reaction center proteins may cause the observed optical effects, we feel that the size and shape of the difference CD spectrum are most simply explained by the presence of exciton interaction.

The model of a photosystem I reaction center containing two chlorophylls should be regarded as a simplest hypothesis. Because of the presence of a large amount of antenna chlorophyll, we do not know the absorption and CD properties of either the reduced or the oxidized reaction center pigments. This is by contrast with the bacterial reaction center of *Rhodospirillum spheroides* (Sauer *et al.*, 1968), where the CD spectrum of the reduced form provides strong evidence for the participation of at least three bacteriochlorophyll molecules. For some bacterial reaction centers the light-minus-dark difference CD spectra (K. D. Philipson and K. Sauer, in preparation) have rather similar shapes to that observed for the HP700 preparation, except that the signs of the long- and short-wavelength components are reversed. Thus, while it is possible that the HP700 preparation reaction centers contain three instead of two participating chlorophyll molecules, the sign reversal demonstrates that the geometries of the bacterial and higher plant reaction centers are substantially different from one another.

Because of its sensitivity to exciton interactions, CD has proved to be particularly useful in the study of certain photosynthetic systems (Philipson and Sauer, 1972). Moreover, the technique of light-minus-dark CD offers unique advantages for examining photoactive components, even in preparations dilute in these components. Other studies (Sauer *et al.*, 1968; K. D. Philipson and K. Sauer, in preparation) on the absorbance and CD properties of purified reaction centers from photosynthetic bacteria imply the presence of exciton interactions within these systems. Thus, it appears that exciton interactions may play a fundamental role in the operation of both bacterial and green plant reaction centers.

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Thermodynamic and Kinetic Studies of the Interconversion of Linear and Circular $\lambda b_2 b_5 c$ Deoxyribonucleic Acid in the Presence of Purine and Native Ribonuclease A†

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ABSTRACT: The melting temperatures and transition widths of the cohesive ends of $\lambda b_2 b_5 c$ DNA have been measured in the presence of varying concentrations of two helix-destabilizing ligands, purine and native ribonuclease A. The depression of the melting point in the presence of purine is in agreement with a model where purine binds only single-stranded DNA with one purine per nucleotide. The enthalpy of the cyclization equilibrium is unaffected by the presence of purine. The depression of the melting point in the presence of ribonuclease A is in agreement with a model of greater binding to denatured than native DNA with one protein molecule bound per cohesive end. The enthalpy of binding of ribonuclease A to denatured DNA is about −25 kcal/mole and to native DNA is about −13 kcal/mole. The rate of cyclization of $\lambda b_2 b_5 c$ DNA was measured in the presence of purine and ribonuclease A. In the presence of purine, the rate is reduced with no change in the enthalpy of activation. In the presence of ribonuclease A, no changes in cyclization rates were observed.

Ligands which bind to DNA may be classified as stabilizers or destabilizers. Stabilizers increase the stability of native DNA relative to denatured DNA, thus raising the melting temperature. Many positively charged planar dyes and positively charged proteins, as histones, are known to be stabi-

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lizers. Destabilizing ligands, which lower the melting temperature of DNA, include nucleosides (Ts'o *et al.*, 1962; Sander and Ts'o, 1970), native ribonuclease A (Felsenfeld *et al.*, 1963; Kosaganov and Lazurkin, 1967), the product of gene 32 of bacteriophage T4 (Alberts and Frey, 1970), and related proteins.

λ DNA may exist as a linear duplex with two complementary cohesive ends or as an open circular duplex (Hershey and Burgi, 1965). Wang and Davidson (1966a,b) have studied the equilibrium and kinetics of the interconversion between linear λb_2b_5c DNA and circular λb_2b_5c DNA. From these studies, they concluded that the cohesive ends contained 10 ± 2 base pairs. Wu and Taylor (1971) have recently determined the sequence of the twelve base pairs of the cohesive ends.

In this work, we describe thermodynamic and kinetic studies of the effect of two weakly binding destabilizing ligands, purine and ribonuclease A, on the interconversion between linear and circular λb_2b_5c DNA. In general, thermodynamic studies of the static structure of DNA give two parameters, the melting point and the transition temperature width at the melting point. These two parameters reflect many properties of DNA structure, such as G + C content, the distribution of nucleotide pairs in the chain, and the interaction of the native and denatured forms with salt, solvent, or ligands (Lazurkin *et al.*, 1970; Crothers, 1971). The advantages of using λb_2b_5c DNA cyclization as a probe to study the effects of ligand binding are that an all or none model may be used for both the kinetics and equilibria, that the sequence of the DNA is known, and that the melting point of the cohesive ends is relatively low, allowing study of protein binding without protein denaturation.

The effects of purine on the melting points of poly(A)·poly(U), calf thymus DNA, and tRNA have been studied (Ts'o *et al.*, 1962; Sander and Ts'o, 1970). Equilibrium dialysis experiments (Ts'o and Lu, 1962) have shown that purine analogs bind almost exclusively to denatured DNA. Nuclear magnetic resonance (nmr) studies (Bangerter and Chan, 1968; Chan *et al.*, 1966) of the binding of purine to poly(U) or to dinucleotides suggest that the mode of purine binding involves intercalation between nucleic acid bases.

