1033.
Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238,* 227.
Sperling, J. (1971), *J. Am. Chem. Soc. 93,* 3839.
Sperling, J. (1976), *Photochem. Photobiol. (in press).*
Sperling, J., and Elad, D. (1971), *J. Am. Chem. Soc. 93,* 967.
Stahl, E. (1969), Thin Layer Chromatography, New York, N.Y., Springer-Verlag, p 899.
Varghese, A. J. (1974), *Photochem. Photobiol. 20,* 339.
von Hippel, P. H., and McGhee, J. D. (1972), *Annu. Rev. Biochem. 41,* 231.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Weintraub, H. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 247.
Wyckoff, H. W. (1968), *Brookhaven Symp. Biol. 21,* 252.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., and Richards, F. M. (1970), *J. Biol. Chem. 245,* 305.

The Nature of Stacking Interactions in Polynucleotides. Molecular States in Oligo- and Polyribocytidylic Acids by Relaxation Analysis†

Dietmar Pörschke

ABSTRACT: The dynamics of the helix–coil transition of single-stranded poly(C) (polyribocytidylate) and CpC (cytidylyl(3′-5′)cytosine) was investigated by an improved cable temperature-jump technique. The single-strand relaxation was characterized by following the ultraviolet (uv) absorbance changes at 248 and 280 nm. Poly(C) and CpC showed single relaxation processes with amplitudes corresponding to those expected from equilibrium melting curves. The relaxation time constants in the range of 25–100 ns were independent of the nucleotide concentration, but strongly dependent upon temperature. Using thermodynamic parameters obtained from circular dichroism (CD) and uv absorbance melting curves, the following rate constants k (at 20 °C, 1.05 M ionic strength, pH 7) and activation enthalpies E_A were calculated for poly(C): helix formation $k_R = 1.11 \times 10^7\ s^{-1}$ ($E_{AR} = 2.6$ kcal); helix dissociation $k_D = 2.1 \times 10^6\ s^{-1}$ ($E_{AD} = 11.9$ kcal). The rate constants obtained for CpC were higher by a factor of about 2 in k_R and 12 in k_D, whereas the activation enthalpies closely corresponded to those found for the polymer. In addition to the single-stranded helix–coil relaxation, poly(C) and CpC exhibit a relaxation process with a time constant below 25 ns and maximum amplitudes at wavelengths $\lambda \geq 285$ nm. The same process is found in cytidine and is attributed to hydration equilibria. The hydration reaction can be considered to be in equilibrium during the entire time range of the helix–coil transition and thus the data obtained for the helix–coil transition can be described by a simple two-state model. The rate parameters indicate the existence of relatively high energy barriers in the helix–coil transition and provide strong evidence against an oscillating dimer model. If there is an ensemble of substates for one of the states (as may be expected for the coil form), the energy difference between the populated substates is small compared with the energy difference between the major conformational states.

The conformation of single-stranded polynucleotides has been studied by nearly all the methods available for the investigation of macromolecular structures. Measurements of uv absorbance, ORD, CD, NMR, light scattering and various other parameters have led to the conclusion that some polynucleotides, like poly(A) and poly(C)[1], exist in a single-stranded helical form with the bases stacked upon each other (Felsenfeld and Miles, 1967; Ts'o, 1974; Bloomfield et al., 1974). The main driving force for the formation of the helix is the stacking interaction between adjacent bases. Data collected for oligonucleotides of various chain lengths showed that the formation of the single-stranded helix is almost uncooperative; i.e., the stacking interaction of two adjacent monomer units is almost independent of the state of their neighbors, and the main interactions leading to the formation of the helix are between consecutive RNA residues (cf. Brahms et al., 1967). A scheme of the single-stranded helix and the coil form is given in Figure 1.

