similar to this site. The pyrophosphate binding sites of DNA polymerase β of ducks, *E. coli* DNA polymerase I, AMV reverse transcriptase, and the α and β polymerases of human Wi-38 cells (Mao et al., 1975), however, appear to be different from this site on the herpesvirus-induced DNA polymerase.

References

Berry, J. P., Isbell, A. F., and Hunt, G. E. (1972), *J. Org. Chem. 37,* 4395.

Boezi, J. A., Lee, L. F., Blakesley, R. W., Koenig, M., and Towle, H. C. (1974), *J. Virol. 14,* 1209.

Chambers, J. R., and Isbell, A. F. (1964), *J. Org. Chem. 29,* 832.

Churchill, A. E., and Biggs, P. M. (1967), *Nature (London) 215,* 528.

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67,* 104.

Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67,* 173.

Deutscher, M. P., and Kornberg, A. (1969), *J. Biol. Chem. 244,* 3019.

Isbell, A. F., Berry, J. P., and Tansey, L. W. (1972), *J. Org. Chem. 37,* 4399.

King, E. L., and Altman, C. (1956), *J. Phys. Chem. 60,* 1375.

Kornberg, A. (1969), *Science 163,* 1410.

Mao, J. C.-H., Robishaw, E. E., and Overby, L. R. (1975), *J. Virol. 15,* 1281.

Marek, J. (1907), *Dtsch. Tieraerztl. Wochenschr. 15,* 417.

McClure, W. R., and Jovin, T. M. (1975), *J. Biol. Chem. 250,* 4073.

Moe, O. A., and Butler, L. G. (1972), *J. Biol. Chem. 247,* 7308.

Nazerian, K., Solomon, J. J., Witter, R. L., and Burmester, B. R. (1968), *Proc. Soc. Exp. Biol. 127,* 177.

Overby, L. R., Robishaw, W. E., Schleicher, J. B., Rueter, A., Shipkowitz, N. L., and Mao, J. C.-H. (1974), *Antimicrob. Agents Chemother. 6,* 260.

Purchase, H. G., Witter, R. L., Okazaki, W., and Burmester, B. R. (1971), *Perspect. Virol. 7,* 91.

Shipkowitz, N. L., Bower, R. R., Appell, R. N., Nordeen, C. W., Overby, L. R., Roderick, W. R., Schleicher, J. B., and VonEsch, A. M. (1973), *Appl. Microbiol. 27,* 264.

Solomon, J. J., Witter, R. L., Nazerian, K., and Burmester, B. R. (1968), *Proc. Soc. Exp. Biol. 127,* 173.

Weissbach, A., Schlabach, A., Fridlender, B., and Bolden, A. (1971), *Nature (London), New Biol. 231,* 167.

Wilkinson, G. N. (1961), *Biochem. J. 80,* 324.

Influence of Electric Dichroism on the Temperature-Jump Relaxation Study of Proflavine–DNA Complexes†

M. Dourlent* and J. F. Hogrel

ABSTRACT: The temperature-jump relaxation kinetics of proflavine–DNA complexes has been reinvestigated with a standard apparatus equipped for absorption detection of plane-polarized light in order to discriminate between chemical relaxation and transient orientation effects. Under low ionic strength conditions (0.015 *M* Na^+), these effects may represent the major contribution to the signal when the T-jump apparatus is used without a polarizer. They have been improperly assigned to chemical relaxation in previous work. The actual relaxation times are smaller than 30 μsec at 10°C. Under medium ionic strength conditions (0.2 *M* Na^+) it is shown that: (i) the "instantaneous" change of transmission reported in earlier work (Li, H. J., and Crothers, D. M. (1969), *J. Mol. Biol. 39,* 461–477; Schmechel, D. E. V., and Crothers, D. M. (1971), *Biopolymers 10,* 465–480) is due to orientation effects; (ii) an intermediate exists whose absorption spectrum resembles somewhat that of proflavine aggregates on a linear polyanion; (iii) the rate constants for outside binding may be significantly larger than previously reported. The new kinetic data are consistent with a modified mechanism derived from equilibrium studies (Ramstein, J., Hogrel, J. F., Dourlent, M., Leng, M., and Hélène, C. (1973), in Dynamic Aspects of Conformation Changes in Biological Macromolecules, Sadron, C., Ed., Dordrecht, Holland, Reidel Publisher, pp 333–347; Dourlent, M., and Hogrel, J. F. (1976), *Biopolymers* (in press)), but, however, do not unambiguously prove it. From these studies, it is inferred that orientation effects can perturb relaxation data on systems containing linear polymers in many cases.

The dynamics of dye–nucleic acid interactions have been extensively studied by means of temperature-jump relaxation and stopped-flow techniques (Li and Crothers, 1969; Schmechel and Crothers, 1971; Ramstein et al., 1972, 1973; Thusius et al., 1973; Steenbergen and Mohr, 1973; Akasaka et al., 1970; Sakoda et al., 1971, 1972; Bittman, 1969; Tritton and Mohr, 1971; Tritton and Mohr, 1973). Simultaneously the mechanism of cooperative binding to other linear polyanions has also been investigated (Hammes and Hubbard, 1966a,b; Schwarz et al., 1970; Schwarz and Balthazar, 1970; Schwarz and Klose, 1972; Vitagliano, 1973). In a recent work (Dourlent et al., 1974) it was suspected that the very fast component of the relaxation signal observed with solutions containing proflavine (Li and Crothers, 1969; Schmechel and Crothers, 1971) could be an artefact resulting from a transient orientation of the polymer molecules in the electric field of the temperature-jump

† From CNRS, Centre de Biophysique Moléculaire, 45045-Orleans, France. *Received June 20, 1975.*