The interaction between native ribonuclease A and DNA has been studied by a number of methods including inhibition of RNA hydrolysis, DNA melting in low salt (Felsenfeld *et al.*, 1963; Kosaganov and Lazurkin, 1967), sedimentation (Kosaganov *et al.*, 1967), and gel filtration (Raju and Davidson, 1969). The results of these studies are summarized below. (1) The binding of ribonuclease to native or denatured DNA is a function of the salt concentration. (2) The binding constant of ribonuclease to DNA is a function of pH. The binding constant is tenfold higher at pH 7 than at pH 8. (3) The ratio of binding constants to native and denatured DNA is a function of ionic strength. At 0.0004 M NaCl or below, ribonuclease binds stronger to native DNA. Above 0.001 M NaCl, this effect is reversed. The ratio of binding constants to denatured and native DNA is about 8 in 0.052 M Na^+, pH 7.5, 25°. (4) Ribonuclease binds to about twelve DNA nucleotides.

Materials and Methods

DNA Preparation. A stock solution of λb_2b_5c phage and a slant of *Escherichia coli* K 12 W3110 were kindly provided by Professor J. C. Wang. Phage were prepared by infection of a logarithmically growing *E. coli* culture ($A_{650} = 0.4$) in tryptone broth plus 0.001 M $CaCl_2$ with one phage per five bacteria. After lysis, 1 ml of chloroform was added per 100 ml of culture and solid NaCl was added to produce a concentration of 2 M. The phage were purified by differential centrifugation and banding in CsCl (density 1.50 g/cm³), 0.01 M Tris-OH plus HCl (pH 7.8), and 0.0025 M $MgSO_4$ in an SW 50 rotor at 40,000 rpm for 18 hr at 20°. The phage band was removed with a syringe and dialyzed against 0.01 M Tris-OH plus HCl (pH 7.8), 0.0025 M $MgSO_4$, and 0.015 M NaCl.

λb_2b_5c DNA was prepared by the phenol extraction procedure of Mandell and Hershey (1960) followed by dialysis against 0.001 M EDTA–0.01 M phosphate buffer (pH 7.2) plus 0.2 or 2.0 M NaCl.

Ligand Concentrations. Purine was purchased from Boehringer Mannheim Corp., N. Y., and used without further purification. Purine is known to self-associate in solution (Ts'o *et al.*, 1963). The self-association constant, K, is about 2.1 M^{-1}. The concentration of purine monomers, P_1, in a solution of stoichiometric molarity, m, may be computed from

$$m = \frac{P_1}{(1 - KP_1)^2} \quad (1)$$

Ribonuclease A was purchased from Worthington Biochemical Corp. and used without further purification. The concentration of ribonuclease was determined from the absorbance at 278 nm. The extinction coefficient at 278 nm is 9800 (Raju and Davidson, 1969).

Incubations. λ DNA at a concentration of 5 μg/ml in 0.2 M NaCl (or 2.0 M NaCl) and 0.001 M Na_3EDTA–0.01 M phosphate buffer (pH 7.2) plus purine at varying concentration was used for all purine incubations. For incubations with ribonuclease, the solvent contained 0.1 M NaCl, 0.01 M Tris-OH plus HCl, and 0.001 M Na_3EDTA (pH 7.02). Solutions were incubated in sealed vessels in a stirred constant-temperature bath. Reactions were stopped by quenching the samples in ice–water.

For purine experiments, the samples were then dialyzed at 4° against 0.001 M Na_3EDTA–0.01 M phosphate buffer (pH 7.2) to remove purine and to prevent any reactions during manipulations at 20° for the subsequent analysis. For ribonuclease experiments, the samples were analyzed directly. Results of band velocity runs have shown that the ratio of circular to linear λb_2b_5c DNA was not affected by storage or dialysis following incubation.

Band Velocity Sedimentation. The relative amounts of linear and circular λb_2b_5c DNA were determined by band velocity sedimentation (Wang and Davidson, 1966a). Velocity runs were performed with 30-mm type III band centerpieces containing 3.0 M CsCl and 0.01 M Tris-OH plus HCl (pH 7.8) in a Beckman Model E analytical ultracentrifuge equipped with a high-intensity light source and monochromator at 26,000 rpm at 20°. Pictures were taken on Kodak commercial film and traced on a Joyce-Loebl micro densitometer with a linear density wedge. The relative amounts of linear and circular λ DNA were calculated from the ratio of the areas under the curves of linear and circular DNA, respectively.

Theoretical

The equilibrium constant for the interconversion of linear, [L], and circular, [C], λb_2b_5c DNA is given by (Wang and Davidson, 1966a,b)

$$K = \frac{[C]}{[L]} = j\sigma S_{A\cdot T}{}^{2} S_{G\cdot C}{}^{10} S_{stack} = j\sigma S_0{}^{n} = j\sigma S_0{}^{12} \quad (2)$$

where $S_{A\cdot T}$ is the equilibrium constant for chain extension of A·T base pairs, $S_{G\cdot C}$ is the same constant for G·C base pairs, σ is the nucleation parameter, j is the Jacobson–Stockmayer factor, and S_{stack} is an additional constant for stacking at the ends of the cohesive ends. S_0 is an average value of the equilibrium constants of the various A·T and G·C pairs plus the stacking interaction. Wang and Davidson (1966a) used this equation to predict, correctly, the number of base pairs in the cohesive ends of λb_2b_5c DNA.