The thermodynamics of the helix–coil transition is usually described by a simple two-state model. However, the validity of the two-state model has been questioned for several reasons and a dynamic structure has been proposed, in which the bases oscillate with respect to one another (Glaubiger et al., 1968; Davis and Tinoco, 1968). Various arguments have been used in favor of one or the other model. However, a clear distinction was not possible on the basis of the available data. This uncertainty simply is due to the fact that all the methods applied hitherto, including NMR, only give an average picture of the polymer conformations since the lifetime of individual conformational states is much shorter than the time resolution of the techniques applied. In order to learn about the number of conformational states and the type of coupling between them, the time resolution of the analyzing technique has to be sufficiently high. In the

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[1] Abbreviations used: CpC, cytidylyl (3′-5′)cytosine; poly(C), polyribocytidylate; CMP, cytidine 5′-monophosphate; poly(A), polyriboadenylate.

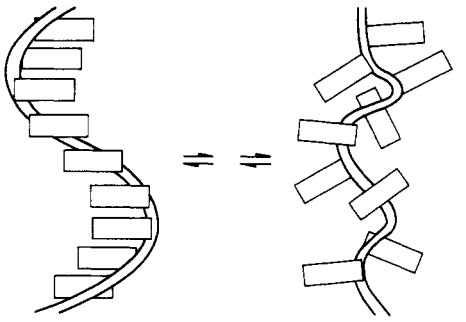

FIGURE 1: Schematic representation of the helix and the random coil form in single-stranded polynucleotides. The transition between these forms is uncooperative. Only the extreme conformations are represented.

case of single-stranded polynucleotides, the time resolution of the conventional temperature-jump technique was not yet sufficient. The development of the cable temperature-jump technique by Hoffman (1971) allowed a higher time resolution. By an improvement of the optical detection system, the sensitivity and time resolution have been further extended (D. Pörschke, in preparation). In the present investigation this technique is applied to the single-stranded helix-coil transition in polyribocytidylate (poly(C)) and cytidylyl(3′-5′)cytosine (CpC). The results clearly demonstrate that an oscillating dimer model for the single-stranded helix-coil transition in cytidylates is not correct.

Materials and Methods

Poly(C) was purchased from Boehringer, Mannheim GmbH (Mannheim, West Germany), and was used after extensive dialysis against 0.1 M sodium cacodylate containing 10^{-3} M EDTA and finally against 0.1 M sodium cacodylate. CpC was obtained from Pharma-Waldhof GmbH (Düsseldorf, West Germany). 3′CMP was the product of Boehringer, Mannheim. All measurements reported in the present investigation were performed in 1 M NaCl, 0.05 M sodium cacodylate, pH 6.9. Nucleotide concentrations were determined spectrophotometrically using the following molar absorption coefficients at 20 °C: poly(C), ϵ_{268} 6300 cm^{-1} M^{-1}; CpC, ϵ_{269} 7900 cm^{-1} M^{-1}.

Absorbance spectra were obtained on a Cary 118 spectrophotometer. Absorbance-temperature profiles were measured by a Zeiss PMQ III spectrophotometer. A Cary 60 spectropolarimeter with CD accessory was used for the CD measurements. Data were corrected for thermal expansion.

The temperature jump relaxation was studied with an improved version of a cable temperature-jump apparatus (D. Pörschke, in preparation). All measurements with the polymer were performed using polarized light with the polarization plane at an angle of 55° with respect to the electric field, in order to avoid any artefacts due to electrodichroism (Labhart, 1961; Pörschke, 1974). The relaxation time constants were evaluated with a simulation box developed by C. R. Rabl. The simulation box allows a very simple and elegant correction of relaxation curves which are obtained in a time range close to the machine or multiplier response time (C. R. Rabl, in preparation).

Results

(*a*) *Uv Spectra and Their Temperature Dependence.* The uv absorbance of cytidine and cytidine phosphates in aqueous solution is strongly dependent upon temperature (cf. Figure 2a). This dependence has been attributed to the existence of a hydrogen-bonded solvent–solute complex at low temperatures and its dissociation at high temperature (Johnson et al., 1971). It would be expected that the same process with a corresponding spectral change is present in all nucleotides containing cytidine.