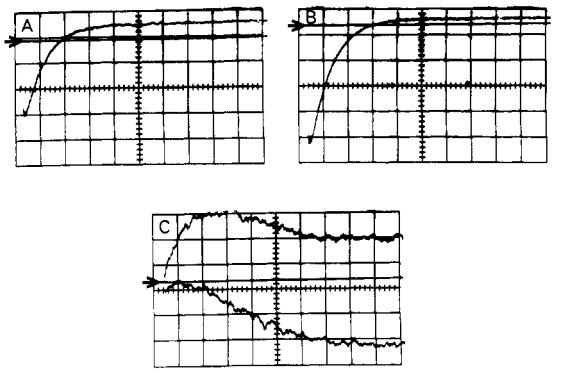

FIGURE 1: Oscilloscope traces of the transmission changes during and after the temperature jump; solvent, 0.1 × SSC. The baseline before the capacitor discharge is shown by an arrow. Upward deviations correspond to a decrease of the optical transmission; C_{DNA} = 400 μM (phosphorus); C_{dye} = 20 μM: (A) λ 425 nm, no polarizer; vertical sensitivity, 0.005 absorbance unit per division; horizontal sweep, 50 μsec per division; (B) λ 425 nm, polarization parallel to the electric field; vertical sensitivity, 0.01 absorbance unit per division; horizontal sweep, 50 μsec per division; (C) polarizer oriented to give an orientation contribution as small as possible (see Appendix); upper trace, λ 435 nm; lower trace, λ 475 nm; vertical sensitivity, 0.004 absorbance unit per division; horizontal sweep, 20 μsec per division; C_{DNA} = 1.6 mM (phosphorus); C_{dye} = 80 μM. For this solution, the transmitted light intensity has a maximum increase of 87% when the polarizer is parallel to the electric field.

cell. The present work is devoted to experiments which confirm this hypothesis and lead to different values of the rate constants for the so-called "outside binding" of proflavine to DNA. The new kinetic data are consistent with a modified mechanism proposed from equilibrium and kinetic studies (Ramstein et al., 1973; Dourlent, 1975; Dourlent and Hogrel, 1976), but, however, do not unambiguously prove it.

Materials and Methods

Calf-thymus DNA was purified by phenol extraction and degraded with a MSC ultrasonicator as already reported (Ramstein et al., 1972). The sedimentation constant $s_{20,w}$ of sonicated samples was 8.2S. This corresponds to a mean molecular weight of approximately 500,000. Highly purified proflavine was a gift from Dr. M. Charlier. The experiments have been performed at a constant polymer to dye ratio (P/D ≃ 20 mol of nucleotides per mol of dye) by appropriate dilutions of a concentrated stock solution in a buffered solvent. The buffer was standard saline-citrate (SSC) (pH 6.9). Two concentrations of this buffer have been used: 0.1 × SSC and 1.5 × SSC. The corresponding ionic strengths are approximately 0.016 and 0.24 M Na^+, respectively. These values are very close to those used in other works (Li and Crothers, 1969; Schmechel and Crothers, 1971).

The kinetic studies were performed with a temperature-jump apparatus purchased from Messanlagen Studiengesellschaft mbH (Göttingen). The rise time for the temperature was 25–30 μsec with 0.1 × SSC and 3–4 μsec with 1.5 × SSC. In order to discriminate between chemical relaxation and orientation effects, the detection of the signals was made with plane polarized light (Dourlent et al., 1974). The final equilibrium temperature was 10°C after a 4°C jump. The corresponding high voltage discharge was 20 kV.

Results

Experimental Data Obtained under Low Ionic Strength

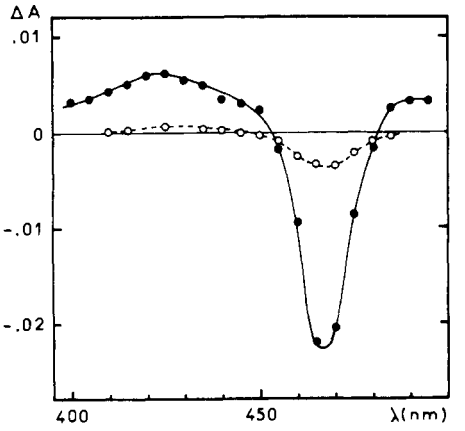

FIGURE 2: Wavelength dependence of the absorbance change between the initial and final equilibrium values in 0.1 × SSC: (solid line) C_{DNA} = 1.6 mM (phosphorus); C_{dye} = 80 μM; (dashed line) C_{DNA} = 0.2 mM; C_{dye} = 10 μM.

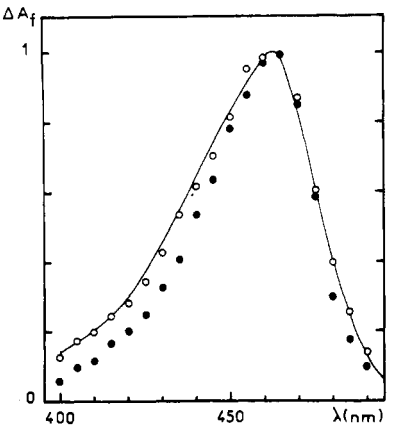

FIGURE 3: Normalized wavelength dependences of the transient absorbance change measured at its extremum amplitude ΔA_m in 0.1 × SSC buffer: (solid circles) no polarizer, C_{DNA} = 1.6 mM (phosphorus), C_{dye} = 80 μM; (open circles) polarization parallel to the electric field, C_{DNA} = 0.2 mM; C_{dye} = 10 μM; (solid line) (for comparison) normalized absorption spectrum of the solution containing 0.4 mM DNA and 20 μM proflavine.

Conditions (0.1 × SSC). When the apparatus is used without polarizer the signals are composed of a very fast and large increase in transmission followed by a slower relaxation of opposite direction (Figure 1A). The differences, ΔA_{tot}, between the final and initial equilibrium absorbances are small compared to the very fast effect. The sign of these differences depends on the wavelength (Figure 2). The amplitude, ΔA_f, of the fast effect measured at its extremum value is unusually large to correspond to a chemical relaxation effect. Its wavelength dependence is similar to that of the absorption spectrum of the intercalated bound dye molecule (Figure 3).

