Volume 4 Number 5 1977 Nucleic Acids Research

An ethidium-induced double helix of poly(dA)·poly(rU)*

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Received I February 1977

ABSTRACT

Equilibrium dialysis, relaxation kinetic, melting, and continuous variation mixing experiments on complexes of poly(dA) and poly(rU) demonstrate that ethidium induces conversion of a 1:1 mixture of these homopolymers (at one molar salt and 19°C) from a three stranded to a two stranded helix. This is the first demonstration of a double helix of poly(dA)·poly(rU) in solution.

INTRODUCTION

Considerable interest attaches to the study of nucleic acid homopolymers because it allows the effects of base composition and sugar residues on nucleic acid properties to be evaluated. Mixtures of complementary homopolymers frequently are capable of forming several complexes that differ in stoichiometry, and much work has been done to determine their phase diagrams, which express the stability of double and triple helical forms as a function of temperature and salt concentration[1-4]. In addition, drug molecules that bind to nucleic acids have been found to exert an influence on the conformation and stoichiometry of homopolymer complexes[5-7].

Mixtures of poly(dA) and poly(rU) have been reported to form the triple helix poly(dA)·poly(rU)$_2$, but not the expected hybrid double helix poly(dA)·poly(rU)[3]. Bresloff and Crothers[7] showed that the binding of ethidium to such mixtures is also anomalous, with a binding isotherm of complex shape that fits neither the standard Scatchard[8] analysis nor the neighbor exclusion model[9]. Therefore, we have undertaken to clarify the interaction of ethidium bromide (EB) with mixtures of poly(dA) and poly(rU) of varying stoichiometry. The techniques used include equilibrium dialysis, thermal denaturation, optical titration by the continuous variation method, using the absorbance bands in both the visible and ultra violet spectral regions, and the temperature-jump technique. The first three of these techniques are standard methods for distinguishing helical forms, while the last, the temperature-jump method, is based on the expectation that double and triple helix may differ in their

dye-binding kinetics even in cases in which the optical or equilibrium binding properties of the two helices are sufficiently alike to make difficult the detection of conversion from one form to the other. (Earlier work had indicated that nucleic acids differ strongly in their rate of reaction with ethidium[10-12].) To test this new approach we measured ethidium binding kinetics to mixtures of poly(rA) and poly(rU), which provide a model system whose phase diagram is well understood[2,4].

Using these four techniques we found that the ethidium binding isotherm for 1:1 mixtures of poly(dA) and poly(rU) may be understood as an EB-induced conversion from triple to double helix. At low extents of binding the triple helix predominates, but ethidium binds more strongly to the double helix, causing a gradual conversion of triple helix plus poly(dA) to two molecules of double helix as dye binding increases. At high levels of ethidium binding, the transition temperature for conversion of double to triple helix in the 1:1 homopolymer mixture rises above 50°C.

EXPERIMENTAL PROCEDURES

Materials

Ethidium bromide was purchased from the Aldrich Chemical Company. Tests for the purity of this sample were described earlier[11]. Poly(rA) and poly(rU) were purchased from P-L Biochemicals, and poly(dA) was purchased both from P-L Biochemicals and from Collaborative Research. The single stranded polymers were dissolved in buffer I (0.006 M Na_2HPO_4, 0.002 M NaH_2PO_4, 0.001 M Na_2EDTA, 0.001 M sodium cacodylate, pH 7.2) and then extracted with phenol, followed by extraction with ether, bubbling with nitrogen, and then extensive dialysis against buffer I. Concentrating the solutions, when necessary, was achieved by dialysis against a 20% solution of polyethylene glycol in buffer I, followed by dialysis against buffer I. Solutions in buffer II were prepared by mixing 4 volumes of solution in buffer I with 1 volume of 4.932 M $NaNO_3$. Nitrate salts were chosen, in conformance with our earlier work[11], because the more common chloride sometimes damages T-jump cell electrodes. However, the UV absorbance of nitrate restricts the wavelengths available for mixing measurement, so buffer III, used for mixing experiments, contains NaCl instead of the $NaNO_3$ in buffer II.