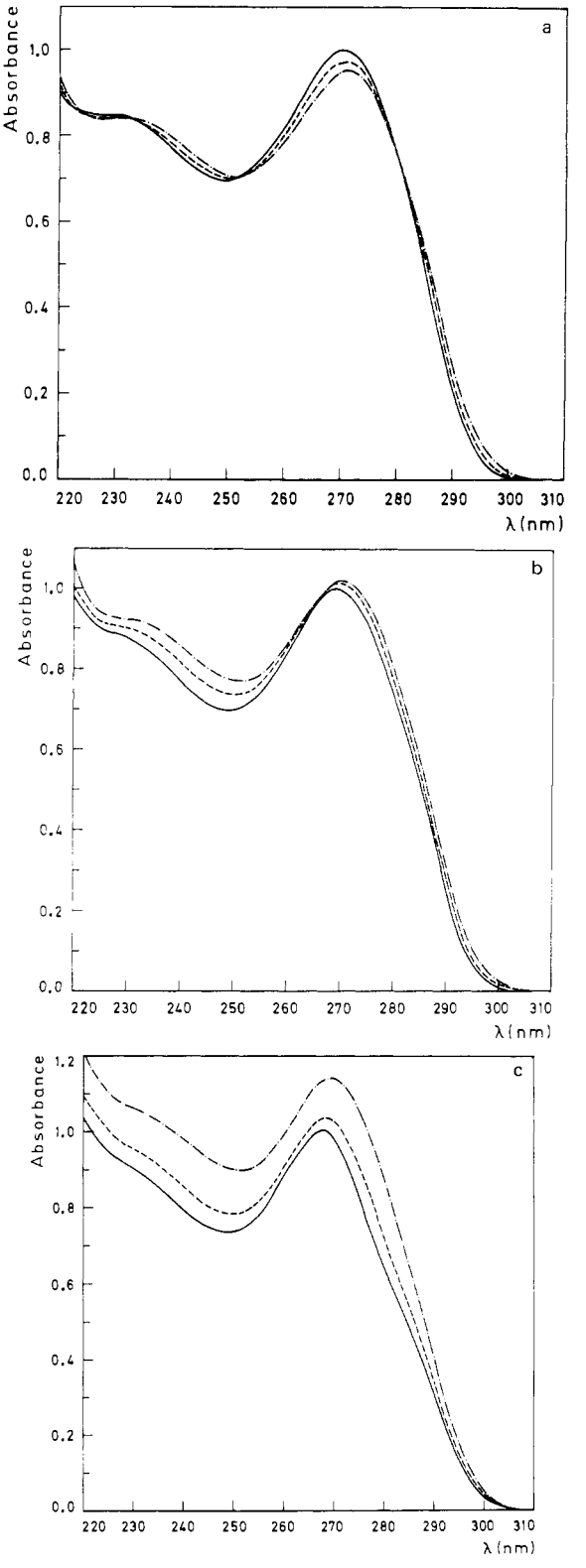

FIGURE 2: Absorbance spectra as a function of the temperature in 1 M NaCl, 0.05 M sodium cacodylate, pH 6.9, 0 °C (-), 30 °C (- - -), 60 °C (-·-). (a) 3′CMP; (b) CpC; (c) poly(C).

The temperature dependence of the CpC and poly(C) ab-

sorbance around 270 nm is opposite to that of CMP (cf. Figure 2b,c). This change in the temperature dependence is due to the interaction between adjacent cytosine bases and the formation of a single-stranded helix. At the wavelengths 252 and 280 nm, corresponding to the isosbestic points in CMP, the absorbance change of CpC and poly(C) should be due to the single-stranded helix–coil transition exclusively. A least-squares analysis of the poly(C) melting curve (cf. Figure 3a) according to a two-state model yielded an enthalpy change of −8.5 kcal/mol and an entropy change of −25.3 eu. For comparison the circular dichroism of poly(C) was also measured as a function of temperature. The temperature dependence of the peak amplitudes around 276 nm (cf. Figure 3b) was analyzed according to a two-state model. The resulting enthalpy change of −9.2 kcal/mol and entropy change of −28.1 eu are in reasonable agreement with the data obtained from absorbance measurements. The accuracy of the thermodynamic data is about ±10%. This uncertainty is mainly due to the rather broad temperature range of the transition and the difficulty in the determination of the reference state parameters.