When the incident light is plane polarized, the value of ΔA_f depends on the angle ψ between the light vector and the transient electric field in a way similar to that previously reported (Dourlent et al., 1974; Figure 7). The value of ΔA_{tot} does not depend on the ψ value. Therefore, it can be concluded that the fast effect results mainly from the orientation build-up of the DNA molecules in the electric field and that the slower part of the signal contains two superimposed effects: orientation decay and chemical relaxation.

When plotted on a semilogarithmic scale over one decade, the slower part of the signal may be accounted for by

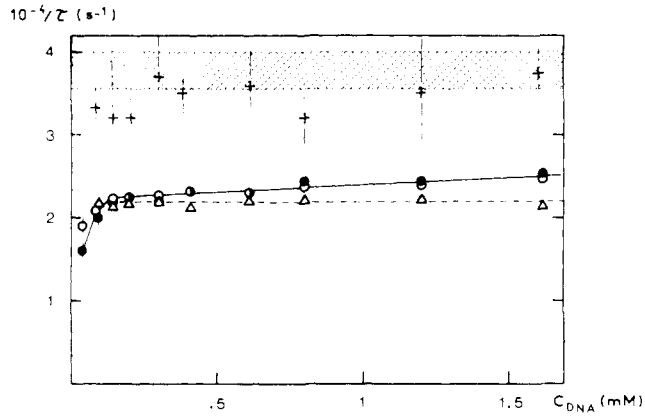

FIGURE 4: Concentration dependence of the apparent reciprocal relaxation time in 0.1 × SSC buffer. The abscissa refers to the total concentration of nucleotides instead of $\bar{X}$ (defined in the text, eq 1). Due to the large P/D value, the error resulting from this approximation is small: (solid circles and solid line) uncorrected data obtained at 420 nm without polarizer; (open circles) uncorrected data obtained at 460 nm with polarization parallel to the electric field (orientation decay); (triangles) corrected data for the orientation decay (for the correction formulas see text); (crosses) apparent relaxation time corresponding to the difference between two signals obtained at 435 and 475 nm as shown in Figure 1C. The hatched area corresponds to the measured rise time of the temperature jump.

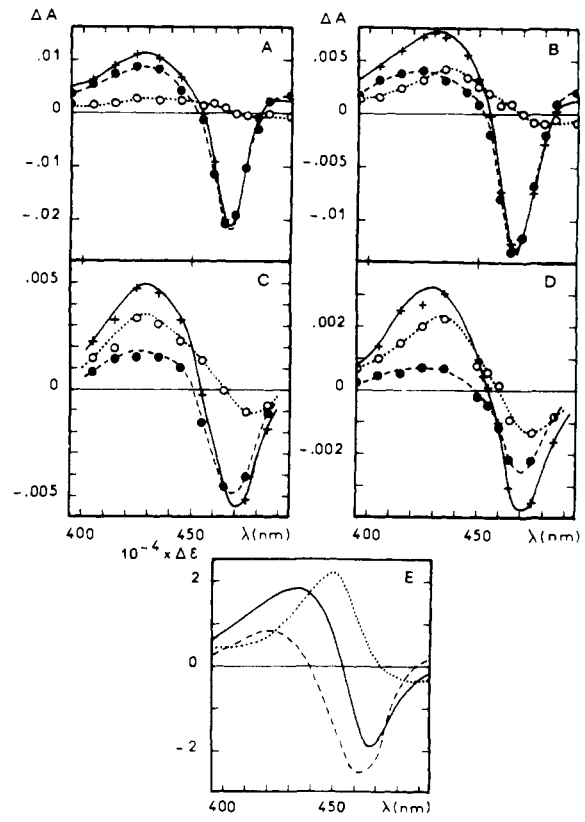

FIGURE 5: Temperature-jump data obtained in 1.5 × SSC buffer, after elimination of the orientation contribution: (crosses and solid lines) total absorbance change between initial and final equilibrium values; (solid circles and broken lines) amplitude of the fast phase; (open circles and dotted lines) amplitude of the slow phase: (A) C_{DNA} = 1.6 mM (phosphorus); C_{dye} = 80 μM; (B) C_{DNA} = 0.75 mM (phosphorus); C_{dye} = 40 μM; (C) C_{DNA} = 0.38 mM (phosphorus); C_{dye} = 20 μM; (D) C_{DNA} = 0.15 mM (phosphorus); C_{dye} = 7.5 μM; (E) difference spectra obtained from Figure 2 of Dourlent and Hélène (1971) (for the definition of the complexes see this reference): (solid line) difference between free proflavine and complex II (intercalated molecule); (broken line) difference between complex I,S (cooperative binding) and complex II; (dotted line) difference between free proflavine and complex I,S.

one single exponential term to a first approximation. The corresponding "apparent" relaxation time, τ_{app}, measured at 420 nm without polarizer depends on the concentration of the solution according to a law similar to that reported by Li and Crothers (1969) (compare Figure 3c of their paper to Figure 4 of the present one). For a total DNA concentration larger than 0.15 mM and experimental conditions maximizing the orientation contribution (i.e., polarization parallel to the electric field and wavelength adjusted to the maximum of absorption of the complex), the "apparent" relaxation time has the same concentration dependence (Figure 4). Thus, it can be inferred that the asymptotic tail of the solid line of Figure 4 is not due to a chemical relaxation effect but to the orientation decay of the macromolecular solution. That the kinetics of this decay seems to be concentration dependent results from a systematic error which is made by using an approximation very common in the analysis of chemical relaxation data. This approximation consists of the assumption that the variation of the transmitted light intensity ΔI is proportional to the variation of the absorbance ΔA. Due to the large amplitude of the orientation signal, the correct relationship ($\Delta A = -\log(1 + \Delta I/I)$ must be used for the analysis of the present data. When this is made, the apparent time constant for the orientation decay becomes independent of the concentrations within experimental accuracy (see Figure 4).

