag{8}$$

with

$$I_0 = I_{0,||} + I_{0,\perp}$$

The quantity $I_0 \exp(-2.3\epsilon cl)$ is the intensity transmitted in the absence of orientation. The relative variation of the absorbance produced by the building up of the anisotropy is then

$$\frac{\Delta A}{A} = \frac{\rho}{3I_0}(2I_{0,||} - I_{0,\perp}) \tag{9}$$

If the incident light is not polarized, $I_{0,||} = I_{0,\perp} = I_0/2$ and eq 9 reduces to

$$\Delta A/A = \rho/6 \tag{10}$$

If the incident light is plane polarized at an angle ψ from the orientation axis (see Figure 1), eq 9 reads

$$\frac{\Delta A}{A} = \frac{\rho}{6}(1 + 3\cos 2\psi) \tag{11}$$

This equation (as eq 8, 9, and 10) is valid in the case of small dichroism only.

(11) D. W. Ding, R. Rill, and K. E. van Holde, *Biopolymers*, **11**, 2109 (1972).

(12) E. Charney, J. B. Milstien, and K. Yamaoka, *J. Amer. Chem. Soc.*, **92**, 2657 (1970).

The comparison of eq 11 and 3 shows that the ϕ and ψ dependences of $\Delta A/A$ are identical. It can be seen that $\Delta A = 0$ for $\psi = 55°$. In this case the total apparent absorption does not depend on the extent of anisotropy, at least to a first-order approximation and provided that the reduced dichroism is small. Such a result is of considerable interest for temperature-jump relaxation studies as it will be shown in section 3.

(2) Kinetics of the Orientation Processes. The orientation build-up and decay which respectively follow the application and the suppression of the electric field are not instantaneous. The kinetics of these transients depend on many parameters,[4,9] and this complicates the analysis of the time dependence of the electrooptical properties of the solutions. The simplest ideal case is that of a monodisperse solution of perfectly rigid rod-like molecules, for which the orientation kinetics may be described by a simple linear differential equation[3]

$$\frac{d\rho}{dt} = k_{rot}(\rho_s - \rho) = \frac{\rho_s - \rho}{\tau_{rot}} \tag{12}$$

where k_{rot} is the orientation rate constant, $\tau_{rot} = 1/k_{rot}$ is the orientation relaxation time, and ρ_s is the steady state value of the reduced dichroism (obtained from eq 3) which is now time dependent. The orientation rate constant is equal to $6D_{rot}$, D_{rot} being the rotation diffusion constant with respect to a transverse axis of the rod.[3]

In Joule heating temperature-jump instruments the applied electric field obeys an exponential decay law

$$\begin{aligned} E &= 0 && \text{for } t < 0 \\ E &= E_0e^{-k_{dis}t} && \text{for } t \geqslant 0 \end{aligned} \tag{13a}$$

in which $k_{dis} = 1/\tau_{dis}$ is the discharge rate constant equal to $1/RC$ (R = resistance of the solution, C = capacitance of the high-voltage capacitor).

The temperature increases according to the following equation

$$\Delta T = \Delta T_0(1 - e^{-2k_{dis}t}) \tag{13b}$$

and ΔT_0 is proportional to E_0^2. The solution of eq 12 depends on the relationship between ρ_s and E (eq 3), *i.e.*, on the orientation function Φ. For rigid rods a quadratic dependence is observed at low field strength ($\rho_s = \beta E^2$), and the solution of eq 12 is then

$$\rho = \frac{\rho_0}{x - 1}(e^{-k_{rot}t} - e^{-xk_{rot}t}) \tag{14a}$$

in which

$$\left.\begin{aligned} &\rho_0 = \beta E_0^2 \\ &k_h = 2k_{dis} = \text{rate constant of the temperature jump} \\ &x = k_h/k_{rot} = \tau_{rot}/\tau_h \neq 1 \end{aligned}\right\} \tag{15}$$

If $x = 1$, eq 14a must read

$$\rho = \rho_0k_hte^{-k_ht} \tag{14b}$$

In the case of a quasilinear relationship between ρ_s and E ($\rho_s = \gamma E$), and assuming that eq 12 remains valid, the solution is of the same type as eq 14a or 14b except that k_h is replaced by k_{dis} and $\rho_0 = \gamma E_0$.

From eq 14 it follows that the reduced dichroism

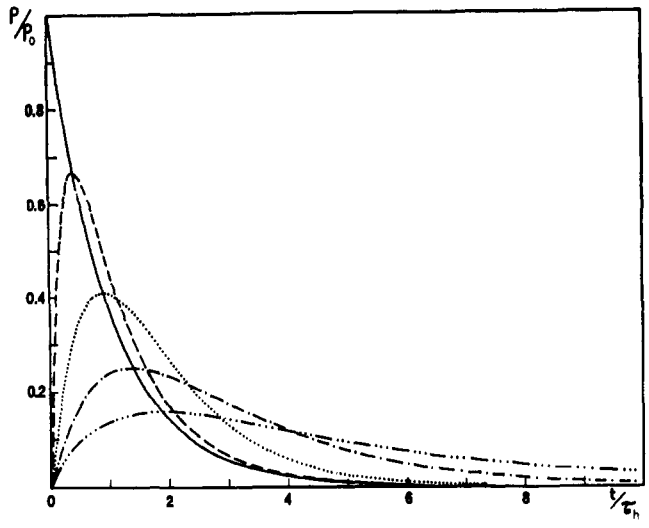

Figure 2. Typical plots of ρ/ρ_0 *vs.* time obtained from eq 14 and the following x values ($x = k_h/k_{rot}$, see text): (—) $x = 0$; (– – –) $x = 0.2$; (· · ·) $x = 0.8$; (—·—) $x = 2$; (· · ·—) $x = 4$.

reaches an extremum value ρ_m at time t_m given by the following equations.

$$\text{for } x \neq 1 \begin{cases} t_m = \tau_{rot}\dfrac{\ln x}{x-1} = \tau_h\dfrac{x \ln x}{x-1} & (16a) \\ \rho_m = \rho_0 x^{x/(1-x)} = \rho_0 e^{-t_m/\tau_h} & (17a) \end{cases}$$

$$\text{for } x = 1 \begin{cases} t_m = \tau_{rot} & (16b) \\ \rho_m = \rho_0/e & (17b) \end{cases}$$

In the case of a quasilinear relationship between ρ_s and E, these equations remain valid provided that $x = k_{dis}/k_{rot}$.