In the presence of ligand, P, the equilibrium constant will be changed. Consider binding to linear λb_2b_5c DNA

$$\begin{gathered} L + P \rightleftarrows LP \\ LP + P \rightleftarrows LP_2 \\ LP_{n-1} + P \rightleftarrows LP_n \end{gathered} \tag{3}$$

If we let K_{1i} denote the equilibrium constant for binding to a class of sites, i, and X_{1i} denote the number of i class binding sites, then the total concentration of linear DNA is given by

$$[L]_{total} = [L](1 + k_{11}[P])^{X_{11}}(1 + K_{12}[P])^{X_{12}} \ldots (1 + K_{1m}[P])^{X_{1m}} \tag{4}$$

where [L] is the concentration of linear DNA with no ligand bound to the cohesive ends and [P] is the concentration of unbound ligand. A similar result holds for circular λ DNA, where K_{2i} is the equilibrium constant and X_{2i} is the number of i class binding sites

$$[C]_{total} = [C](1 + K_{21}[P])^{X_{21}}(1 + K_{22}[P])^{X_{22}} \ldots (1 + K_{2n}[P])^{X_{2n}} \tag{5}$$

where [C] is the concentration of circular λ DNA without any ligand bound to the helical cohesive ends. The equilibrium constant is then given by

$$K' = \frac{[C]_{total}}{[L]_{total}} = \frac{[C](1 + K_{21}[P])^{X_{21}}(1 + K_{22}[P])^{X_{22}} \ldots (1 + K_{2n}[P])^{X_{2n}}}{[L](1 + K_{11}[P])^{X_{11}}(1 + K_{12}[P])^{X_{12}} \ldots (1 + K_{1m}[P])^{X_{1m}}} \tag{6}$$

Since

$$\frac{[C]}{[L]} = j\sigma S_0{}^{12} \tag{7}$$

if we assume j does not change in the presence of ligand

$$K' = j\sigma S_0{}^{12}\, y^{12} = j\sigma S_1{}^{12} \tag{8}$$

where a new base pairing constant in the presence of ligand, S_1, is defined as the product of S_0 and y, where

$$y^{12} = \frac{(1 + K_{21}[P])^{X_{21}} \ldots (1 + K_{2n}[P])^{X_{2n}}}{(1 + K_{11}[P])^{X_{11}} \ldots (1 + K_{1m}[P])^{X_{1m}}} \tag{9}$$

At the melting temperature without ligand, $T_m{}^0$

$$j\sigma S_0{}^{12} = 1 \text{ or } S_0 = \left(\frac{1}{j\sigma}\right)^{1/12} \tag{10}$$

At the melting temperature with ligand, $T_m{}^1$

$$j\sigma S_0{}^{12} y^{12} = 1 \text{ or } S_0 = \left(\frac{1}{j\sigma}\right)^{1/12} \frac{1}{y} \tag{11}$$

If Δh_0 is the average enthalpy of base pair formation of λb_2b_5c DNA cohesive ends without ligand, then

$$\frac{\partial \ln S_0}{\partial T} = \frac{\Delta h_0}{RT^2} \tag{12}$$

We may calculate

$$\int_{T_m{}^0}^{T_m{}^1} d\left(\frac{1}{T}\right) = \frac{-R}{\Delta h_0} \int_{\left(\frac{1}{j\sigma}\right)^{1/12}}^{\left(\frac{1}{j\sigma}\right)^{1/12}\frac{1}{y}} d \ln S_0 \tag{13}$$

giving

$$\frac{1}{T_m{}^1} - \frac{1}{T_m{}^0} = \frac{R}{12\Delta h_0} \ln y^{12} \tag{14}$$

$$\frac{1}{T_m{}^1} - \frac{1}{T_m{}^0} = \frac{-R}{12\Delta h_0} \times \ln \frac{\{(1 + K_{11}[P])^{X_{11}}(1 + K_{12}[P])^{X_{12}} \ldots (1 + K_{1m}[P])^{X_{1m}}\}}{\{(1 + K_{21}[P])^{X_{21}}(1 + K_{22}[P])^{X_{22}} \ldots (1 + K_{2n}[P])^{X_{2n}}\}} \tag{15}$$

A study of the change in melting temperature of the ends of λb_2b_5c DNA in the presence of ligand should give the number and strength of the ligand binding sites.

Another kind of information which, in principal, may be obtained is the enthalpy of the binding reaction between ligand and denatured DNA. The transition width, $\Delta T_{1/2}$ may be defined in terms of θ, the fraction of linear molecules

$$\Delta T_{1/2} = \left(\frac{1}{\partial\theta/\partial T}\right)_\theta = \frac{1}{2} \tag{16}$$

Since

$$\theta = \frac{j\sigma S_0{}^n}{1 + j\sigma S_0{}^n} \tag{17}$$

$$\Delta T_{1/2} = \frac{1}{\left(\frac{\partial \ln S_0}{\partial T}\right)\left(\frac{\partial\theta}{\partial \ln S_0}\right)} = \frac{4RT_m{}^2}{n\Delta h_0} \tag{18}$$

In the presence of ligand

$$\Delta T_{1/2} = \frac{1}{\left(\frac{\partial \ln S_1}{\partial T}\right)\left(\frac{\partial\theta}{\partial \ln S_1}\right)} = \frac{4RT_m{}^1}{n\Delta h_1} \tag{19}$$

where

$$\frac{\Delta h_1}{RT^2} = \frac{\partial \ln S_1}{\partial T} = \frac{\partial}{\partial T} \ln S_0 y \tag{20}$$

For $n = 12$

$$\Delta h_1 = \Delta h_0 - \frac{1}{12}\left(\sum_{i=1}^{m}\left(\frac{K_{1i}[P]}{1 + K_{1i}[P]}\right) X_{1i}\Delta h_{1i}\right) + \frac{1}{12}\left(\sum_{i=1}^{n}\left(\frac{K_{2i}[P]}{1 + K_{2i}[P]}\right) X_{2i}\Delta h_{2i}\right) \tag{21}$$