In order to measure the kinetics of the chemical relaxation, the orientation contribution must be cancelled or separately measured. In the present case, the reduced electric dichroism is too large to allow the use of the method previously proposed (Dourlent et al., 1974). However, it is still possible to obtain an estimate of the chemical contribution by taking advantage of the fact that the reduced dichroism is nearly constant along the whole absorption band of the complex (Houssier and Kubbal, 1971). Then, for any pair of wavelengths (λ_1, λ_2) satisfying to the condition of equal absorbances ($\epsilon(\lambda_1) = \epsilon(\lambda_2)$), it can be assumed that the differences between the two corresponding signals reflect the chemical contribution. Since any differential method lowers the signal-to-noise ratio, it is obviously better to adjust ψ to a value giving an orientation contribution as small as possible (see Appendix). Typical data obtained under these conditions and for λ_1 435 nm and λ_2 475 nm are shown in Figure 1C. The analysis of the difference signal shows that the time dependence of the chemical effect obeys a single exponential law within experimental accuracy. The corresponding time constant τ_{ch} does not depend on the concentrations and falls into the range of the temperature-jump rise time, $\tau_h = RC/2$ (see Figure 4). The logical conclusion of this finding is that the correct chemical relaxation times of the system are much smaller than 30 μsec in 0.015 M Na^+ at 10°C (except for very small concentrations). Returning to the corresponding original data (Li and Crothers, 1969), it seems therefore very likely that the calculated values of the rate constants under these low ionic strength conditions were very much underestimated.

Experimental Data Obtained under Medium Ionic Strength Conditions (1.5 × SSC). Although the lifetime of the electric field is only 8–10 μsec under these conditions, the transient electric dichroism is not negligible as revealed by the fact that the signals are not identical with parallel and perpendicular polarization. However, the amplitude of the orientation contribution is small and it is possible to

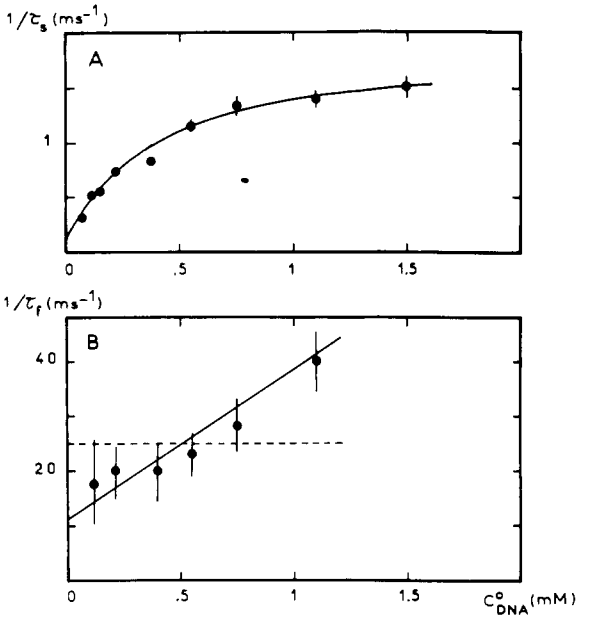

FIGURE 6: Concentration dependence of the measurable relaxation times. The total concentration of DNA has been used instead of X as in Figure 4: (A) relaxation time associated to the slow phase (τ_s); the solid line corresponds to a curve fitting according to eq 2 or 5 or 9; (B) measurable relaxation time associated with the fast phase (τ_f); the solid line corresponds to a curve fitting according to eq 4 or 8; the dotted line corresponds to the mean apparent relaxation time for the orientation decay.

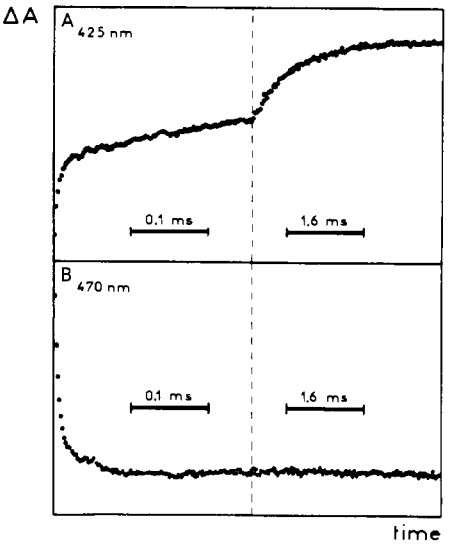

FIGURE 7: Typical relaxation signals obtained after elimination of the orientation contribution in 1.5 × SSC buffer: C_{DNA} = 0.8 mM (phosphorus); C_{dye} = 40 μM; (A) λ 425 nm; (B) λ 470 nm. The vertical scale is in arbitrary units.

cancel this component by an appropriate orientation of the polarizer (Dourlent et al., 1974). In order to take into account a possible depolarization by the windows of the temperature-jump cell and a fast chemical effect, an empirical adjustment of the ψ angle was made. For this adjustment, a proflavine-poly(A) mixture (P_0/D_0 = 1) in 10 mM Na^+ was used because of its small and slow chemical contribution at 475 nm (Dourlent et al., 1974).