Nucleic acid concentrations were determined spectrophotometrically in buffer I using the following molar extinction coefficients in terms of nucleotide phosphate: $\epsilon_{257} = 10.5 \times 10^3\ M^{-1}cm^{-1}$ for poly(rA) and $\epsilon_{261} = 9.5 \times 10^3\ M^{-1}cm^{-1}$ for poly(rU)[13]; and $\epsilon_{257} = 8.6 \times 10^3\ M^{-1}cm^{-1}$ for poly(dA)[3]. Helical complexes were formed by mixing, and the product was dialyzed for 24 hours

against buffer I at 90°C.

RNA was stored frozen at -20°C; DNA and DNA-RNA hybrids were stored at 4°C. Stock EB solutions in buffer I, as well as all nucleic acid complexes with EB, were stored at 4°C in the dark until use.

Methods

Determination of binding isotherms in buffer II by equilibrium dialysis has been described by Bresloff and Crothers[7,11,14]. We verified by their ethanol precipitation technique that the extinction coefficient of dye bound to a triple helical structure is the same as that for binding to double helix, ϵ_B = 4150 $M^{-1}cm^{-1}$, buffer II, 19°C, 520 nm[14]. To correct the total free dye concentration, C_F, for EB dimerization, we found a slight modification of their equations to be convenient:

$$\epsilon_D K_D C_M^2 - \epsilon_M C_M - A_F/\ell = 0 \qquad (1)$$

in which ℓ is the path length of the cell, A_F is the measured absorbance of free dye at 520 nm determined from the dialysate, ϵ_D = 6510 $M^{-1}cm^{-1}$ [14] is the extinction coefficient of ethidium dimer (buffer II, 19°C, 520 nm), ϵ_M = 5820 $M^{-1}cm^{-1}$ [14] is the corresponding extinction coefficient for the monomeric free dye, and K_D = 287 M^{-1} [14] is the EB dimerization constant. The quadratic equation (1) was solved to determine the concentration of monomeric free dye, C_M.

Binding data were plotted as is appropriate for the Scatchard equation[8]

$$\frac{r}{C_M} = K_{ap}(B_{ap} - r) \qquad (2)$$

in which r is the ratio of the concentration of bound dye, C_B, to the concentration of nucleic acid, C_N^o, the latter expressed in terms of moles of adenosine nucleotides per liter, unless otherwise indicated. K_{ap} and B_{ap} are respectively the apparent binding constant and number of binding sites per adenosine nucleotide, but these parameters have little significance when the binding isotherm is complex in shape, as in the present case.

Temperature-jump relaxation kinetic studies were performed as previously described, using a 4.6 degree rise to a final temperature of 19.0°C[11,15].

Melting curves were obtained on a Cary 14 recording spectrophotometer, using the 0 - 0.2 OD slidewire. The temperature was controlled by a circulating bath, with ten minutes allowed for equilibration at each point. Cell temperatures were calculated from the bath temperature using a calibration determined in a separate experiment. Melting curves were measured for each sample in both the visible and ultraviolet. 1-cm cuvettes were used for the visible melting curves, with paraffin oil layered over the sample in a water

jacketed, stoppered cuvette, but the high nucleic acid concentrations required for reasonable dye binding forced use of 0.1 mm cylindrical quartz cuvettes for UV melting curves. In such short path-length cells the nitrate absorbance is negligible. The demountable cylindrical cell was lined with silicone grease to prevent leakage and evaporation, and suspended in a thermostatted cell block, whose internal air temperature was monitored with a thermistor. Buffer II was used in all cases as a blank because the free dye concentration changes with temperature, so there is no advantage to using a blank of EB-buffer II at some fixed C_F.