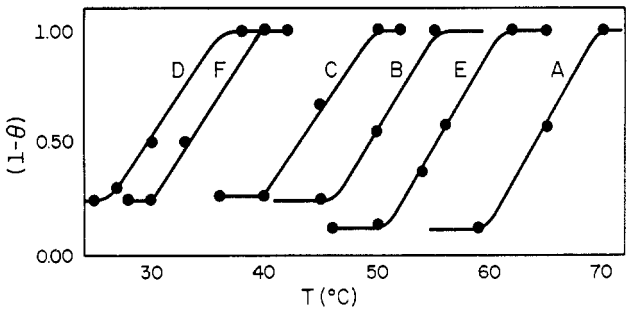

FIGURE 1: The mole fraction of linear λb_2b_5c DNA ($1 - \theta$) as a function of temperature. Graphs A, B, C, and D were obtained in 2.0 M NaCl with purine concentrations 0, 0.104, 0.208, and 0.416 M, respectively. Graphs E and F were obtained in 0.2 M NaCl with purine concentrations 0 and 0.208 M, respectively.

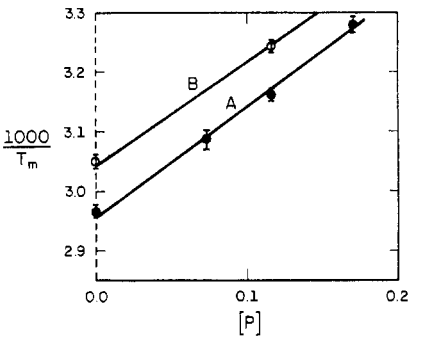

FIGURE 2: The inverse of the melting temperature of λb_2b_5c DNA cohesive ends as a function of purine concentration. Graphs A and B were obtained in 2.0 and 0.2 M NaCl, respectively.

where Δh_{1i} or Δh_{2i} is the enthalpy of binding of ligand to a class i site of helix or coil cohesive ends, respectively. A study of the transition widths in the presence of varying concentrations of ligand should give the enthalpies of binding of ligand to native and denatured DNA.

From eq 12 and 13 we may calculate the concentration of free ligand at which $(\Delta T_{1/2}/T_m^2)$ is maximum by calculating the extreme values of Δh_1.

For the special case

$$K_{2i} = K_2,\ n = 1,\ X_{2i} = 2,\ \Delta h_{2i} = \Delta h_2$$
$$K_{1i} = K_1,\ m = 1,\ X_{1i} = 2,\ \Delta h_{1i} = \Delta h_1 \qquad (22)$$

the concentration at which $(\Delta T_{1/2}/T_m^2)$ is maximum is given by

$$[P] = \frac{(rq)^{1/2} - 1}{K_2(r - (rq)^{1/2})} \qquad (23)$$

where

$$r = K_1/K_2,\ q = \Delta h_1/\Delta h_2 \qquad (24)$$

Equation 23 may be used to check the binding properties obtained from measurements of melting points and transition widths.

Finally, we should mention that Applequist and Damle (1965) have defined the transition width for oligonucleotide binding and that a similar result may be obtained, as is applicable in the case of λ DNA dimerization, that

$$\Delta T_{1/2} = \frac{6RT_m^2}{n\Delta h_0} \qquad (25)$$

Equation 21 would still hold for this case.

Results and Discussion

Equilibrium Effects of Purine. Figure 1 shows the mole fraction of linear λb_2b_5c DNA as a function of temperature for various concentrations of purine in solutions containing 0.2 or 2.0 M Na^+. All points shown were obtained at equilibrium as judged by separate kinetic measurements of the slowest rates. The melting point of λb_2b_5c DNA cohesive ends is defined as the temperature at which one-half of the total DNA, excluding some DNA which cannot cyclize, is present as circular DNA. The fraction which cannot cyclize depends on the process of preparation and the time of storage. Table I shows the melting point data. The concentration of purine monomer was calculated using eq 1 and assuming the association constant of purine to be 2.1 M^{-1}.

Figure 2 shows $(1/T_m)$ for various purine monomer concentrations. If we assume that purine binds only to single-stranded DNA with a single class of binding constant, we may expand eq 15 to give

$$\Delta\left(\frac{1}{T_m}\right) = \frac{xR}{12\Delta h_0}K_1[P_1]\ldots \qquad (26)$$

From the straight line fit in Figure 2, we may calculate the product of the number of binding sites, x, and the binding constant of purine with denatured DNA, K_1, knowing 12 Δh_0 to be 88 ± 10 kcal (Wang and Davidson, 1966a). With a weak interaction of this type, x and K_1 cannot be obtained separately from the melting temperature data with much precision. The product of K_1 x is 87 ± 10. If we assume that there are 24 sites, one per nucleotide in the cohesive ends, then the binding constant would be 3.6 ± 0.6. This value is close to the value estimated by Sander and Ts'o (1970).

The depression of the melting point of λb_2b_5c DNA cohesive ends is similar in 0.2 or 2.0 M Na^+. This result is consistent with a model of purine binding between DNA bases due to hydrophobic stacking interactions.

The melting point of tRNA is lowered by 20, 25, and 30° in the presence of 0.2, 0.3, and 0.4 M purine (Sander and Ts'o,

TABLE I: Melting Point of λb_2b_5c DNA Cohesive Ends in the Presence of Varying Concentration of Purine.