The results obtained with proflavine–DNA solutions after cancelling of the orientation contribution indicate that the chemical contribution is composed of two relaxation "phases". The faster phase can be resolved in one or two exponentials, depending on the wavelength (see below). The wavelength dependence of the amplitude ΔA_F associated to the fast phase is very similar to that of the total change obtained in 0.1 × SSC (compare Figure 5 and Figure 2). The slower phase is in the millisecond range. The relative amplitude $\Delta A_{slow}/\Delta A_{total}$ varies significantly with the concentration (compare Figures 5A, B, C, and D). It is worth noting that: (i) the wavelength dependence of ΔA_f is not exactly similar to that originally reported (Li and Crothers, 1969) (we believe that this was due to the presence of a residual orientation contribution, in this work); (ii) any of the two relaxation difference spectra (i.e., the wavelength dependences of ΔA_f and ΔA_{slow}) are similar to the difference spectrum between a free proflavine molecule and the intercalation complex; (iii) the difference spectrum of the fast phase displays some analogy with the difference spectrum between the intercalation complex and the cooperative aggregation to a linear polyanion (see Figure 5E); the wavelength dependence of ΔA_{slow} displays some resemblance to the difference spectrum between the aggregated dye species and the free one (Figure 5E).

The time dependence of the "slow" phase can be accounted for with one single relaxation time, τ_s, to a first approximation. The variations of $1/\tau_s$ with the concentration are exactly those previously reported (Li and Crothers, 1969) (see Figure 6A). For wavelengths where the relative amplitude of the "slow" phase is not small (λ <465 nm), the fast phase appears as a "very fast" process whose apparent relaxation time, $\tau_{v.f.}$, is in the range 5–10 μsec (e.g., Figure 7A). Since the temperature rise time was 3–4 μsec and the smallest time constant of the detection unit was 2 μsec in our experiments, it seems very likely that the correct relaxation time(s) involved in the "very fast" phase is (are) smaller than 5 μsec. For wavelengths where the relative amplitude of the "slow" phase is very small or even zero (e.g. in Figure 7B), the "fast" phase appears as a two-step process within experimental accuracy. The very rapid initial step has an apparent relaxation time equal to $\tau_{v.f.}$. The second step is characterized by a small relative amplitude. Consequently, the associated relaxation time τ_f cannot be measured with high accuracy. Its value is found between 20 and 50 μsec and seems to depend on the concentration (see Figure 6B).

Discussion

The amplitudes of the "very fast" and "slow" components of the signal may be negative or positive, depending on the chosen wavelength. This shows that $\tau_{v.f.}$ and τ_s are associated with chemical relaxation processes. As regards the "fast" component, the time and amplitude resolutions of our apparatus are not sufficient to obtain accurate data. Moreover, preliminary data show that the kinetics of the orientation decay of the pure DNA solution in 1.5 × SSC can be described with two rotational relaxation times whose values are 15 and 80 μsec and whose associated normalized amplitudes are 0.6 and 0.4, respectively. The resulting mean relaxation time is about 40 μsec. Taking account of the large error size on τ_f, this value can fit approximately the data shown in Figure 6B (broken line). Thus, at least two plausible hypotheses can be made about the origin of τ_f: either this time corresponds to a residual contribution of the orientation decay, or τ_f is actually a true chemical relaxation time. We shall discuss briefly the consequences of each possibility.

τ_f Is Not a Chemical Relaxation Time. In this case, the simplest reaction scheme is a two-step mechanism. If the model of Li and Crothers (1969) is taken as a reference, the very fast step must be ascribed to the outside binding because the concentration dependence of τ_s is not consistent with the hypothesis of a slow bimolecular step. From the reaction scheme:

$$\text{DNA} + \text{dye} \underset{k_{2,1}}{\overset{k_{1,2}}{\rightleftarrows}} (\text{D})_{\text{outside}} \underset{k_{3,2}}{\overset{k_{2,3}}{\rightleftarrows}} (\text{D})_{\text{inside}}$$

the two relaxation times obey the relationships shown in eq 1 and 2, where $\bar{X} = \bar{C}_{\text{DNA}} + \bar{C}_{\text{Dye}}$ (equilibrium values of free binding sites and ligand concentrations, respectively).

$$1/\tau_{\text{v.f.}} = k_{1,2}\bar{X} + k_{2,1} = k_{1,2}(\bar{X} + 1/K_1) \quad (1)$$

$$1/\tau_s = k_{3,2} + k_{2,3}[\bar{X}/(1/K_1 + \bar{X})] \quad (2)$$

The experimental data can fit eq 2 with the following values: $K_1 = k_{1,2}/k_{2,1} = 2500\ M^{-1}$, $k_{2,3} = 1800\ \text{sec}^{-1}$, and $k_{3,2} = 110\ \text{sec}^{-1}$. Since the chemical relaxation time $\tau_{\text{v.f.}}$ is probably smaller than 5 μsec, the lower limit of the association rate constant, $k_{1,2}$, can be estimated to be $10^{10}\ M^{-1}\ \text{sec}^{-1}$ approximately from eq 1. This result suggests that outside binding is typically diffusion controlled. However, due to the competition with the solvent counterions, a smaller value should be expected, as in the case of cooperative binding to linear polyanions (Schwarz et al., 1970; Schwarz and Balthazar, 1970; Schwarz and Klose, 1972; Vitagliano, 1973; Dourlent et al., 1974). Accordingly, it seems that this first possibility is rather unlikely.

τ_f Corresponds to a Chemical Relaxation Time. In this case, the reaction scheme must involve at least three independent steps. The simplest plausible model corresponds to a sequential mechanism recently proposed (Dourlent and Hogrel, 1976):

$$\text{DNA} + \text{dye} \underset{k_{1,0}}{\overset{k_{0,1}}{\rightleftarrows}} \text{C}_{\text{I}} \underset{k_{2,1}}{\overset{k_{1,2}}{\rightleftarrows}} \text{C}_{\text{II},1} \underset{k_{3,2}}{\overset{k_{2,3}}{\rightleftarrows}} \text{C}_{\text{II},2}$$

If $\tau_{\text{v.f.}}$ is associated with the bimolecular step, the considerations of the above section are still relevant to this case. They lead to the conclusion that $k_{0,1} \simeq 10^{10}\ M^{-1}\ \text{sec}^{-1}$, which seems a rather large value as discussed above. If τ_f is mainly governed by the bimolecular step, there are two remaining possibilities.