(*b*) *Relaxation Analysis.* The analysis of temperature-jump relaxation in the fast time range requires the existence of a rather high temperature dependence of the absorbance. Furthermore the intensity of the light used for detection of relaxation processes must be very high, in order to arrive at reasonable signal-to-noise ratios. In the present investigation, the existence of strong mercury lines at the wavelengths of interest (248, 280, and 290 nm) greatly assisted an accurate determination of relaxation times. These favorable conditions together with an improved optical detection system (D. Pörschke, to be published) allowed the characterization of relaxation processes, which belong to the fastest measured by temperature-jump techniques.

The single-stranded helix–coil relaxation was analyzed at the wavelengths 248 and 280 nm close to the isosbestic points observed for the cytosine hydration equilibria. CpC and poly(C) showed relaxation processes in the time range of 25–100 ns, which could be fitted by single exponentials within experimental accuracy. The time constants observed at 248 and 280 nm were the same within experimental accuracy. Relaxation amplitudes corresponded to those expected from equilibrium melting curves. An analysis of the relaxation at 24 °C over a range of nucleotide concentrations from 1.4×10^{-4} to 1.45×10^{-3} M did not reveal any concentration dependence. All these results demonstrate that the observed relaxation process is associated with the intramolecular single-stranded helix–coil transition. Furthermore relaxation time constants determined at pH 8.7 (1 M NaCl, 0.05 M $Na_2B_4O_7$) corresponded to those measured at pH 6.9 (1 M NaCl, 0.05 M sodium cacodylate) within experimental error. Thus there is no pH dependence of the measured relaxation process, indicating that this process is not associated with any protolytic reaction.

In addition to the relaxation process observed at 248 and 280 nm, there is a second one with optimal amplitudes at wavelengths around 290 nm. The second process was found in CpC and poly(C) as well as in cytidine and CMP. Its time constant is beyond the limit of resolution of the present cable temperature-jump technique and thus is smaller than 25 ns. Apparently this process is associated with the hydration of the cytosine residue, which is expected to be a very fast reaction.

At the present state of knowledge, the relaxation observed in CpC and poly(C) may be represented by the following reaction scheme:

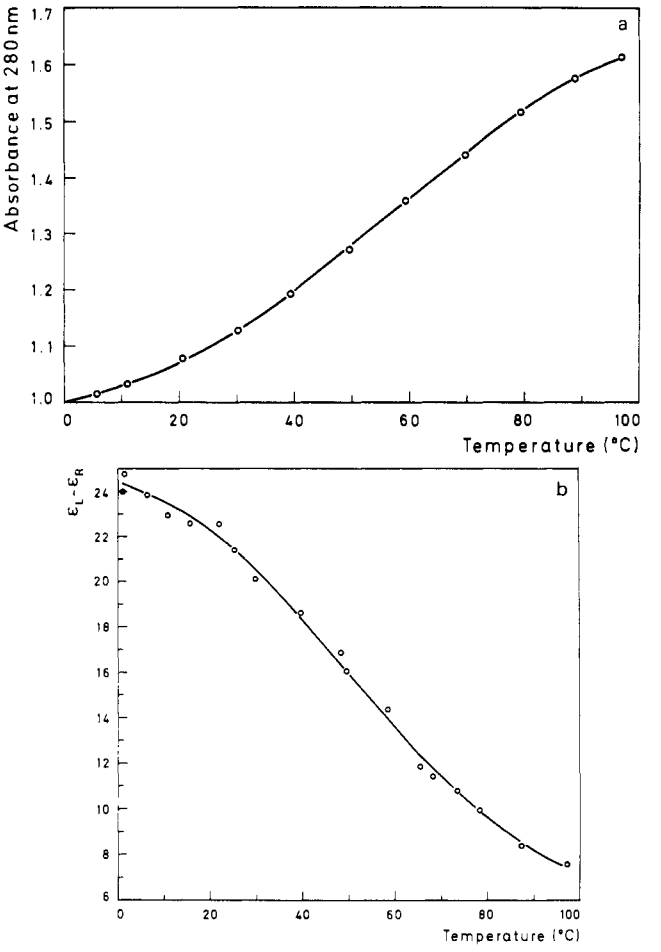

FIGURE 3: Melting curves of poly(C) in 1 M NaCl, 0.05 M sodium cacodylate, pH 6.9. (a) Absorbance at 280 nm as a function of temperature; (b) molar circular dichroism $\epsilon_L - \epsilon_R$ at the maximum around 276 nm as a function of temperature.

$$\begin{array}{ccc} C_{coil} + H_2O & \rightleftharpoons & C_{helix} + H_2O \\ \Updownarrow & & \Updownarrow \\ C_{coil}{\times}H_2O & \rightleftharpoons & C_{helix}{\times}H_2O \end{array}$$

The different hydration states of the coil form need not revert to the helix form with equal facility. There is no information about these details. However, the relaxation measurements demonstrate that the hydration reactions are much faster than the helix–coil reaction. Thus the hydration can be considered to be in equilibrium at all stages of the helix–coil reaction and the reaction scheme may be simplified to:

$$C_{coil} \underset{k_D}{\overset{k_R}{\rightleftharpoons}} C_{helix}$$

The rate constants defined in this way contain preequilibrium terms from hydration reactions. The equilibrium constant $K = k_R/k_D$ corresponds to that obtained from a two-state analysis of melting curves.

(*c*) *Rate Constants and Their Dependence upon Temperature and Chain Length.* The helix–coil relaxation of poly(C) was studied over a broad range of temperatures from 4 to 64 °C. At increasing temperatures, the relaxation rate increases, corresponding to an apparent activation enthalpy of 5.2 kcal/mol. The same temperature dependence is observed for the dimer CpC, although the relaxation at a

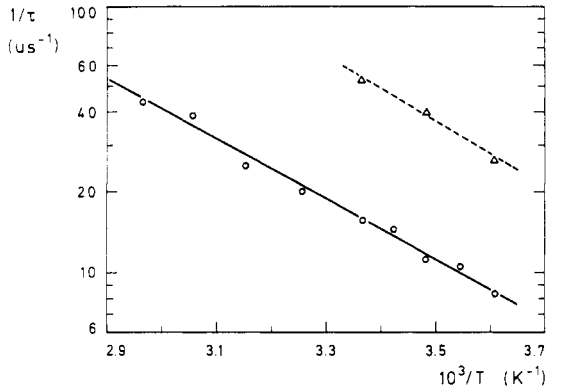

FIGURE 4: Reciprocal relaxation times $1/\tau$ as a function of the reciprocal absolute temperature, poly(C) (○); CpC (△).

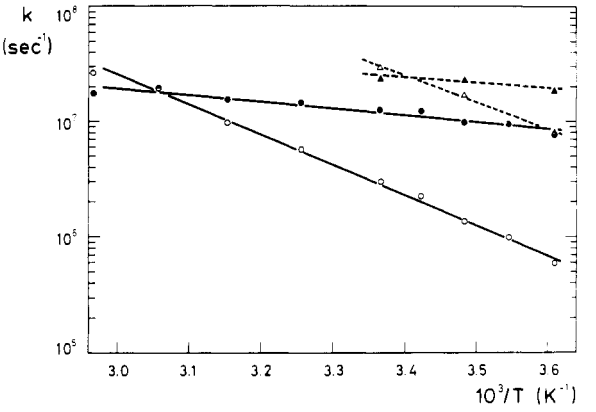

FIGURE 5: Arrhenius plot of the rate constants of helix formation [poly(C) (●); CpC (▲)] and of helix dissociation [poly(C) (○); CpC (△)].