Na^+ (M)	Purine (M)	Purine Monomer (M)	T_m (°C)	$10^3/T_m$
2	0.416	0.17	31.5 ± 1	3.28 ± 0.01
2	0.208	0.116	44 ± 1	3.16 ± 0.01
2	0.104	0.073	51 ± 1	3.08 ± 0.01
2	0	0	64 ± 1	2.97 ± 0.01
0.2	0.208	0.116	35 ± 1	3.24 ± 0.01
0.2	0	0	55 ± 1	3.05 ± 0.01

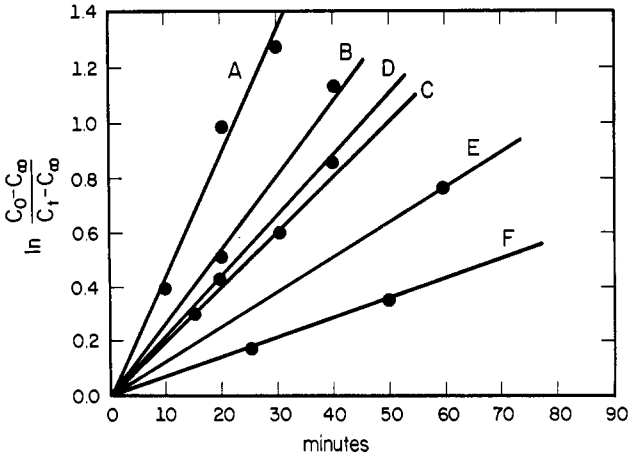

FIGURE 3: Rate plots for λb_2b_5c DNA cyclization with purine in 2.0 M NaCl. Graphs A, B, and C were obtained with 0.05 M purine at 50.0, 45.8, and 40.6°, respectively. Graphs D, E, and F were obtained with 0.1 M purine at 45.8, 40.6, and 35°, respectively.

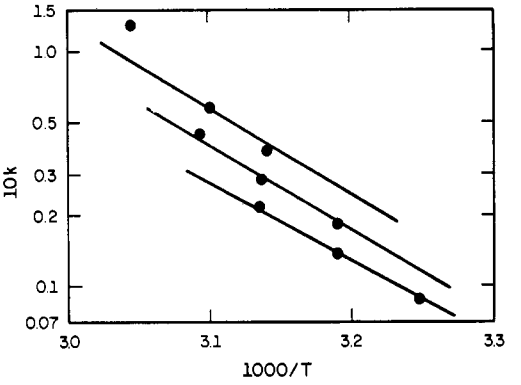

FIGURE 4: Activation energy determination for λb_2b_5c DNA cyclization rates in the presence of purine in 2.0 M NaCl. Graphs A, B, and C show rates obtained in 0, 0.05, and 0.1 M purine, respectively. Control data points in graph A are taken from Wang and Davidson (1966a).

1970). Our results are similar to the tRNA results, suggesting that DNA and RNA molecules behave similarly in the presence of purine. Most tRNA molecules have eight nucleotide bases in the longest helical region. Both tRNA melting (Yang *et al.*, 1972) and the melting of the ends of λb_2b_5c DNA may be considered to be all-or-none processes. These results differ from results of melting of long native DNA molecules (Ts'o *et al.*, 1962; Sander and Ts'o, 1970), where purine has less effect on melting temperatures than in either of these cases.

The value of the transition width, $\Delta T_{1/2}$ does not change in the presence of purine. $\Delta T_{1/2}$ is about 9°, in agreement with Wang and Davidson (1966a) for a length of 12. Wu and Kaiser (1968) have decreased the length of the ends to 8 or 9 and have observed an increase in the transition width to about 13°, in agreement with the width calculated using eq 17. The observed widths in this work confirm that partial degradation of the ends of λb_2b_5c DNA has not occurred during preparation or storage. We might also mention that the transition width for the bimolecular reaction between half-molecules of λb_2b_5c DNA studied by Wang and Davidson (1966b) is about 13.5°, in agreement with results predicted by comparison of eq 25.

Kinetics of λb_2b_5c DNA Cyclization in the Presence of Purine. Wang and Davidson (1966a) have studied the rate of cyclization of λb_2b_5c DNA. The equilibrium between linear and circular molecules may be given by

$$L \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C \qquad (27)$$

where k_f and k_b are the forward and reverse rate constants. Let C_t be the concentration of circular molecules at time t. A plot of ln $(C_0 - C_\infty)/(C_t - C_\infty)$ *vs.* time gives a slope $(k_f + k_b)$ which is almost equal to k_f at temperatures well below the melting point of the cohesive ends. Figure 3 shows data for the determination of several rate constants, excluding the fraction which will not cyclize. The calculated rate constants are given in Table II. Figure 4 shows the temperature dependence of λb_2b_5c DNA cyclization rates in the presence of 0.0, 0.05, and 0.1 M purine. The kinetic rate was reduced by the addition of purine with no change in the activation energy of 17 kcal/mole. The reduction in rate was 30% in 0.05 M purine and 50% in 0.1 M purine. This reduction in rate may be due to purine interference with nucleation. Lee and Wetmur (1972) have shown that the formation of the second base is the rate-determining step. The probability of purine binding to each nucleation site under the conditions studied is comparable to the reduction in rate. We cannot, however, rule out other explanations for the effect of purine on cyclization rates.

Equilibrium Effects of Ribonuclease A. Melting curves for the cohesive ends of λb_2b_5c DNA in the presence of varying concentrations of ribonuclease A are shown in Figure 5. A plot of change in melting temperature, ΔT_m, versus ribonuclease concentration is shown in Figure 6. The change in melting temperature approaches a constant value as the concentration of ribonuclease goes to infinity. From this result, we may conclude that ribonuclease binds to both native and denatured cohesive ends of λb_2b_5c DNA with the same number of ribonuclease molecules bound per nucleotide. Ribonuclease is a kidney-shaped molecule with dimensions 38 × 28 × 22Å (Kartha *et al.*, 1967). Raju and Davidson (1969) found that ribonuclease binds to 12 nucleotides, which is reasonable considering the size of the molecule. In the case of the ends of λb_2b_5c DNA, this means that one ribonuclease binds per single-stranded cohesive end in either the denatured or native state.