The solvent for mixing curves was buffer III, containing chloride as anion, formed by addition of 2.966 M NaCl to buffer I in a volume ratio of 1:2. Solutions of poly(dA) and poly(rU) containing the same nucleotide concentration (1.2 mM) and the same total ethidium concentration were prepared. Small aliquots (10, 20, or 50 $\mu\ell$) of these polymers were added to a solution of the complementary strand. Following mixing, solutions were allowed to stand at least 6 hours in the dark at 19-20°C. Absorbance values were measured in a 1 mm quartz cuvette, with the cell housing compartment of the spectrophotometer thermostatted at 19.0°C.

RESULTS

Equilibrium binding studies

Figure 1 shows the results of our equilibrium dialysis studies on mixtures of poly(dA) and poly(rU). At low r, the 1:2 mixture binds almost precisely twice as much EB as does the 1:1 mixture at the same free dye concentration. (Notice that on a Scatchard plot a straight line through the origin of slope $1/C_M$ passes through points of constant free dye concentration.) This result suggests that half of the poly(dA) in the 1:1 mixture forms triple helix, with the remainder present as single strand, which does not bind EB appreciably under these conditions. Thus, binding calculated in terms of moles of EB per mole of adenosine nucleotide is only half as large in the 1:1 mixture because the number of binding sites is only half as large as it is in the 1:2 mixture. At high r, however, the 1:2 mixture binds somewhat less dye than the 1:1 mixture at a given C_M, implying that each mixture contains a different species of helix. Therefore, the binding data for the 1:1 mixture are consistent with the interpretation that the local maximum in the isotherm reflects an EB-induced conversion from triple to double helix as binding increases.

In contrast to the complex binding equilibria exhibited in Figure 1, the simpler Scatchard plot for binding EB to triple helical poly(rA)·poly(rU)$_2$ is

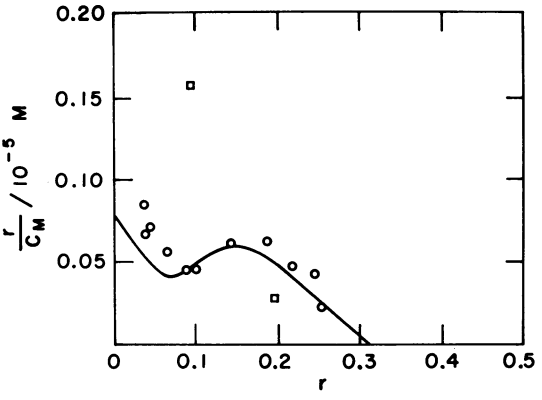

Figure 1. Scatchard plot for equilibrium binding of EB to mixtures of poly(dA) and poly(rU). The solid line is the Scatchard plot determined by Bresloff and Crothers.[7] Circles are the points obtained in this study for a 1:1 mixture, and the squares are points for a 1:2 mixture.

shown in Figure 2. Comparison with the isotherm for binding EB to double helical poly(rA)·poly(rU) under the same conditions[7,14] shows that the double helix binds about 14 times as much dye as the triple helix at the same free dye concentration. Since the triple helix binding isotherm never rises to the level of binding observed for double helix, we can conclude that addition of EB to triple helical poly(rA)·poly(rU)$_2$ in a 1:2 mixture does not cause conversion to the double helix, as also did not occur for triple helical poly(dA)·poly(rU)$_2$ in a 1:2 mixture. Conversion of triple helix to double helix by EB is observed only in the 1:1 mixture of poly(dA) and poly(rU). In the poly(rA) and poly(rU) system this conversion is spontaneous even in the absence of EB.

Melting experiments

Thermal denaturation studies were performed on both the 1:1 and 1:2 mixtures of poly(dA) and poly(rU) at various extents of ethidium binding, with typical results as shown in Figure 3a for a 1:1 mixture and in Figure 3b for a 1:2 mixture. Generally, the melting curves sloped upward at low temperatures because the drug complex dissociates as temperature increases, and the EB absorbance consequently increases at the wavelengths studied.