The variations of $\rho(t)$, $t_m(x)$, and $\rho_m(x)$ are plotted in Figures 2 and 3. As x approaches zero, t_m approaches zero and ρ_m approaches ρ_0. Then, for $x \ll 1$ and $t > t_m$ the orientation decay may be represented by the following approximations.

$$\rho \sim \beta E_0^2 e^{-k_h t} \text{ for quadratic field dependence} \quad (18)$$

$$\rho \sim \gamma E_0 e^{-k_{dis} t} \text{ for linear field dependence} \quad (19)$$

On the opposite, the extent of anisotropy is always small for $x \gg 1$ ($\rho_m \sim \rho_0/x$) and the rate limiting parameter for the orientation decay is k_{rot}. For $t \gg t_m$ and $x \gg 1$, eq 14a may be approximated by

$$\rho \sim \frac{\rho_0}{x-1} e^{-k_{rot} t} \quad (20)$$

The physical content of eq 18, 19, and 20 may be intuitively extended to systems which have more than one orientation relaxation time; if all these times are much smaller than the field lifetime, the maximum anisotropy is large and $\rho \sim \rho_s$ as soon as the orientation build-up is achieved; if the orientation relaxation times are much longer than the field lifetime, the anisotropy is always weak and its decay is rate limited by the larger orientation relaxation times.

(3) Orientation Effects in the Presence of Chemical Relaxation. Although a general treatment could be done, the present study will be limited to a simple case which emphasizes the main properties of the phenomenon. Let us consider a chemical equilibrium between a small molecule L and a linear polymer P whose binding sites are equivalent and independent. Assuming only one kind of complex C, the reaction may be described by a single step mechanism

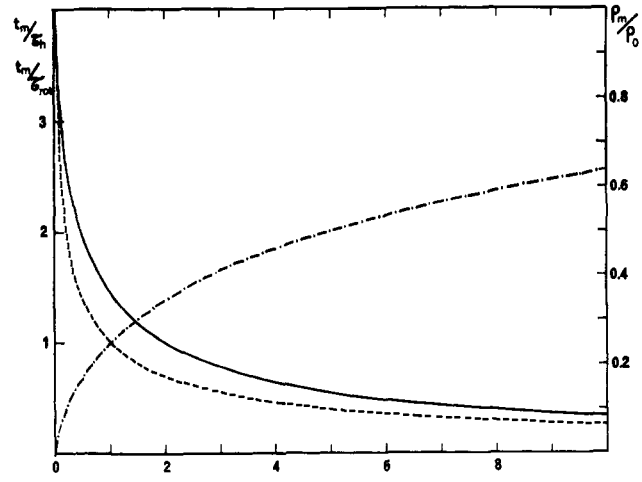

Figure 3. Variation of ρ_m/ρ_0 (——), t_m/τ_h (–·–), and t_m/τ_{rot} (- - -) *vs.* x calculated from eq 14, 16, and 17.

$$P + L \underset{k_D}{\overset{k_R}{\rightleftharpoons}} C \qquad K = k_R/k_D$$

in which the association equilibrium constant K refers to the fixation of one molecule L to one binding site P. If L and C are the only absorbing species, the apparent extinction coefficient is

$$\epsilon_{app} = \epsilon_C \xi + \epsilon_L(1 - \xi) \quad (21)$$

where ϵ_C and ϵ_L are the extinction coefficients of the species indicated by the subscripts, and ξ is the fraction of L molecules which are bound. When a temperature jump is produced by the electric field defined by eq 13a, all the quantities involved in eq 21 become time dependent either because of orientation effects (ϵ_L, ϵ_C) or because of chemical relaxation (ξ). If $\bar{\xi}$, $\bar{\epsilon}_L$, and $\bar{\epsilon}_C$ refer to the time-independent *initial equilibrium* values, each time-dependent parameter (*e.g.*, ξ) may be written as the sum of the initial equilibrium value plus a time-dependent small variation (*e.g.*, $\xi(t) = \bar{\xi} + \delta\xi(t)$).

Setting $\bar{\Delta\epsilon} = \bar{\epsilon}_C - \bar{\epsilon}_L$, eq 21 reads to a first-order approximation

$$\delta\epsilon(t) \simeq \bar{\Delta\epsilon}\delta\xi(t) + \bar{\xi}\delta\epsilon_C(t) \quad (22)$$

In addition to the second-order terms (*e.g.*, $\delta\epsilon_C\delta\xi$), the first-order quantity $\delta\epsilon_L(t)$ may be omitted because the dipole moment of the free L molecule is generally too small to give observable anisotropy effects. On the contrary, $\delta\epsilon_C(t)$ reflects the orientation of the polymer chains if the bound L molecule is rigidly fixed to the binding site. In this case, the time dependence of $\delta\epsilon_C$ will be given by an equation similar to eq 14 in which $\rho = \delta\epsilon_C/\bar{\epsilon}_C$.

In eq 22, the first term of the sum corresponds to the chemical relaxation while the second term corresponds to the orientation relaxation.