TABLE II: Rate Constant for Cyclization of λb_2b_5c DNA in 2 M NaCl in the Presence of Purine.

Purine (M)	T (°K)	k (min^{-1})
0.1	308	0.00825
0.1	313.6	0.0136
0.1	318.8	0.022
0.05	313.6	0.018
0.05	318.8	0.028
0.05	323	0.046
0.0[a]	318	0.038
0.0	323	0.057
0.0	328	0.13

[a] From Wang and Davidson (1966a).

TABLE III: Thermodynamic Parameters from the Interconversion of Linear and Circular $\lambda b_2 b_5 c$ DNA in the Presence of Ribonuclease.

RNase (M)	T_m (°C)	$10^3/T_m$	ΔT_m	K_1[a]	K_2[a]	$\Delta T_{1/2}$	$\frac{\Delta T_{1/2}}{T_m^2} \times 10^5$	$12\Delta h_1$[b] (kcal)	Calcd $12\Delta h_1$[c] (kcal)
0	48.7 ± 0.2	3.108	0			9.3	9.05	88	88
1.16×10^{-4}	47.4 ± 0.2	3.121	1.3	3.4×10^3	9.7×10^2	11	10.7	74	78
5.8×10^{-4}	45.5 ± 0.2	3.140	3.2	4.15×10^3	1.05×10^3	13.5	13.3	59.5	62
1.74×10^{-3}	43.2 + 0.2	3.162	4.5	5.6×10^3	1.23×10^3	13	13	61	61

[a] K_1 and K_2 are the binding constants between ribonuclease and each cohesive end in linear and circular $\lambda b_2 b_5 c$ DNA, respectively. The binding enthalpies are −25 and −13 kcal per mole, respectively. [b] Total experimental enthalpies of cyclization of $\lambda b_2 b_5 c$ DNA calculated from the melting point and transition width using eq 17 and 18. [c] Calculated total enthalpies of cyclization of $\lambda b_2 b_5 c$ DNA obtained by using the binding parameters and eq 20.

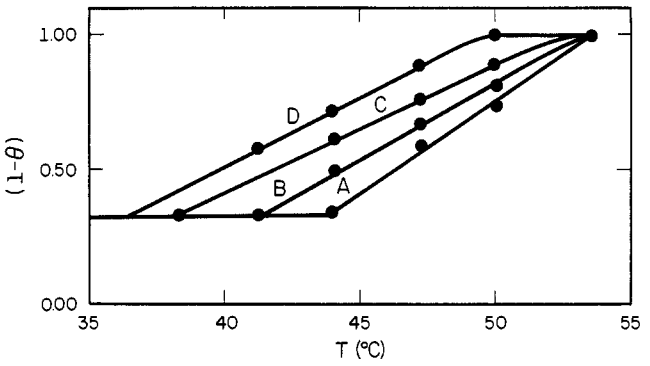

FIGURE 5: The mole fraction of linear $\lambda b_2 b_5 c$ DNA ($1 - \theta$) as a function of temperature. All solutions were 0.1 M NaCl, 0.001 M EDTA, and 0.01 M Tris-OH plus HCl (pH 7.02). Graphs A, B, C, and D were obtained with 0, 1.16×10^{-4}, 5.8×10^{-4}, and 1.74×10^{-3} M ribonuclease A, respectively.

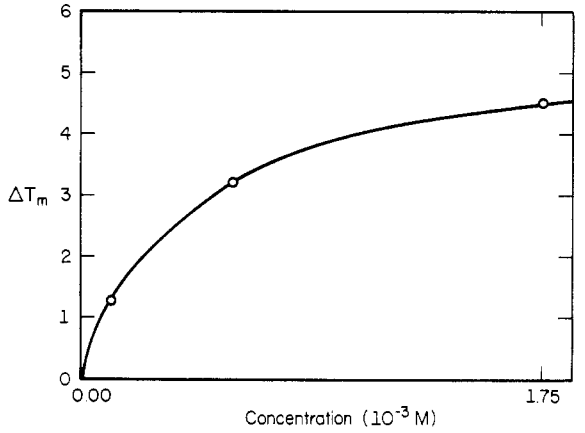

FIGURE 6: The change in the melting temperature of the cohesive ends of $\lambda b_2 b_5 c$ DNA as a function of ribonuclease A concentration.

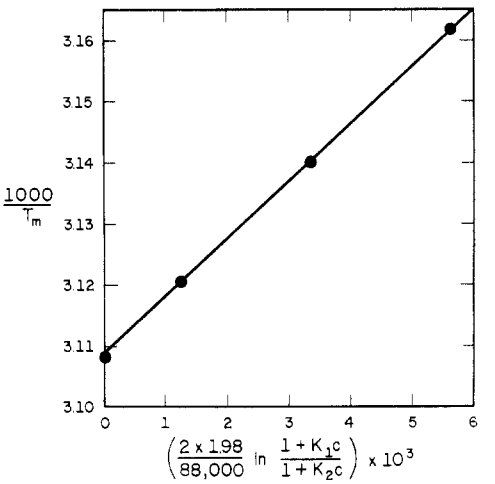

FIGURE 7: The inverse of the melting temperature of $\lambda b_2 b_5 c$ DNA cohesive ends as a function of ribonuclease A concentration using the calculated binding constants (see text).