The 1:1 mixture of the two homopolymers clearly shows a biphasic melting transition, whereas only one transition can be identified for the 2:1 mixture. The upper transition temperatures are the same in the two mixtures, implying that the last structure to melt is the triple helix. The first transition, present in the 1:1 mixture, must therefore be conversion of double helix to the triple helix, which has greater thermal stability. Furthermore, at

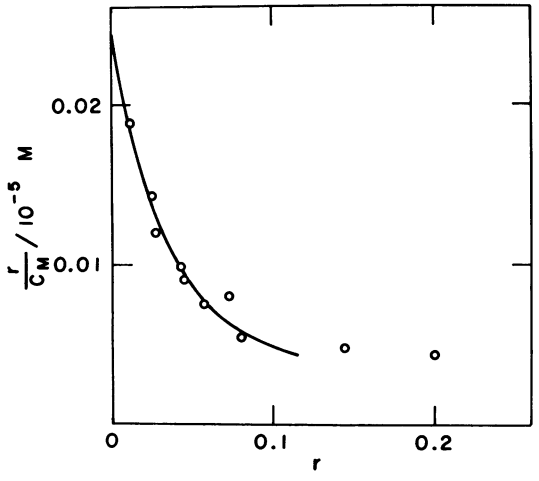

Figure 2. Scatchard plot for equilibrium binding of EB to triple helical poly(rA)·2poly(rU) at 19.0°C in buffer II.

280 nm (near the UV absorbance maximum of EB), and in the visible absorbance band at 430 nm, a decrease in EB binding is observed at the lower transition temperature. This is as expected from the equilibrium binding isotherms, because the triple helix plus poly(dA) binds EB more weakly than two molecules of double helix do. In summary, the transition behavior of mixtures of poly(dA) + poly(rU) in the presence of EB in 1M Na^+ is closely analogous to that observed for poly(rA) + poly(rU) in high salt solutions without ethidium[2].

We found that only the lower transition temperature shows appreciable dependence on ethidium concentration. Qualitatively, we observed that as r decreases that transition both begins and ends at lower temperatures. It also broadens somewhat, and the magnitude of the absorbance change decreases since less EB is being released. We found a good correlation of the melting behavior with the 19°C binding isotherm shown in Figure 1. For example, at r = 0.14 (measured at 19°C), melting begins just below 19°C, indicating, in agreement with the binding isotherm, that the sample is predominantly in the form of double helix at 19°C and r values above 0.14.

Quantitative description of the variation of T_m with ethidium binding requires knowledge of the free ethidium concentration at the melting transition temperature. Specifically, for the transition

$$\underset{\underset{r_d(EB)}{|}}{\text{poly(dA)}\cdot\text{poly(rU)}} \rightarrow \tfrac{1}{2}\ \underset{\underset{r_t(EB)}{|}}{\text{poly(dA)}\cdot\text{poly(rU)}_2} + \tfrac{1}{2}\ \underset{\underset{r_s(EB)}{|}}{\text{poly(dA)}} \qquad (3)$$

$$+\ [r_d - (r_t + r_s)/2]\ (EB)$$

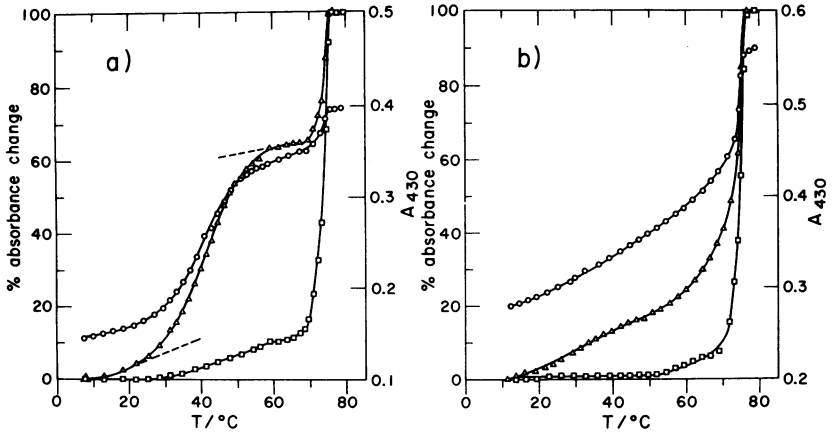

Figure 3. Melting curves for (a) a 1:1 mixture and (b) a 1:2 mixture of poly(dA) and poly(rU) in buffer II. For both cases, r = 0.19 and C_N° = 8.4 x 10^{-4} M at 19°C. Curves at 260 nm (□) and 280 nm (Δ) are in % absorbance change, while that at 430 nm (○) is in absolute OD units. Dashed lines in part (a) indicate the observable beginning and end base lines of the transition.