For a small perturbation of the chemical equilibrium, $\xi(t)$ is obtained from a linear relaxation equation[1] similar to the orientation equation (eq 12).

$$\frac{d\xi}{dt} = \frac{\bar{\xi}_s(t) - \xi}{\tau_{ch}} \quad (23)$$

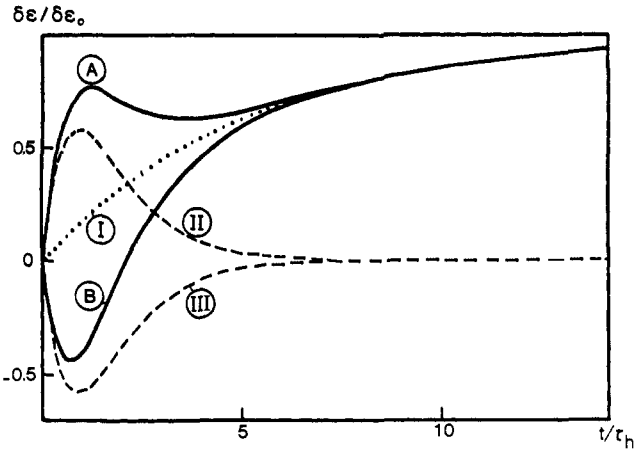

Figure 4. Calculated relaxation signals corresponding to a single-step reaction according to eq 14, 22, and 26, with $\tau_{rot}/\tau_h = 0.8$ and $\tau_{ch}/\tau_h = 5$. $\delta\epsilon_0$ is the variation of the apparent extinction coefficient (eq 21) produced by the temperature jump ΔT_0: (···) chemical contribution I; (- - -) orientation contribution (II positive dichroism, III negative dichroism); (—) total signal (A = I + II; B = I + III).

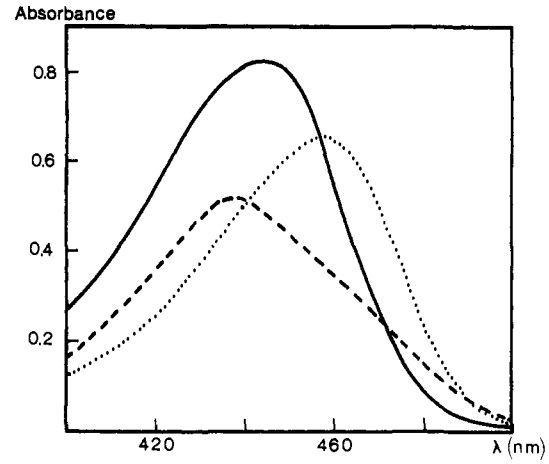

Figure 5. Visible absorption spectra of poly A–proflavine mixtures: (—) $P/D = 0$; (- - -) $P/D = 1$; (···) $P/D = 130$. Total dye concentration = 20 μM, temperature = 11°, 10 mM NaCl, 1 mM Na cacodylate, pH 6.8.

τ_{ch} is the chemical relaxation time and $\bar{\xi}_s(t)$ is the equilibrium value at time t. For the above reaction mechanism the reciprocal chemical relaxation time is[1]

$$\frac{1}{\tau_{ch}} = k_{ch} = k_R([P] + [L]) + k_D \qquad (24)$$

For a small perturbation, the variation of ξ_s and the temperature variation ΔT are proportional to a first-order approximation. Then eq 13b leads to

$$\bar{\xi}_s(t) = \bar{\xi} + \delta\xi_0(1 - e^{-k_h t}) \qquad (25)$$

The solution of eq 23 is then

$$\delta\xi(t) = \delta\xi_0\left[1 - \frac{k_h e^{-k_{ch}t} - k_{ch}e^{-k_h t}}{k_h - k_{ch}}\right] \text{for } k_h \neq k_{ch} \qquad (26)$$

$$\delta\xi(t) = \delta\xi_0[1 - (1 + k_{ch}t)e^{-k_{ch}t}] \text{ for } k_h = k_{ch} \qquad (26a)$$

Under the usual conditions of temperature-jump relaxation studies, τ_h is much smaller than τ_{ch}, so that the temperature jump may be approximated by a step function. In this case, eq 26 can be simplified and reads

$$\delta\xi(t) \simeq \delta\xi_0(1 - e^{-k_{ch}t}) \qquad (27)$$

If $\tau_{ch} \ll \tau_h$, a similar approximation yields

$$\delta\xi(t) \simeq \delta\xi_0(1 - e^{-k_h t}) \qquad (28)$$

Conclusion

To summarize the simplified theoretical approach, let us return to eq 14, 22, and 26 which contain the major results. These equations show how the relaxation signal corresponding to a single-step mechanism is complicated by the presence of orientation effects. At least three time constants (τ_{ch}, τ_{rot}, τ_h) are involved in the total relaxation function which is the sum of the orientation and chemical contributions. As an illustration of these results, Figure 4 shows a typical example corresponding to $\tau_{rot}/\tau_h = 0.8$ and $\tau_{ch}/\tau_h = 5$. If the orientation is very fast as compared to the temperature rise time, then the orientation build-up gives rise to a very fast effect occurring at the early beginning of the discharge. Only two relaxation times (τ_{ch}, τ_h) are needed to account for the measurable remaining part of the signal. In the case of multistep reactions, a higher level of complexity must be expected.

Figures 2 and 3 show that ρ_m/ρ_o increases as x decreases. Then, for a given orientation relaxation time, the amplitude of the orientation dichroism increases as the lifetime of the electric field increases. Since this time decreases as the ionic strength increases, it can be expected that the orientation effects will have a larger amplitude in solutions containing low salt concentrations than in solutions containing high salt concentrations.

Experimental Section

In order to obtain an experimental test of the predictions of the above simplified theory, poly A–proflavine mixtures represent a convenient chemical system. Recent equilibrium and kinetic studies[13,14] have led us to propose the existence of two complexes between this dye and the single-stranded stacked form of the polymer. One of these complexes (complex I) results from cooperative binding of the ligand to the linear polyanionic chain which serves as a template for dye aggregation.[15] The other complex (complex II) involves van der Waals–London stacking interactions between the aromatic rings of one or two consecutive adenine residues and that of a proflavine molecule. At constant ionic strength and temperature, the relative amounts of the two complexes depend on the total dye concentration D and the polymer to dye ratio P/D (where P is the polymer concentration expressed in nucleotide equivalents per liter). For not too high ionic strength conditions (<0.2 M Na) and $D \simeq 20$ μM, complex I is strongly predominant at low P/D values ($0 < P/D < 20$). For P/D values close to unity, the polymer binding sites are nearly all saturated by long stacks of bound dye molecules and the sedimentation coefficient of the complex is very similar to that of the double-stranded acidic form of poly A ($s_{20,w} = 13$ S). The visible absorption spectrum of the proflavine molecule belonging to one of these stacks has an isosbestic point at 472 nm with that of the free dye (see Figure 5).