given temperature is faster by a factor of about 3 (cf. Figure 4). For the calculation of the rate constants according to

$$1/\tau = k_R + k_D$$

the equilibrium constant $K = k_R/k_D$ is needed. The equilibrium constants for the polymer were calculated according to the thermodynamic parameters derived from the CD-melting curve (cf. Results, section a), whereas the corresponding data for the dimer were taken from the investigation of Powell et al. (1972; $\Delta H = -8.5$ kcal/mol, $\Delta S = -29.0$ eu). The resulting rate constants are given in Figure 5. For both the dimer and the polymer, the rates of helix formation are characterized by a relatively small temperature dependence, corresponding to activation enthalpies of 2.1 and 2.6 kcal/mol, respectively. However the rates of dissociation are strongly dependent upon temperature, corresponding to activation enthalpies of 10.6 kcal/mol for the dimer and 11.9 kcal/mol for the polymer.

The chain-length dependence is much higher for the rate of helix dissociation than for the rate of helix formation. In CpC the rate of helix dissociation is higher by a factor of about 12 than in poly(C), whereas the corresponding factor for the rate of helix formation is only about 2.

Discussion

The main driving force for the formation of the single strand helix in poly(C) is considered to be the stacking interaction between adjacent cytosine bases. Thus the rates of the helix–coil transition may be compared with the stacking rate of monomer bases in aqueous solution. The data available indicate a very high rate for the simple stacking reaction with relaxation times in the 1–10 ns time range (Pörschke and Eggers, 1972; Garland and Patel, 1974). Compared with these high rates close to the limit of diffusion control, the rate of the helix–coil transition in CpC and poly(C) is rather low, although the transition in CpC and poly(C) is an intramolecular reaction with a high "local concentration" of the reaction partners. The relatively low rates in CpC and poly(C) suggest the existence of considerable energy barriers in the helix–coil transition.

Such energy barriers are clearly not consistent with an oscillation dimer model, which is a currently favored model in the description of the single-stranded helix–coil transition. An oscillating dimer model implies the existence of a continous distribution of molecular conformations starting from the helix to the coil form without any discrete energy barriers. The arguments given previously in favor of the oscillating dimer model turn out to be rather weak upon close inspection. Some authors preferred the oscillating dimer model, because a two-state model is "too simple". It was also argued that the results obtained from the analysis of different system parameters according to a two-state model are not consistent. However, this problem seems to be mainly due to the general difficulty in the analysis of conformation changes extended over broad temperature ranges, which do not allow the derivation of model parameters with high accuracy. In addition Powell et al. (1972) have demonstrated that reagents used in some measurements of melting curves like LiCl at high concentrations may introduce complications due to complexing with the oligo- and polynucleotides. Evidence against a two-state model for the single-stranded helix–coil transition comes from NMR data. NMR measurements were mainly performed with oligoadenylates, where the polymerization shifts of different base protons cannot be explained on the basis of a two-state model (Ts'o, 1974; Kroon et al., 1974). However, these data certainly do not prove an oscillation dimer model. The NMR data may be explained as well by a three-state model. In fact it has been demonstrated that the oscillating dimer model is not consistent with relaxation data obtained for oligoadenylates of various chain lengths. The relaxation data obtained for oligoadenylates suggest a three-state model for the description of their helix–coil transition (Pörschke, 1973).

In summary it must be concluded that the oscillating dimer model does not give a correct description of the single-stranded helix–coil transition. Nevertheless the major conformational states in the transition may be characterized by various substates with an oscillation type of conversion between these substates. However, the energy difference between the major conformational states is much larger than the energy difference between the populated substates within one of the major states.

For the interpretation of the present results, it may be important to consider the hydration process. Although there are no exact data available, it may be expected that the hydration energies are not negligible. There are probably defined hydration spheres (around polar groups) which could favor a certain conformation and suppress the existence of others, although some of the suppressed conformations may be favored in vacuo. Thus the number of states in the conformation space may be reduced by specific hydration.