in which r_x is the moles of EB bound per mole of adenosine in species x, the variation of T_m with the free EB concentration at the T_m, C_{Fm}, is given by the equation[16]

$$\frac{dT_m}{dC_{Fm}} = \frac{-[(r_t + r_s)/2 - r_d]_m}{C_{Fm}\,(\Delta H_F)_m} RT_m^2 \qquad (4)$$

in which the subscript m indicates that all quantities are taken at the transition midpoint. The heat of Reaction (3), which depends on the free ethidium concentration, is symbolized by ΔH_F.

The free ethidium concentration at T_m can be determined with good accuracy by using the assumption that the absorbance of bound dye is negligible at 430 nm, so that all of the absorbance at 430 nm is due to free dye. Determination of the concentration of free dye then requires knowledge of the dye extinction coefficient at all temperatures, which we found could be summarized for ethidium at low concentration in buffer II by the equation

$$\epsilon_{F_{430}} = 2630 - 4.7\,T \qquad (5)$$

when temperature is expressed in °C and the extinction coefficient is given in $M^{-1}cm^{-1}$.

The results of this analysis are shown in Figure 4, which gives T_m for both upper and lower transitions as functions of the free ethidium concentration at T_m. Notice that the lower transition line slopes sharply downward as low r values are approached, confirming the earlier conclusion of Riley, Maling and Chamberlin[3] that the double helix does not exist in a 1:1 mixture (in the absence of ethidium).

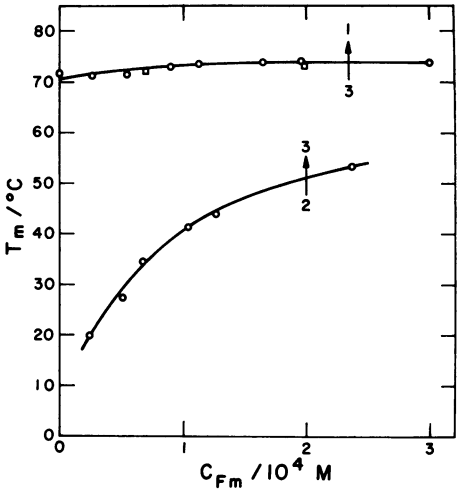

Figure 4. Melting temperature as a function of free dye concentration at the melting point in buffer II. The lower line represents the transition from poly(dA)·poly(rU) to poly(dA)·poly(rU)$_2$, while the upperline is the transition from triple helix to single strands. ○, 1:1 mixture; □, 1:2 mixture.

The transition heat at 19°C can be estimated using Equation (4) and the data in Figures 1 and 4. The left side of Equation (4) is determined graphically by drawing the (approximate) tangent to the curve in Figure 4 at 19°C; C_{Fm} also can be read from this graph. Then, using the assumption that the Scatchard plot for 1:1 mixtures is divisible into regions, representing binding to triple helix plus single strand ($r < 0.06$) and binding to double helix ($r > 0.18$), we made a linear extrapolation of each region of the isotherm into the transition zone. A straight line through the origin, of slope $1/C_{Fm}$, intersects the extrapolated isotherms at $[(r_t + r_s)/2]_m$ and $(r_d)_m$ respectively. Substituting these results in Equation (4) gives $(\Delta H_F)_m = 1.5$ kcal mol^{-1} for the reaction as written in Equation (3), at 19°C in buffer II. This result is close to the value of 1.7 kcal mol^{-1} obtained by Krakauer and Sturtevant[4] for melting of poly(rA)·poly(rU) to the triple helix. Notice that our value includes the heat of releasing approximately 0.07 moles of ethidium, which could contribute as much as half a kilocalorie to the measured heat. Hence it is likely that the enthalpy that stabilizes the double helix of poly(dA)·poly(rU) relative to the triple helix is significantly less than the comparable value for poly(rA)·poly(rU), thus providing a possible thermodynamic basis for the failure to find the double helix in the dye-free system.