As P/D increases the fraction of complex I decreases while the fraction of complex II increases. For $P/D > 100$, complex II is largely predominant and the visible absorption spectrum is very similar to that of the intercalation complex between DNA and proflavine (Figure 5) with an isosbestic point at 457 nm.

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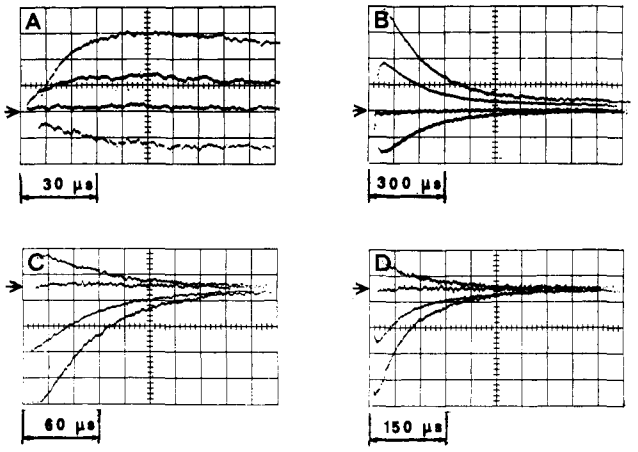

Figure 6. Orientation signals at constant charge voltage (18 kV) and various positions of the polarizer in the case of poly A–proflavine mixtures (same conditions as in Figure 5). Vertical sensitivity, 0.005 absorbance unit per division. The arrows indicate the position of the base line before the temperature jump: (A and B) $P/D = 1$, λ_i 472 nm, top to bottom $\psi = 0°$, no polarizer, $\psi = 70°$, $\psi = 90°$; (C and D) $P/D = 130$, λ_i 457 nm, top to bottom $\psi = 90°$; $\psi = 70°$; no polarizer; $\psi = 0°$.

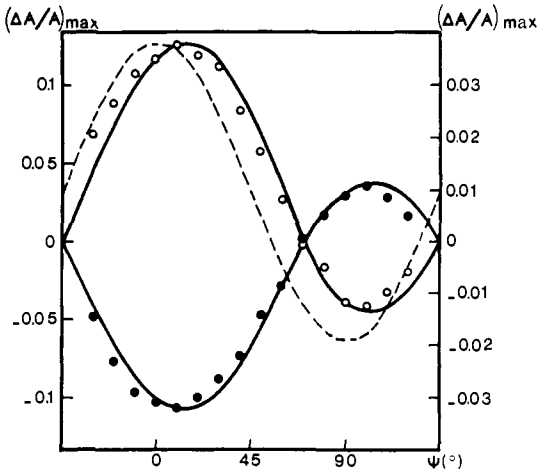

Figure 7. Variation of $(\Delta A/A)_{max}$ as a function of ψ: (○ and left vertical scale) $P/D = 1$, λ_i 472 nm; (● and right vertical scale) $P/D = 130$, λ_i 457 nm. (—) theoretical curves calculated from eq 9 and Figure 1 (correction for depolarization and rotation of the temperature-jump cell window) with the assumption that the measured and calculated values are equal for $\psi = 10°$. (- - -) theoretical curve calculated from eq 11 for $P/D = 1$ and assuming that the experimental value obtained at $\psi = 10°$ is equal to the theoretical value at $\psi = 0°$.

In order to emphasize the orientation effect the experiments have been performed under low ionic strength conditions (~10 m*M* Na^+, corresponding to a cell resistance of 1700 ohms).

(1) Study of the Orientation Effect. To observe pure orientation signals there must exist an isosbestic wavelength λ_i between the absorption spectrum of the free dye and that of the poly A–proflavine mixture. Since the two complexes have different λ_i, the experimental conditions must be adjusted to obtain a negligible amount of one of the complexes. This can be achieved either at low P/D ($P/D = 1$; λ_i 472 nm) or at very large P/D ($P/D = 130$ λ_i 457 nm).

In both cases, the shape of the signal obtained under plane polarization conditions (Figure 6) is in very good agreement with the theoretical prediction (eq 14 and Figure 2). Absorbance variations depend on both electric field strength and polarization angle.

(a) **Angular Dependence.** At a constant charge voltage of the capacitor, the angular dependence of the signal has been analyzed by plotting the relative absorbance change (measured at its extremum value $(\Delta A/A)_{max}$) *vs.* the polarization angle ψ measured at the front of the entrance window of the temperature-jump cell (see Figure 1). As shown in Figure 7 (dashed line), the experimental results are not in agreement with the predictions of eq 11. In particular, the experimental value of the angle at which the orientation signal vanishes is found close to 70° while the theoretical value derived from eq 11 is 55°. This discrepancy arises from the fact that incident light is partly depolarized during its passage through the window of the temperature-jump cell (see Material and Methods). Then, the general relation corresponding to eq 9 must be used (eq 11 is only valid for plane polarized light). After having checked that the solution has no appreciable effect on the polarization properties of the incident light (at least under our experimental conditions), the $I_{0,||}/I_0$ and $I_{0,\perp}/I_0$ values have been taken equal to those measured in the study of the optical properties of the cell (see Material and Methods and Figure 1). The calculated curves obtained from these values and eq 9 are in satisfactory agreement with the experimental results (Figure 7, solid lines). Therefore, it can be concluded that the observed angular dependence is consistent with the simplified theory.