The relaxation data obtained for CpC and poly(C) show

an interesting contrast to the data obtained for oligo(A) and poly(A) (Pörschke, 1973). In the former system only one relaxation process is associated with the helix–coil transition, whereas two relaxation processes were observed in the latter system. It cannot be excluded that the second process observed in the $(A)_n$ system simply did not show up in the $(C)_n$ system, because it is characterized by a much lower absorbance change in the cytosine chromophore. However, it is more likely that there is a real difference in the number of conformational states. It may be, for example, that the adenylate residues stack upon each other both in the anti and the syn conformation, whereas the cytidylate residues are more strictly bound to the anti conformation. Evidence obtained by various methods indicates that the probability for the formation of syn conformers is lower in cytidylates than in adenylates (Ts'o, 1974; Bloomfield et al., 1974). Apparently the syn conformation in cytidylates is disfavored because of some steric hindrance by the carbonyl group in the 2-position of the cytosine base. Thus it is conceivable that the absence of a second relaxation process in CpC and poly(C) is due to the relatively low population of syn conformers in cytidylates. It is also possible that the different relaxation processes observed in adenylates and cytidylates are due to some difference in the sugar ring puckering. Further experimentation is required to obtain more information about these processes.

The $(C)_n$ and the $(A)_n$ systems are similar with respect to the time range of their helix–coil relaxation, indicating the existence of considerable energy barriers in both helix–coil transitions. From the comparison to the stacking rate of monomer bases, it is apparent that the energy barriers are due to the coupling of base stacking with the folding process of the ribose–phosphate backbone. The relatively low chain-length dependence, in particular, of the rate of helix formation demonstrates that the energy barriers come mainly from the interactions between nearest neighbors. The detailed nature of these barriers remains for further investigation.

At the end of the discussion it should be mentioned that the thermodynamic and kinetic parameters derived in the present investigation are supported by the results of field jump experiments. These experiments were performed at various ionic strengths and demonstrate a coupling of ion binding to the single-stranded helix–coil transition in poly(C) and various other single-stranded polynucleotides (D. Pörschke, in preparation).

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References

Bloomfield, V. A., Crothers, D. M., and Tinoco, I. Jr. (1974), Physical Chemistry of Nucleic Acids, New York, N.Y., Harper & Row.

Brahms, J., Maurizot, J. C., and Michelson, A. M. (1967), *J. Mol. Biol. 25,* 465.

Davis, R. C., and Tinoco, I., Jr. (1968), *Biopolymers 6,* 223.

Felsenfeld, G., and Miles, H. T. (1967), *Annu. Rev. Biochem. 36,* 407.

Garland, F., and Patel, R. C. (1974), *J. Phys. Chem. 78,* 848.

Glaubiger, D., Lloyd, D. A., and Tinoco, I., Jr. (1968), *Biopolymers 6,* 409.

Hoffman, G. W. (1971), *Rev. Sci. Instrum. 42,* 1643.

Johnson, W. C., Jr., Vipond, P. M., and Girod, J. C. (1971), *Biopolymers 10,* 923.

Kroon, P. A., Kreishman, G. P., Nelson, J. H., and Chan, S. I. (1974), *Biopolymers 13,* 2571.

Labhart, H. (1961), *Chimia 15,* 20.

Pörschke, D. (1973), *Eur. J. Biochem. 39,* 117.

Pörschke, D. (1974), *Nucleic Acids Res. 1,* 1601.

Pörschke, D., and Eggers, F. (1972), *Eur. J. Biochem. 26,* 490.

Powell, J. T., Richards, E. G., and Gratzer, W. B. (1972), *Biopolymers 11,* 235.

Ts'o, P. O. P. (1974), in Basic Principles in Nucleic Acid Chemistry, Vol. II, Ts'o, P. O. P., Ed., New York, N.Y., Academic Press, p 305.