(a) The Second Step is Very Fast, the First One Is Less Fast, and the Third One Is Slow. The relationships between the relaxation times and the rate constants are then:

$$1/\tau_{\text{v.f.}} = k_{1,2} + k_{2,1} \quad (3)$$

$$1/\tau_f = k_{0,1}\bar{X} + k_{1,0}^* \quad (4)$$

$$1/\tau_s = k_{3,2} + k_{2,3}^*\bar{X}/(\bar{X} + 1/K_1^*) \quad (5)$$

with

$$k_{1,0}^* = k_{1,0}/(1 + K_1) \quad (6a)$$

$$k_{2,3}^* = k_{2,3}K_1/(1 + K_1) \quad (6b)$$

$$K_1^* = K_0(1 + K_1) \quad (6c)$$

The experimental data can fit this model with the following numerical values: $k_{0,1} = 2.8 \times 10^7\ M^{-1}\ \text{sec}^{-1}$; $k_{1,0}^* = 1.1 \times 10^4\ \text{sec}^{-1}$; $K_1^* = 2500\ M^{-1}$; $k_{2,3}^* = 1800\ \text{sec}^{-1}$; $k_{3,2} = 110\ \text{sec}^{-1}$.

According to this mechanism, the association constant for the first step (K_0) is smaller than the apparent bimolecular constant K_1^* associated with τ_f. This is in agreement with the model proposed by Dourlent and Hogrel (1976) where it is assumed that the first stage of the dye–DNA association corresponds to a very weak binding in which the dominant interactions are the Coulombic forces between the negative phosphate groups and the positive charge of the dye molecule. The second step of the mechanism could then correspond to a very fast isomerization of this external nonspecific complex to another form ($C_{II,1}$) in which base–dye interactions are located in the groove of the DNA helix without intercalation. In agreement with previous findings (Ramstein et al., 1972), a base selectivity could exist in this mode of binding, due to the nature and the position of the heteroatoms in the grooves. Finally, the last step corresponds to the intercalation reaction which is relatively slow due to the activation barrier associated with the distortion of the DNA double helix.

(b) The Third Step Is Very Fast, the First Is Less Fast, and the Second One is Slow. In this case, the equations for the relaxation times are:

$$1/\tau_{\text{v.f.}} = k_{2,3} + k_{3,2} \quad (7)$$

$$1/\tau_f = k_{0,1}\bar{X} + k_{1,0} \quad (8)$$

$$1/\tau_s = k_{2,1}/(1 + K_2) + k_{1,2}\bar{X}/(\bar{X} + 1/K_0) \quad (9)$$

The experimental data can fit this model with the following numerical values: $k_{0,1} = 2.8 \times 10^7\ M^{-1}\ \text{sec}^{-1}$; $k_{1,0} = 1.1 \times 10^4\ \text{sec}^{-1}$; $K_0 = 2500\ M^{-1}$; $K_1(1 + K_2) = 16.3$.

If the last step of the reaction scheme corresponds to the intercalation process, its very fast kinetics suggests that a favorable distortion of the DNA helix is present when the dye is bound in the $C_{II,1}$ form (otherwise, the activation barrier that exists when the DNA structure is not distorted should decrease the rate of intercalation). This hypothesis is in agreement with the fact that the formation of $C_{II,1}$ is the slower step of the mechanism.

An alternative interpretation for the very fast kinetics of the last step should be that a rapid conformational equilibrium of the polymer exists as already suggested for the ethidium–transfer RNA complexes (Tritton and Mohr, 1971). However, this seems less probable for DNA which is devoid of tertiary structure.

At the present time, neither of the possibilities a or b can be ruled out. Moreover, other reaction schemes could be considered to interpret the experimental data (e.g., the shape of the difference spectrum of the fast phase could suggest that a very rapid equilibrium exists between bound "dimers" and intercalated monomers as already proposed (Armstrong et al., 1970). In any case, at medium ionic strength, the rate constants for the bimolecular step are at least a factor of two larger than those originally reported (Li and Crothers, 1969).

Conclusion

The present study provides some new information on the kinetics of proflavine binding to DNA: (i) the instantaneous change of transmission results from an orientation effect by the transient electric field; (ii) an intermediate exists, whose absorption spectrum resembles more or less that of proflavine aggregates; (iii) the rate constants for outside binding are larger than previously reported, especially at low ionic strength. In particular, it is not possible to obtain chemical relaxation data on this system with standard temperature-jump apparatus when the ionic strength is on the order of $10^{-2}\ M\ \text{Na}^+$.

To date, no definite picture of the mechanism can be pro-

posed, but it seems that the original model needs to be refined. This requires the use of equipment more sensitive than ours. Nevertheless, the present data clearly demonstrate that orientation effects can perturb strongly the analysis of temperature-jump experiments. This is particularly true for the studies performed under low ionic strength conditions, but even under usual salt conditions (e.g., 0.2 *M* Na^+) these orientation effects can still play a nonnegligible role. The sensitivity of the temperature-jump measurements to orientation effects is due to the fact that very small dichroic effects can yield to absorption changes comparable to those originating from chemical relaxation. For instance, from eq 10 of the article by Dourlent et al. (1974), it can be computed that a reduced dichroism equal to 0.006 corresponds approximately to a change of 0.001 absorbance unit for a solution of one absorbance unit when excited with unpolarized light.

In light of the present conclusions, it seems worthwhile to restart some previous works, including ours, with special care to the correction of possible orientation effects. In the case of recently studied nonintercalative ligands (Muller et al., 1973; Sturm, 1974), electrooptic measurements (Sturm, 1974; Bontemps et al., 1974) show that the ligand is rigidly fixed with respect to the helix axis. The value of the angle Φ between the transition moment of the bound chromophore and the helix axis depends on the nature of the ligand. When this value is close to that corresponding to vanishing electric dichroism ($\Phi_0 \simeq 55°$), the temperature-jump relaxation measurements are not significantly perturbed by the orientation effects (Sturm, 1974). In other cases, where Φ differs from Φ_0, orientation effects may perturb the relaxation data as suggested by Bontemps et al. (1974). Valuable data on these systems and intercalative dyes might be obtained with laser heating or cable discharge which have nanosecond rise time (Hammes, 1974). Furthermore, laser heating would avoid the electric-field-induced orientation effect altogether.