It is very important to note that an orientation signal is obtained even in the absence of a polarizer in front of the temperature-jump cell (Figure 6): the $(\Delta A/A)_{max}$ values obtained under this condition are +0.06 and −0.016 for $P/D = 1$ and $P/D = 130$, respectively. When the polarizer is removed, the light emerging from the monochromator is partly polarized and $I_{0,||} = 1.5I_{0,\perp}$. Using this relation, the theoretical values computed from eq 9 are +0.065 and −0.015 for $P/D = 1$ and $P/D = 130$, respectively. They agree very well with the experimental results. This remark demonstrates that orientation effects can be nonnegligible under the usual conditions of temperature-jump relaxation studies.

From Figure 6 and eq 9 it can be concluded that the orientation signals obtained at $P/D = 1$ correspond to positive linear dichroism, while those obtained at $P/D = 130$ correspond to negative linear dichroism. Since the dipole moment of the polymer molecule is in the direction of the long axis, it can be inferred that the mean value of the angle ϕ between the transition moment of the bound dye molecules and the polymer axis is smaller than 55° in the first case ($P/D = 1$) and larger than this value in the second one ($P/D = 130$). These results provide direct experimental evidence for the existence of at least two different possible positions of the bound proflavine molecule, thus confirming the conclusions which have been drawn from previous studies.[13,14] Furthermore, the fact that $\phi > 55°$ in complex I indicates that the molecular plane of the bound dye belonging to a stack is significantly inclined toward the polymer axis. This is a direct experimental confirmation of recent ideas concerning the structure of the aggregation complexes between proflavine and various linear polyelectrolytes.[16] The present experimental data do not allow a quantitative evaluation of the tilting angle because complete orientation of the polymer molecules is not achieved (see next section) and because the orientation kinetics of pure poly A differs strongly from that of the complex (see also next section).

At large P/D value, the sign of the reduced dichroism is changed. This result shows that the direction of the visible transition moment of the bound molecule is less inclined toward the polymer axis in complex II than in complex I. However, this is not sufficient to conclude about the orientation of the molecular plane of the dye molecule in complex II. In order to get more insight into this problem, it would be necessary to determine the orientation with respect to the polymer axis of the long-wavelength uv transition moment which is perpendicular to the visible one.[17] Unfortunately, this is not possible because the uv absorption is mainly that of the polymer at large P/D value. Nevertheless, the negative dichroism observed in the visible range and the absorption spectrum of complex II are both qualitatively consistent with the hypothesis of either a sandwich complex between two consecutive adenine residues or dye stacking onto only one of them.

At relatively low P/D values ($P/D \leq 10$), Acridine Orange–poly A complexes exhibit parallel electric dichroism[18] while perpendicular dichroism ($\phi > 55°$) is obtained with DNA under similar conditions.[18] The finding of both types of dichroism in poly A–proflavine interactions suggests that perpendicular dichroism could also be expected with Acridine Orange–poly A solutions provided that the polymer concentration is large enough to prevent the forma-

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(18) D. F. Bradley, N. C. Stellwagen, C. T. O'Konski, and C. M. Paulson, *Biopolymers*, **11**, 645 (1972).

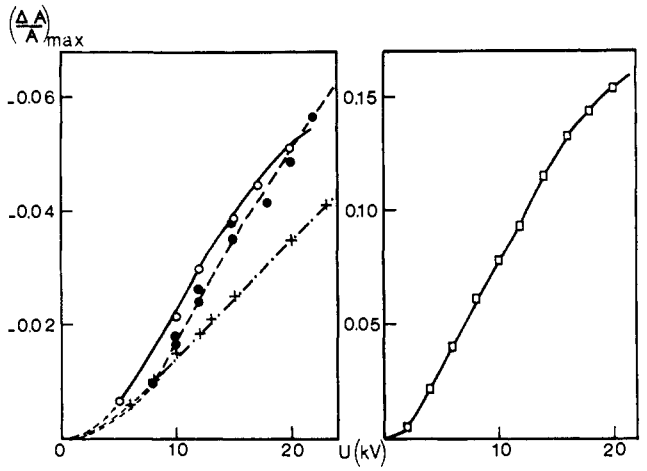

Figure 8. Field strength dependence of the orientation signal (measured at its extreme value and for $\psi = 0°$). Initial temperature 11°, ionic strength 11 mM Na^+: (○ and (—)) poly A, pH 4.2 (acetate buffer), λ 260 nm; (● and (– – –)) poly A, pH 6.8 (cacodylate buffer), λ 280 nm; (+ and (–·–)) poly A–proflavine (P/D = 130), pH 6.8, λ 457 nm; (□ and (—)) poly A–proflavine (P/D = 1), pH 6.8, λ 472 nm.

tion of long stacks of bound dye molecules[15] and that the binding equilibrium constant relative to complex II is significantly larger than the nucleation binding constant relative to complex I.[13] Since the cooperativity of the electrostatic binding is generally stronger with Acridine Orange than with proflavine,[15] it may be expected that the inversion of the dichroism occurs at larger P/D for Acridine Orange than for proflavine (with the same D value in both cases). In the case of DNA, recent data[19] have also shown that the reduced dichroism of bound Acridine Orange becomes less negative as the number r of bound molecules per nucleotide increases.

(b) Field Dependence and Orientation Kinetics. The field strength dependence of the orientation effect has been studied by plotting $(\Delta A/A)_{max}$ as a function of the charge voltage of the capacitor and for light polarized parallel to the electric field. For P/D = 1 and 130, the observed dependence is not quadratic but rather approximately linear except at very low U values where accurate measurements are no longer possible (Figure 8). The results suggest that poly A molecules do not behave as rigid rods and are significantly longer than their persistence length.[5] Since no plateau is reached at the larger U values, it may be concluded that only partial orientation occurs. Complete parallel orientation of the macromolecules would probably require that the field strength be higher and that the electric field be applied for a longer period of time.