The binding constants and enthalpies were calculated by iteration using eq 15 and 19 and assuming that the enthalpies were constant. The results are presented in Table III. Figure 7 shows the results of such a fit to eq 15. The slope of the line in Figure 7 is about 1, in agreement with the binding of one ribonuclease molecule per cohesive end. Figure 8 shows the value of $(\Delta T_{1/2}/T_m^2)$ versus ribonuclease concentrations. This curve passes through a maximum at a concentration of ribonuclease of about 0.001 M. At 45°, the binding constant to native DNA is 1.05×10^3 M^{-1} and to denatured DNA is 4.15×10^3 M^{-1}. The binding enthalpy is −25 kcal/mole for denatured DNA and −13 kcal/mole for native DNA. Using the values $r \approx 4$, $q \approx 2$, and $K_2 \approx 10^3$ M^{-1}, we may calculate the maximum in $(\Delta T_{1/2}/T_m^2)$ with eq 22 to occur at 1.2×10^{-3} M ribonuclease, a result consistent with Figure 8.

Raju and Davidson (1969) have measured the binding constants for ribonuclease A plus native and denatured calf thymus DNA at 25° in 0.053 M Na^+ at pH 7.00. The ratio of binding constants was found to be $r = 8$, with higher binding constants than we observed in 0.1 M Na^+ at 45°. These differences in ratio and magnitude may be accounted for by considering the enthalpies of binding, with a small salt effect effecting the magnitudes.

The arguments above were based on the assumption that the Jacobson–Stockmayer factor, j, does not change in the presence of ribonuclease concentrations up to 1.74×10^{-3} M. Under the most extreme conditions, about 60% of the entire native $\lambda b_2 b_5 c$ DNA molecule is covered with ribonuclease. No data exist on sedimentation of DNA–ribo-

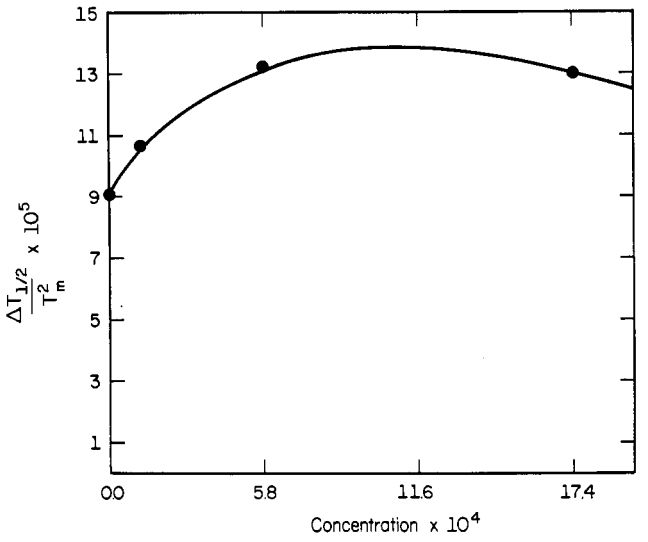

FIGURE 8: The dependence of transition width on ribonuclease concentration.

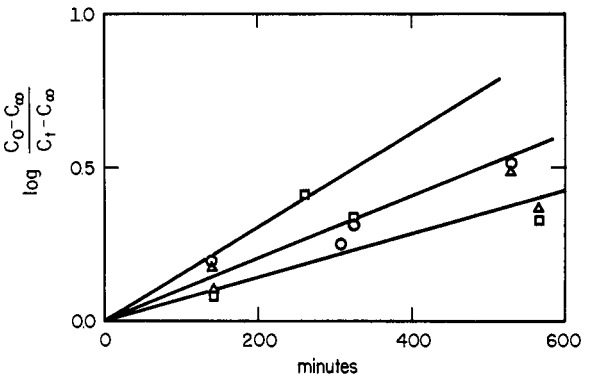

FIGURE 9: Rate plots for λb_2b_5c DNA cyclization with ribonuclease A at three temperatures. Graphs A, B, and C were obtained at 43, 40, and 37°, respectively. (□) No ribonuclease; (○) 0.58×10^{-4} M ribonuclease; (△) 1.16×10^{-4} M ribonuclease. The rate constants obtained were 0.035, 0.025, and 0.016 min^{-1} for each ribonuclease concentration at 43, 40, and 37°, respectively.

nuclease complexes in 0.1 M salt, although sedimentation experiments have been performed in low salt with apparently no change in sedimentation other than that due to changes in partial specific volume and mass (Felsenfeld *et al.*, 1963). The most convincing results are the kinetic results presented in the next section. The rate of cyclization is a strong function of DNA segment length. Bound ribonuclease does not have an effect on the rate constant for cyclization.

Kinetics of λb_2b_5c Cyclization in the Presence of Ribonuclease A. There are three possible linear λb_2b_5c DNA molecules which may cyclize: DNA with ribonuclease bound to both cohesive ends (rate constant k_3), DNA with ribonuclease bound to one of the cohesive ends (rate constant k_2), and DNA with no ribonuclease bound to the cohesive ends (rate constant k_1). Using eq 4, we may calculate the total concentration in terms of the concentration, [L], of DNA with no ribonuclease bound

$$[L]_{total} = [L](1 + K_1[P])^2 \quad (28)$$

The overall rate for the three types of molecules is

$$-\frac{d[L]}{dt}\text{ total} = \frac{k_1 + 2k_2K_1[P] + k_3K_1^2[P]^2}{(1 + K_1[P])^2}[L]_{total} \quad (29)$$

with the measured rate constant, k, given by

$$k = \frac{k_1 + 2k_2K_1[P] + k_3K_1^2[P]^2}{(1 + K_1[P])^2} \quad (30)$$

If the rate of cyclization were changed in the presence of ribonuclease A, the values of the three rate constants could be calculated. However, we find that even with very high saturation of the DNA molecules with bound ribonuclease, the cyclization rate is unchanged. Cyclization rate plots in the presence of 0, 0.58×10^{-4} ($K_1[P] = 0.35$), and 1.16×10^{-4} M ($K_1[P] = 0.7$) ribonuclease are shown for three temperatures in Figure 9. As the rate is the same for all ribonuclease concentrations at each temperature, the activation energy for cyclization is not changed with ribonuclease binding to the cohesive ends. Although we do not know the exact mechanism of ribonuclease binding, we may conclude that the DNA bases are still available for reaction in a DNA–ribonuclease complex.