Appendix

Under plane polarized excitation, the intensity of the transmitted light (I_t) depends on the angle ψ between the light vector and the applied electric field according to eq A-1 (Dourlent et al., 1974), where I_0 is the incident light intensity, $\epsilon_\parallel$ and $\epsilon_\perp$ are the parallel and perpendicular extinction coefficients respectively, c is the molar concentration of the chromophore, and l is the optical path length.

$$I_t = I_0(10^{-\epsilon_\parallel cl}\cos^2\psi + 10^{-\epsilon_\perp cl}\sin^2\psi) \qquad \text{(A-1)}$$

Defining the reduced dichroism as $\rho = 3(\epsilon_\parallel - \epsilon_\perp)/(\epsilon_\parallel + 2\epsilon_\perp)$ and the absorbance of the isotropic solution as $A = (1/3)(\epsilon_\parallel + 2\epsilon_\perp)cl$, eq A-1 reads:

$$I_t = I_0 10^{-A}(10^{-2A\rho/3}\cos^2\psi + 10^{A\rho/3}\sin^2\psi) \qquad \text{(A-2)}$$

The relative change of the transmitted light intensity which results from anisotropy is then:

$$\Delta I/I_t = 10^{-2A\rho/3}\cos^2\psi + 10^{A\rho/3}\sin^2\psi - 1 \qquad \text{(A-3)}$$

When the dimensionless quantity $D = A\rho$ is much smaller than 1/2.3, it can be shown (Dourlent et al., 1974) that ΔI approaches zero for a critical value of (ψ_c) such that $\cos 2\psi_c = -\frac{1}{3}$($\psi_c \simeq 55°$). This property can be used to obtain pure chemical relaxation signals when D is very small. When this condition is not fulfilled, the orientation contribution cannot be cancelled because (1) the ψ value corresponding to $\Delta I = 0$ depends on the D value (see below) and (2) the D value is time dependent since the applied electric field has an exponential decay.

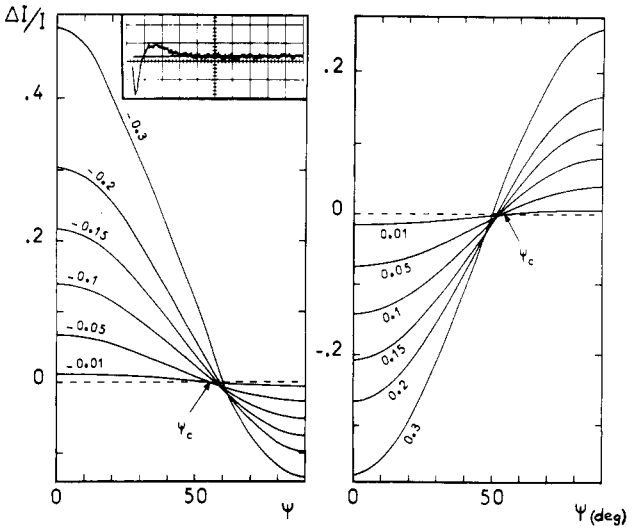

FIGURE 8: Relative variations of the transmitted light intensity ($\Delta I/I_t$) vs. the angle ψ between the light vector and the electric field for some values of D (indicated on the figure), calculated according to eq A-3: (left) negative dichroism; (right) positive dichroism; (insert) typical oscilloscope trace of a damped oscillation corresponding to a value between $\psi_{0,max}$ and ψ_c; same solution as in Figure 1C; vertical sensitivity, 0.005 absorbance unit per division; horizontal sweep, 50 μsec per division.

Defining ψ_0 as the (time-dependent) value of ψ for which $\Delta I = 0$ in those cases where the small dichroism approximation does not apply, eq A-3 yields eq A-4 and A-5:

$$\cos 2\psi_0 = (\alpha^3 - 2\alpha^2 + 1)/(\alpha^3 - 1) \qquad \text{(A-4)}$$

with

$$\alpha = 10^{D/3} \qquad \text{(A-5)}$$

For a solution having a typical absorbance $A = 1$, the ψ_0 value ranges between 47 and 74° for the maximum and the minimum values of the reduced dichroism, respectively (+3 and $-\frac{3}{2}$). In Figure 8, the angular dependence of $\Delta I/I$ has been plotted for several values of D.

After the anisotropy of the solution has reached its maximum ($D = D_{max}$), the time dependence of the signal corresponding to the orientation decay may have three typical shapes, depending on the ψ value. Let us first consider a solution having negative dichroism, and define $\psi_{0,max}$ as the ψ_0 value corresponding to D_{max}.

(1) For $\psi < \psi_c$, the transmitted light intensity decreases continuously toward its initial isotropic value. (2) For $\psi_c < \psi < \psi_{0,max}$ the transmitted light intensity varies according to a damped oscillation law (see insert to Figure 8). The ΔI value is positive at the beginning of the anisotropy decay and negative after the D value has reached the value for which $\psi = \psi_0$. (3) For $\psi > \psi_{0,max}$ the transmitted light intensity increases continuously toward its initial value.

In the case of positive dichroism, the continuous increase is observed for $\psi < \psi_{0,max}$, the damped oscillation for $\psi_{0,max} < \psi < \psi_c$, and the continuous decrease for $\psi > \psi_c$.

This brief analysis shows that there is no possibility for obtaining pure chemical relaxation signals under plane polarization excitation when the D value is not small. However, it is possible to minimize the size of the orientation contribution by choosing the best value between ψ_c and $\psi_{0,max}$.