The field dependence observed in the visible range in the presence of proflavine is similar to that obtained in the ultraviolet without added dye (see Figure 8). The poly A solutions exhibit negative dichroism either at pH 6.8 (single-stranded form) or at pH 4.2 (double-stranded form). The reduced dichroism measured at maximum orientation is *apparently* very similar for both conformations. However, this point requires further confirmation because the quality of the polarizer used in this study becomes poorer and poorer as the wavelength decreases below 285 nm. Moreover, the apparent similarity of the extreme $\Delta A/A$ values does not imply that the angle ϕ between the base planes and the polymer long axis is identical for both conformations because, at constant U and ψ values, $(\Delta A/A)_{max}$ depends not only on ϕ (eq 3) but also on the kinetics of the molecular orientation (see Figure 2).

Figure 9 shows that the orientation of the single-stranded form is faster than that of the double-stranded structure. The analysis of the time dependence of the signal at constant U and ψ values leads to the following remarks.

(i) For single-stranded stacked poly A, the apparent rate constant for orientation decay is equal to the rate constant (k_{dis}) for the decay of the applied field (see Table I). Provided that $x \ll 1$, this result is fully consistent with the quasilinear field dependence of the orientation maximum (eq 19). A quadratic field dependence

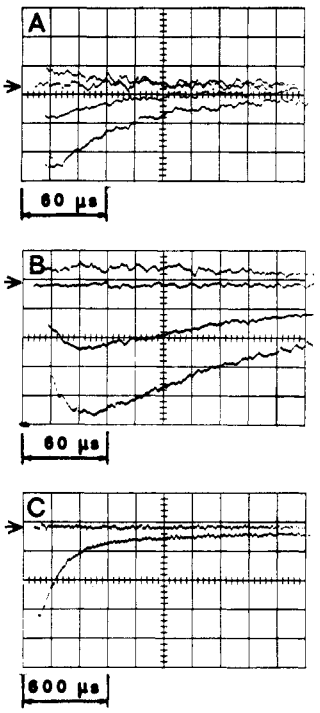

Figure 9. Typical orientation signals obtained with pure poly A. Initial temperature 11°; charge voltage 18 kV; ionic strength 11 mM Na^+. The arrows indicate the position of the base line before the temperature jump. (A) Single-stranded stacked form (pH 6.8), λ 280 nm, top to bottom $\psi = 90°$; $\psi = 70°$; no polarizer; $\psi = 0°$, vertical sensitivity 0.005 absorbance unit per division. (B and C) Double-stranded helical form (pH 4.2, acetate buffer), λ 260 nm, top to bottom same as in A, vertical sensitivity 0.01 absorbance unit per division.

Table I

	Kinetics (α_i, τ_i)[a]				Delay[b]
	α_1	τ_1, μsec	α_2	τ_2, μsec	(t_m), μsec
Single-stranded poly A	1	80	0		20
Double-stranded poly A	0.82	130	0.18	1300	50
Poly A + proflavine $P/D = 1$	0.85	140	0.15	1000	50
Poly A + proflavine $P/D = 130$	1	85	0		20

[a] Analysis of the orientation decay kinetics in terms of exponential functions: $S = \Sigma_i \alpha_i \exp(-t/\tau_i)$; $\Sigma_i \alpha_i = 1$. [b] Delay time (t_m) between the application of the electric field and the maximum amplitude of the orientation signal. The measured lifetime τ_{dis} of the electric field is 85 μsec.

should yield a decay rate equal to $k_h = 2k_{dis}$ (eq 18). Maximum orientation occurs about 20 μsec after the application of the field and then $t_m = \tau_{dis}/4$. Under the assumption of only one orientation relaxation time (τ_{rot}) the above relationship and Figure 3 allow us to estimate that τ_{rot} is close to 8 μsec. Since the assumption of only one orientation relaxation time is probably incorrect, this value must be considered only as an order of magnitude of the orientation kinetics.

(ii) In the case of double-helical poly A the decaying part of the orientation signal may be considered as the sum of two exponentially decaying terms (Figure 10A) whose respective lifetimes and normalized amplitudes are given in Table I. Although this decomposition may have no real physical meaning, it can be nevertheless concluded that the rate-limiting processes are slower than the field decay.

The time t_m at which maximum orientation occurs is also de-

(19) E. Fredericq and C. Houssier, *Biopolymers*, **11**, 2281 (1972).

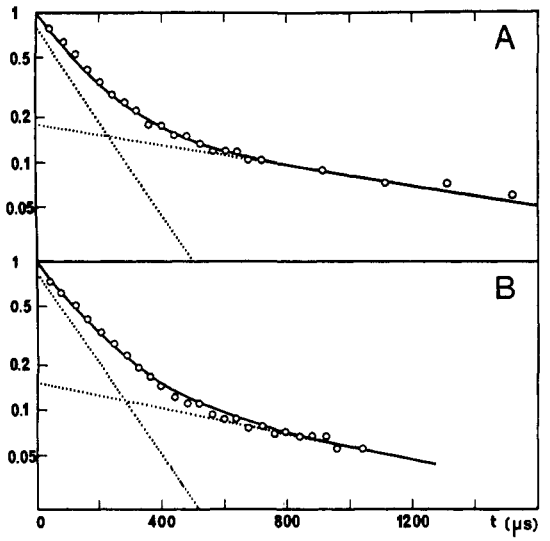

Figure 10. Semilogarithmic analysis of the orientation decay for: (A) double-stranded poly A (the corresponding signal is that of Figure 9C); (B) proflavine–poly A, $P/D = 1$ (the corresponding signal is that of Figure 6B top). The solid line is the sum of the two dotted lines.

pendent on the conformation of the polymer molecule (Table I), and the results suggest that the orientation build-up of the double helix is slower than that of the single-stranded form. This agrees with the conclusions drawn from the analysis of the decays.