Continuous variation experiment

If double helix indeed exists at high extents of ethidium binding, as implied by our equilibrium dialysis and melting experiments, it should be possible to demonstrate the point definitively with a continuous variation experiment[1,2] when ethidium is added to an appropriate concentration. Figure 5 shows the results of such an experiment, performed within the high binding region of the Scatchard plot (r = 0.23 for the 1:1 mixture). The figure shows that the equivalence points observed in the continuous variation titration depend on the wavelength chosen, as frequently is the case[2]. At 520 nm, the absorbance maximum of bound dye, the observed absorbance should be greatest when the amount of double helix is a maximum, because the double helix binds EB more strongly than the triple helix (Figure 1). Indeed there is a broad maximum in A_{520} (Figure 5c) near 50% rU; the data, though somewhat scattered, can be fitted to three lines with intersection points near 50% and 67% rU. At 465 nm the only equivalence point detected is between 40% and 50% rU, whereas at 260 nm only the equivalence point for triple helix, near 67% rU, is evident. Data at 260 nm were also recorded, showing a broad minimum near 50% rU. In all cases the reversibility of the ethidium binding equilibrium causes the titration end points to be less sharply defined than is usually found for polynucleotide strand displacement reactions.

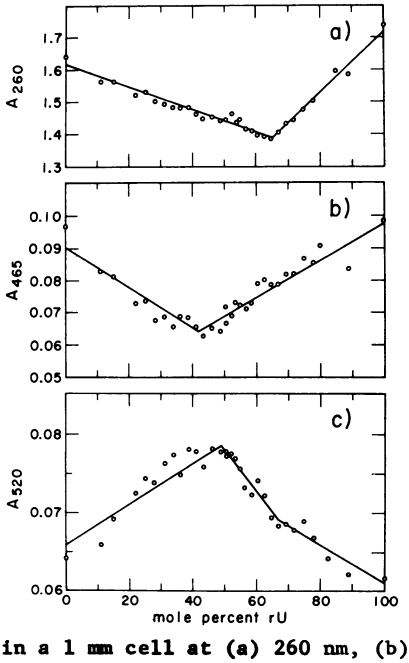

Figure 5. OD measured in a 1 mm cell at (a) 260 nm, (b) 465 nm, and (c) 520 nm for mixtures of poly(dA) and poly(rU). Buffer III, 19.0°C.

Perturbation kinetic studies

Tables 1 and 2 summarize data from the kinetic experiments. In mixtures of poly(rA) and poly(rU), the relaxation times for triple helix are considerably longer, by a factor of 8, than those for double helix at identical nucleotide concentrations. Both the double and triple helices show the two closely coupled relaxation times characteristic of many other nucleic acids, and the very fast relaxation also frequently observed[11,12,14]. Table 1 gives the values of the two slower relaxation times τ_2 and τ_3, along with the ratio of their amplitudes, A_3/A_2, for poly(rA)·poly(rU) and poly(rA)·poly(rU)$_2$.

Table 1. Temperature-jump Results for Mixtures of Poly(rA) and Poly(rU).

	solution	r	relaxation times (msec)	A_3/A_2
(A)	100% double helix	.24	τ_3 = 30.4 τ_2 = 9.1	4.9
(B)	100% triple helix	.03	τ_3 = 252.8 τ_2 = 58.2	10.7
(C)	10% double helix, 90% triple helix	–	τ_ℓ = 183.6	–
(D)	90% double helix, 10% triple helix	–	τ_ℓ = 30.7	–
(E)	50% double helix, 50% triple helix	–	τ_ℓ = 86.7	–

For all solutions, $C_N^\circ = 1.1 \times 10^{-3}$ M in adenosine. $C_M = 2.24 \times 10^{-5}$ M, buffer II, $T_f = 19.0°$, $\Delta T = 4.6°$, $\ell = 0.7$ cm. Solutions (C) and (D) were formed by mixing (A) and (B) in the proportions shown. The nucleic acids for solution (E) were mixed before addition of EB to serve as a control.