References
Akasaka, K., Sakoda, M., and Hiromi, K. (1970), *Biochem. Biophys. Res. Commun. 40,* 1239–1245.
Armstrong, R. W., Kurucsev, T., and Strauss, U. P. (1970), *J. Am. Chem. Soc. 92,* 3174–3181.
Bittman, R. (1969), *J. Mol. Biol. 46,* 251–268.
Bontemps, J., Houssier, C., and Frederic, E. (1974), *Biophys. Chem. 2,* 301–315.
Ding, D. W., Rill, R., and Van Holde, K. E. (1972), *Biopolymers 11,* 2109–2124.
Dourlent, M. (1975), *Biopolymers 14,* 1717–1738.
Dourlent, M., and Hélène, C. (1971), *Eur. J. Biochem. 23,* 86–95.
Dourlent, M., and Hogrel, J. F. (1976), *Biopolymers* (in press).
Dourlent, M., Hogrel, J. F., and Hélène, C. (1974), *J. Am. Chem. Soc. 96,* 3398–3406.
Hammes, G. G., and Hubbard, C. D. (1966a), *J. Phys. Chem. 70,* 1615–1622.
Hammes, G. G., and Hubbard, C. D. (1966b), *J. Phys. Chem. 70,* 2889–2895.
Hammes, G. G. (1974), Investigation of Rates and Mechanism of Reaction. Part II, 3rd ed, New York, N.Y., Wiley-Interscience, pp 151–158.
Houssier, C., and Kubbal, H. G. (1971), *Biopolymers 10,* 2421–2433.
Li, H. J., and Crothers, D. M. (1969), *J. Mol. Biol. 39,* 461–477.
Muller, W., Crothers, D. M., and Waring, M. J. (1973), *Eur. J. Biochem. 39,* 223–234.
Ramstein, J., Dourlent, M., and Leng, M. (1972), *Biochem. Biophys. Res. Commun. 47,* 874–882.
Ramstein, J., Hogrel, J. F., Dourlent, M., Leng, M., and Hélène, C. (1973), in Dynamic Aspects of Conformation Changes in Biological Macromolecules, Sadron, C., Ed., Dordrecht, Holland, D. Reidel Publisher, pp 333–347.
Sakoda, M., Hiromi, K., and Akasaka, K. (1971), *Biopolymers 10,* 1003–1012.
Sakoda, M., Hiromi, K., and Akasaka, K. (1972), *J. Biochem. 71,* 891–896.
Schmechel, D. E. V., and Crothers, D. M. (1971), *Biopolymers 10,* 465–480.
Schwarz, G., and Balthazar, R. (1970), *Eur. J. Biochem. 12,* 461–467.
Schwarz, G., and Klose, S. (1972), *Eur. J. Biochem. 29,* 249–256.
Schwarz, G., Klose, S., and Balthazar, R. (1970), *Eur. J. Biochem. 12,* 454–460.
Steenbergen, C., and Mohr, S. C. (1973), *Biopolymers 12,* 791–798.
Sturm, J. (1974), Ph.D. Thesis, Strasbourg.
Thusius, D., Foucault, G., and Guillain, F. (1973), in Dynamic Aspects of Conformation Changes in Biological Macromolecules, Sadron, C., Ed., Dordrecht, Holland, D. Reidel Publisher, pp 271–284.
Tritton, T. R., and Mohr, S. C. (1971), *Biochem. Biophys. Res. Commun. 45,* 1240–1249.
Tritton, T. R., and Mohr, S. C. (1973), *Biochemistry 12,* 905–914.
Vitagliano, V. (1973), *J. Phys. Chem. 77,* 1922–1924.

Circular Dichroism Studies on the α-D-Galactopyranosyl Binding Lectin Isolated from the Seeds of *Bandeiraea simplicifolia*†

Jörgen Lönngren,‡ Irwin J. Goldstein,* and Robert Zand*

ABSTRACT: The conformation of the α-D-galactopyranosyl binding lectin isolated from *Bandeiraea simplicifolia* seeds has been investigated over a broad range of pH in the presence of various solvents by circular dichroism (CD) spectroscopy in the region 200–300 nm. Analyses of the spectra obtained on the native protein show the lectin to contain a considerable proportion of β structure (30–40%). The native conformation was found to be largely insensitive to changes in pH, but was influenced by sodium dodecyl sulfate or trifluoroethanol. Alterations in conformation in the presence of these agents were reflected in the CD spectra and show the presence of α helix under these conditions. These changes in conformation are accompanied by a loss in polysaccharide-precipitating activity. The protein is irreversibly denatured in 8 M urea. Neither removal of the intrinsic calcium ions from the protein nor addition of methyl α-D-galactopyranoside induces any appreciable change in the CD spectra of the protein although the former treatment abolishes the polysaccharide-precipitating capacity of the lectin. The conformational data obtained in the present study are compared with data available from conformational studies of other lectins and leads to the hypothesis that most lectins probably contain β structure as the predominant conformational feature.

The isolation and characterization of an α-D-galactopyranosyl binding lectin from the seeds of *Bandeiraea simplicifolia* were recently reported from this laboratory (Hayes and Goldstein, 1974). The lectin consists of four similar subunits (molecular weight 28 500) held together by noncovalent forces. Each subunit contains one sulfhydryl group and two subunits contain one Ca(II). Both of these structural features are necessary for the lectin to exhibit polysaccharide precipitating activity. The protein molecule has been shown to contain four binding sites (Hayes and Goldstein, 1975).

† From the Department of Biological Chemistry, and Biophysics Research Division of the Institute of Science and Technology, The University of Michigan, Ann Arbor, Michigan 48104. *Received August 12, 1975.* This research was supported in part by U.S. Public Health Service Grants AM 10171 and Rol NS 11923.

‡ On leave from Stockholm University, Arrhenius Laboratory, Department of Organic Chemistry, S-10405 Stockholm, Sweden.