The differences which are observed in the kinetics of the orientation processes may be assigned to the differences between the flexibilities of the two structures. The persistence length (which is a measure of the stiffness) of the double-helical conformation is larger than that of the single-stranded stacked one. Therefore, the former is expected to have a slower rotational diffusion than the latter.

The two proflavine complexes exhibit also strong differences in their orientation kinetics. For $P/D = 130$, the field lifetime is the rate-limiting parameter for the orientation decay (Figures 6C and 6D and Table I) as in the case of single-stranded pure poly A. The interpretation of this result is straightforward; for large P/D values the fraction r of occupied binding sites is very small ($r \leq 0.01$), and, therefore, the structure and the rigidity of the polymer chains are not strongly perturbed by the presence of so small an amount of statistically bound molecules.

On the contrary, r is found to be close to 0.8 at $P/D = 1$ (as revealed by the analysis of the absorption spectrum according to ref 13), and the kinetics of the orientation decay is significantly slower than that of the field decay (Figure 6B). The semilogarithmic plot (Figure 10B) yields similar lifetimes and normalized amplitudes for double-helical poly A (see Table I). This finding suggests that dye stacking along the polyelectrolyte chain increases the stiffness of the single strand to about the same level as does double-helix formation.

(2) Chemical Relaxation in the Presence of Orientation. As the excitation wavelength is changed from that of an isosbestic point between the spectra of the free and bound dye species, the asymptotic tail of the oscilloscope trace shifts either above or below the base line (compare Figure 6 to Figure 11). Since the difference between the final and the initial absorbances does not depend on the orientation of the polarizer, it can be concluded that this deviation corresponds to the amplitude of the chemical relaxation signal. Before the limiting equilibrium value is reached, the oscilloscope trace results from the simultaneous presence of a chemical relaxation signal (independent of the ψ value) and an orientation relaxation signal (whose amplitude and direction depend on the ψ value). The shapes of the oscilloscope traces are very similar to the theoretical curves shown in Figure 4. Moreover, the fast initial deviation vanishes as ψ approaches 70° as expected for an orientation dichroism (see the previous section). Therefore, it can reasonably be concluded that the very rapid initial deviation from the base line corresponds to the orientation build-up. For $\psi = 70°$, it may be considered that only the chemical relaxation effect is observable. This is not the case if there is no polarizer in front of the temperature-jump cell (Figure 11D).

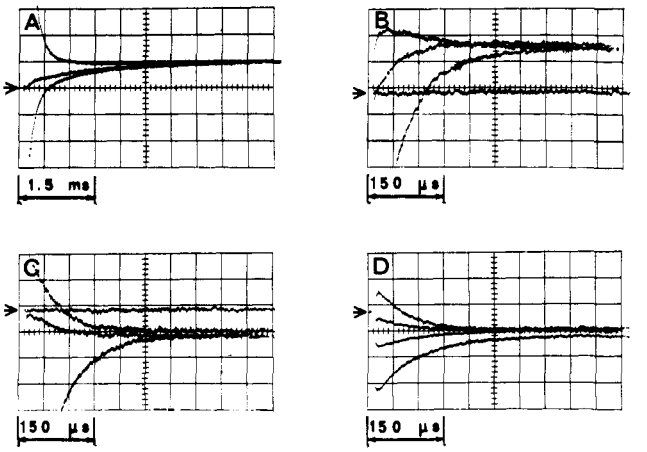

Figure 11. Typical relaxation signals (orientation + chemical) observed with poly A–proflavine mixtures, Initial temperature 11°; charge voltage 18 kV; ionic strength 11 mM Na^+; pH 6.8 (cacodylate buffer). The arrow indicates the position of the base line before the temperature jump. (A) $P/D = 1$, λ 430 nm, top to bottom $\psi = 0°$; $\psi = 70°$; $\psi = 90°$, vertical sensitivity 0.005 absorbance unit per division. (B) $P/D = 130$; λ 430 nm, top to bottom $\psi = 90°$; $\psi = 70°$; base line; $\psi = 0°$, vertical sensitivity 0.002 absorbance unit per division. (C) $P/D = 130$; λ 475 nm, top to bottom $\psi = 90°$; base line; $\psi = 70°$; $\psi = 0°$, vertical sensitivity 0.002 absorbance unit per division. (D) $P/D = 130$, λ 475 nm, top to bottom $\psi = 0°$; $\psi = 70°$; no polarizer; $\psi = 0°$, vertical sensitivity 0.005 absorbance unit per division.

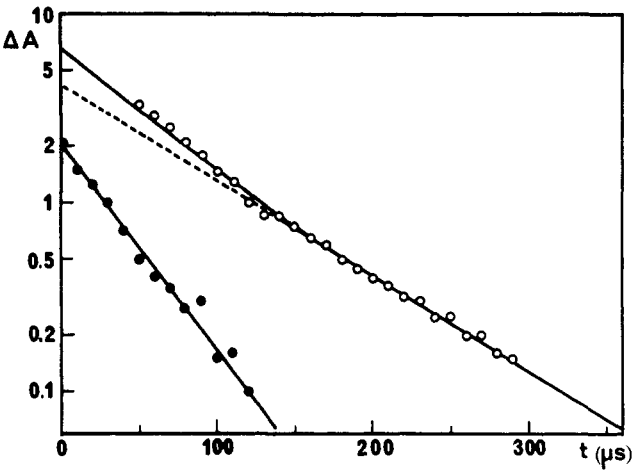

Figure 12. Semilogarithmic analysis of some signals shown in Figure 11: (●) signal corresponding to Figure 11B ($\psi = 70°$); (○) signal corresponding to Figure 11B ($\psi = 0°$).