λb_2b_5c DNA Cyclization as a Probe of Binding Equilibria. In the presence of ligands, the activities of helical or coil DNA may be changed, depending on the affinity of the DNA for the ligands. From changes in DNA melting temperatures, the binding properties of the ligands may be determined. In principle, changes in the melting point of the cohesive ends of λb_2b_5c DNA should be the same as changes in native DNA melting temperatures. We do find, however, that the effect of purine on λb_2b_5c DNA cyclization equilibria is comparable to the effect on tRNA melting but not the same as the effect on DNA melting. This difference could be due to a difference in experimental conditions, the unusual base composition of the cohesive ends of λb_2b_5c DNA or to a difference between all or none processes and DNA melting.

An advantage of using cyclization equilibria instead of DNA melting may be seen in the determination of binding enthalpies. The transition width for DNA melting is very sharp compared to the melting of the cohesive ends of λb_2b_5c DNA. Changes in transition widths of cyclization equilibria, the result of coupling to ligand binding equilibria, may be interpreted without ambiguity.

Another advantage of using cyclization involves kinetic studies, particularly for the case of proteins binding to a large number of nucleotides. Interpretation of cyclization kinetics results will small numbers of bound protein molecules is relatively simpler than interpretation of renaturation kinetics results with bound proteins.

The disadvantage of using λb_2b_5c DNA cyclization as a probe involves the time and effort necessary to obtain data relative to DNA melting studies in the presence of ligands. However, the potential for studying very small quantities of ligands which bind strongly to denatured DNA, the relatively low-melting temperature of the cohesive ends in high-salt solutions, as well as the known sequence of the cohesive ends, the simple theory of the all-or-none model and the large transition width, make this probe quite useful and versatile.

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A Random Sequential Mechanism for Arginyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*†

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ABSTRACT: Using purified arginyl-tRNA synthetase and purified $tRNA^{Arg}$ (*Escherichia coli*) we determined kinetic patterns according to Cleland. Inverse plots of initial velocities at varying concentrations of arginine and different fixed levels of $tRNA^{Arg}$ gave a family of converging lines. Similar intersecting plots were also obtained when arginine was varied at several fixed levels of ATP. These studies as well as those involving the inhibitors homoarginine and 5′-adenylyl(methylenediphosphonate) indicate that all three substrates (ATP, arginine, and $tRNA^{Arg}$) interact in a random order with the enzyme but that all substrates must be bound before any product is released. Verification of the nature of substrate addition as well as the order of product release was investigated by studies of isotope exchange rates ($PP_i \leftrightarrow$ ATP; arginine $\leftrightarrow$ arginyl-$tRNA^{Arg}$; AMP $\leftrightarrow$ ATP; tRNA $\leftrightarrow$ arginyl-$tRNA^{Arg}$) at chemical equilibrium. Increasing concentrations of a variety of reactant pairs without perturbing the equilibrium led to an increase in exchange rate up to a plateau level. The lack of inhibition of the various exchanges at high concentrations of reactants indicate that not only do the substrates interact randomly with the enzyme but that the products are also released in a random order. The various exchange rates vary over a factor of 100-fold, therefore the rate-limiting step in the overall reaction involves release of some reactant or product, not interconversions of enzyme-bound quaternary complexes.

Most aminoacyl-tRNA synthetases catalyze an amino acid dependent ATP–pyrophosphate-exchange reaction in the absence of added tRNA. According to the nomenclature of Cleland (1963a–c), these enzymes operate by a Ping-Pong mechanism whereby tRNA adds to the enzyme subsequent to the release of pyrophosphate. The aminoacyl-tRNA synthetases for arginine (Mitra and Mehler, 1966, 1967; Mehler and Mitra, 1967), glutamine, and glutamic acid (Ravel *et al.*, 1965) have the unique property of requiring tRNA for the ATP–pyrophosphate-exchange reaction. This property of the arginine-activating enzyme to show an absolute requirement for a specific tRNA for the ATP–pyrophosphate-exchange reaction prompted us to look at the mechanism of the reaction by following the kinetic procedures proposed by Cleland (1963a–c) and Boyer (1959). Initial velocity studies were carried out at varying concentrations of the different reactants.[1] Similar experiments were also performed in the presence of the dead-end inhibitors AMPPCP[2] and homoarginine. A further study was carried out to verify the random sequential addition of reactants and to elucidate the mechanism of product release by using Boyer's method for measuring isotope exchange at chemical equilibrium. The data obtained sup-

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[1] In this paper, we have chosen to use the terminology of Wedler and Boyer (1972). *Substrates* refer to ATP, arginine, $tRNA^{Arg}$, AMP, PP_i, and arginyl-$tRNA^{Arg}$. *Reactants* refer to the first three and *products* to the latter three.

[2] Abbreviation used is: AMPPCP, 5′-adenylyl(methylenediphosphonate).