Table 2. Temperature-jump Results for Mixtures of Poly(dA) and Poly(rU).

single strands mixed*	C_N°, M	r	τ_ℓ (msec)
(A) 2:2	1.7×10^{-3}	.04	22.3
(B) 1:1	8.4×10^{-4}	.04	31.9
(C) 1:1	8.4×10^{-4}	.24	14.9
(D) 1:2	8.6×10^{-4}	.20	52.5
(E) 1:2	8.6×10^{-4}	.09	32.8
(F) 1:2	4.3×10^{-4}	.09	55.7

Buffer II, $T_f = 19.0°$, $\Delta T = 4.6°$.

*Ratio of mixing, where 1 is 8.4×10^{-4} M, and the relative amount of poly(dA) is given first.

Because one finds multiple relaxations for mixtures of the two- and three-stranded complexes, we report only the longest relaxation time τ_ℓ. This serves as a qualitative indicator of the helix forms in solution, since τ_ℓ continues to decrease as the percent double helix increases.

In contrast, the relaxation times for mixtures of poly(dA) and poly(rU) do not vary so widely (Table 2). However, the results support our earlier conclusions, since the relaxation times tend to be longer for both the 1:2 mixture and under conditions of dye binding where we expect to find the three-stranded structure in a 1:1 mixture.

DISCUSSION

Our results both support and extend several earlier findings. For example, LePecq and Paoletti[17] observed that the fluorescence enhancement of EB in the presence of poly(rA)·poly(rU)$_2$ was less than in the presence of poly(rA)·poly(rU). Similarly, Waring[6], working with several ribohomopolymers at salt concentrations slightly below 0.1 M, has reported that EB stabilizes double helix relative to triple helix. Our results on the hybrid helix show an analogous preference of ethidium for the double helix in comparison to the triple helix. However, the ratio of the EB binding affinities of double and triple helices is much smaller for the hybrid system. Ethidium-induced conformational changes of nucleic acids also are known, for example from the work of Pohl et al.[5] on poly(dG)·poly(dC).

Addition of ethidium means that the physical state of mixtures of poly(dA) and poly(rU) depends on the three variables, temperature, salt concentration and ethidium concentration. Therefore, the complete phase diagram would be a surface in a three-dimensional coordinate system. Figure 4 is the intersection of that surface with a plane of constant salt concentration, at 1M Na^+.

CONCLUSION

Our results indicate that the poly(dA)·poly(rU) double helix can exist in high salt solutions when ethidium is added. Therefore, there is no structural influence that prohibits the hybrid double helix, which is consequently free to form in such processes as transcription from poly(dA) tracts in DNA. The reason that the double helix is not found in homopolymer mixtures is that the thermodynamic balance is shifted slightly in favor of the triple helix plus a single strand of poly(dA), by comparison with mixtures of poly(rA) and poly(rU). The basis for this thermodynamic shift is a more subtle question. It appears from our results that the enthalpy stabilizing the double helix relative to triple helix plus single strand may be smaller for

the hybrid helix. Since that enthalpy is already small (1.5 kcal mol^{-1}) for poly(rA)·poly(rU), an even smaller value will cause the strand equilibrium in poly(dA) + poly(rU) to become nearly independent of temperature, so one cannot force double helix formation by lowering the temperature. It is probably premature to seek structural reasons for the small shift in the relative free energies of double and triple helix in the hybrid system, since the magnitudes involved, which need be only a kilocalorie per mole, are a small fraction of the total interaction energies.

ACKNOWLEDGEMENTS

This work was supported by United States Public Health Service grant number CA 15589 from the National Cancer Institute. E.A.L. was the recipient of summer support from the National Science Foundation Undergraduate Research Participation program.

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