Although part of the chemical relaxation is slower than the orientation decay for $P/D = 1$, Figure 11 shows that a proper analysis of the whole chemical contribution is not easy without the aid of polarized light. As the orientation contribution is canceled ($\psi = 70°$), fast components appear in the signal and give evidence for a rather broad chemical relaxation spectrum. This agrees with other published data on temperature-jump studies of the cooperative dye binding to linear polyelectrolytes, which have been performed in higher ionic strength conditions.[15,16,20]

In the case of large P/D values, the analysis of the signal obtained at $\psi = 70°$ yields an apparent relaxation time of 42 μsec (Figures 11 and 12). This value is nearly equal to the temperature-jump rise time ($\tau_h = \tau_{dis}/2$). In this case it may be inferred that the true chemical relaxation times are probably much shorter than τ_h as shown by eq 28. Therefore, it can be concluded that the temperature-jump method is not appropriate to study the reaction mechanism under these special conditions, because the reaction proceeds

(20) G. G. Hammes and C. D. Hubbard, *J. Chem. Phys.*, 70, 2889 (1966).

at least as fast as the heating of the solution. Such a conclusion could not be drawn from the study of the signal observed without polarizer, whose semilogarithmic plot gives an apparent relaxation time only slightly smaller than τ_{dis}. This comes from the fact that the amplitude of the orientation signal is still larger than that of the chemical component in the absence of polarizer and because the orientation decay is rate limited by the field lifetime, as previously shown.

According to eq 19, 22, and 28, and for $t > t_m$, the signal can be accounted for by the following equation

$$S(t) - S_{ch} = S_{rot}e^{-t/2\tau_h} - S_{ch}e^{-t/\tau_h} \qquad (29)$$

in which $S(t)$ is the deviation from the initial base line, S_{ch} is the amplitude of the chemical effect, and S_{rot} is the amplitude of the orientation effect. S_{ch} and τ_h are known from the signal observed at $\psi = 70°$ (for which $S_{rot} = 0$). For any other ψ value, or in the absence of polarizer, the only unknown quantity is S_{rot} which may be adjusted to obtain good curve fitting between the experimental curve and eq 29. A typical example ($\psi = 0°$, Figure 12) shows that no unique decomposition could be found out by simple inspection of the signal, without any previous knowledge of the relaxation times. For instance, a satisfying approximate fitting could be obtained by the assumption of only one relaxation time between 70 and 80 μsec. If the total signal is misinterpreted in terms of chemical relaxation only, this could give the false impression that the relaxation time is slightly larger than the temperature rise time.

Discussion and Conclusion

The results presented in the previous section show that anisotropy effects may have considerable influence on the shape of the temperature-jump relaxation signals obtained in the presence of linear polymers. In the present study, only the changes in optical absorption caused by molecular orientation have been considered. Since many other optical properties are sensitive to the space symmetry of the medium (*e.g.*, fluorescence intensity and polarization), it may be expected that the orientation caused by the electric field produces observable effects when any of these properties is used for the detection of temperature-jump relaxation.

The maximum amplitude of the orientation contribution has been found considerably larger than that of the chemical relaxation in the experiments reported in the previous section. However, due to the low salt concentration, the voltage decay time was much larger than commonly used in temperature-jump measurements. According to the theory (eq 15, 16, and 17 and Figures 2 and 3), it may be expected that the amplitude of the orientation signal becomes smaller under higher ionic strength conditions (as long as the orientation kinetics is not much more rapid at higher salt concentrations). Therefore, the question arises as to what extent the present analysis is relevant to the usual utilization of temperature-jump apparatus. For example, previous reports on the relaxation kinetics of poly A–proflavine complexes at ionic strength corresponding to 0.1[14] and 0.5 *M*[20] monovalent salt did not mention any very fast optical change followed by slower relaxation of opposite direction at the beginning of the relaxation signal. In the latter case[20] the voltage decay time was close to 4 μsec. According to the present study and assuming that τ_{rot} does not depend on the ionic strength, it can be estimated that the maximum amplitude of the orientation signal should be about three times smaller in 0.5 *M* than in 0.01 *M* Na^+ (see Figures 2 and 3). In fact, the experimental study reveals that the orientation contribution is quite negligible in 0.5 *M* Na^+ (this may be due to a decrease of the apparent electrical moment of the polyelectrolyte molecule as the ionic strength increases (see ref 11) and to the large error size on τ_{rot}) but becomes worth considering in 0.1 *M* Na^+, depending on the wavelength.

In addition, it can be mentioned that very fast optical changes followed by slower relaxation have been previously reported in several studies where the voltage decay time was much smaller than that used in the present work. They have not been assigned to rotatory orientation of the polyions in the electric field. For example, the fast changes observed in the relaxation kinetics of DNA–proflavine complexes[21] have been attributed to a temperature dependence of the absorption spectrum of the intercalated dye molecule. In order to test this interpretation we have studied this system under linear polarized excitation and found that the angular dependence of the fast effect is in satisfactory agreement with the hypothesis of a transient orientation. A more detailed study is now undertaken whose result will be published later.

It might also be suggested that the occurrence of a transient anisotropy due to orientation could be a relevant hypothesis to explain the rapid initial transmission decay in metastable poly A–2poly U solutions submitted to electric impulses.[22] If this is confirmed by an experimental study, it might be possible that this anisotropy should not be neglected in the quantitative analysis of the data.

In conclusion, the present approach shows that orientation effects can play an important role in temperature-jump relaxation studies on systems containing linear polymer molecules. Special care must be taken in the analysis of the data obtained under low ionic strength conditions. Nevertheless, in the case of absorption detection, pure chemical relaxation signals can be obtained with the use of plane polarized light whose polarization direction is in principle oriented at 55° from the direction of the electric field. This value must be modified and adjusted empirically if additional optical effects (rotation, depolarization) are produced by the windows of the temperature-jump cell.

Finally, it may also be mentioned that the existence of isosbestic wavelengths allows one to collect pure orientation signals. Qualitative informations can thus be obtained on the structure of the polymer or its complexes with other molecules, at ionic strengths which are generally too large to allow the use of a conventional electric dichroism apparatus.

Acknowledgments. We are very grateful to Professor Ch. Sadron for stimulating suggestions about this work. We wish to acknowledge the Ligue Nationale Française Contre le Cancer for financial support to one of us (J. F. H.).

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