Thermodynamic Properties of Superhelical DNAs†

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ABSTRACT: Binding isotherms of ethidium to the superhelical DNA from phage PM2, and to PM2 DNA containing ~1 single-chain scission per molecule, have been determined at six temperatures from 2.5 to 50°, in 3M CsCl–0.01 M Na_3EDTA. Spectrophotometric measurements in both the visible and ultraviolet (uv) regions were used to obtain the binding isotherms. The isotherm at 20° was also obtained by determining the free ethidium concentrations in equilibrium with DNA–ethidium complexes in boundary sedimentation experiments. A simple thermodynamic analysis shows that for a superhelical DNA with ν bound ethidium per nucleotide, the change in free energy per unit change in the number of superhelical turns τ, $dG_\tau/d\tau$, is directly related the ratio of the free ethidium concentrations, $c_{f,\nu}$* and $c_{f,\nu}$, in equilibrium with the ethidium complexes of the superhelical and the nicked DNA, respectively, at the same values of ν. The relationship is $dG_\tau/d\tau = (360/\phi_e)RT\ln(c_{f,\nu}^*/c_{f,\nu})$, where ϕ_e is the unwinding angle of the DNA helix per bound ethidium molecule. Experimentally, it was found that $\ln(c_{f,\nu}^*/c_{f,\nu}) = a_1(\nu - \nu_c)$, where a_1 is a constant at a given temperature and ν_c is the value of ν at which the originally superhelical DNA is completely relaxed, *i.e.*, containing no superhelical turns. The equation relating dG_τ/d_τ and ν, upon transformation and integration, gives $\Delta G_{\tau,\nu=0} = -a_1 NRT\nu_c^2/2$, where $\Delta G_{\tau,\nu=0}$ is the free energy of superhelix formation of the superhelical DNA in the absence of ethidium and N is the number of nucleotides per DNA molecule. This equation is independent of the unwinding angle ϕ_e. The values of a_1 are 11.2 ± 0.9, 11.2 ± 0.7, 11.2 ± 0.6, 10.7 ± 0.8, 10.0 ± 1.0, and 9.8 ± 1.4 at 2.5, 10, 20, 30, 40, and 50°, respectively. Measurements with λb2b5c DNA, and monomeric and trimeric λdv DNA, indicate that the constant a_1 is insensitive to the molecular length of DNA. The present results are compared with the previous results of Bauer and Vinograd (Bauer, W., and Vinograd, J. (1970), *J. Mol. Biol. 47,* 419), obtained by a statistical mechanical analysis of the ethidium binding isotherms (determined by density gradient centrifugation measurements) to two forms of simian virus 40 DNA at 25°. The effects of superhelical turns on a number of processes, such as the binding of a ligand which unwinds or winds the DNA helix, the denaturation of a DNA segment, and the formation of hair-pinned structures from base sequences with a twofold rotational symmetry, are discussed from the thermodynamic point of view, based on the present results.

For a superhelical DNA, unwinding (or winding) of the double helix results in a concomitant change of the number of superhelical turns. Thermodynamically, the supercoiling of a DNA is unfavorable. Thus, any process which reduces the absolute number of superhelical turns is favored for the superhelical DNA, relative to the same process for the same DNA containing at least one swivel point (a single-chain scission for example) per molecule. The free energy associated with the superhelical turns is termed the free energy of superhelix formation, and is of paramount importance in discussing the equilibrium properties of a covalently closed DNA.

A quantitative treatment of the free energy of superhelix formation has been done by Bauer and Vinograd (1970). These authors analyzed the binding of ethidium, which unwinds the DNA helix, to superhelical and nicked SV 40 DNAs by density gradient centrifugation in CsCl at 25°. The binding isotherms were calculated from the buoyant densities of the ethidium–DNA complexes at various free ethidium concentrations. A statistical mechanical treatment of the two binding isotherms gave the free energy of superhelix formation.

In the present paper, we describe results obtained spectrophotometrically for the binding of ethidium by the native superhelical PM2 DNA and PM2 DNA containing one single-chain scission per molecule (nicked PM2 DNA), at six temperatures ranging from 2.5 to 50°. A simple thermodynamic analysis is used to evaluate the free energy of superhelix formation at these temperatures. The possibility that the free energy of superhelix formation is dependent on molecular length as well as superhelix density has also been tested by measurements with monomeric and trimeric λdv DNA and λb2b5c DNA.

Thermodynamic Analysis

The binding of ethidium by DNA can be formally represented by the reaction:

$$\text{free site} + \text{ethidium} \rightleftharpoons \text{occupied site}$$

For a nicked DNA, the free-energy change (per mole of DNA molecules) corresponding to the binding of $d\nu$ moles of ethidium (Et) per nucleotide to a DNA–dye complex with ν bound ethidium per nucleotide is:

$$dG = (\mu_{\text{occ. site}} - \mu_{\text{free site}} - \mu_{\text{free Et}})N d\nu \qquad (1)$$

where μ is the chemical potential and N is the number of nucleotides per DNA molecule. For an equilibrium system at constant temperature and pressure, $dG = 0$.

The quantity $\mu_{\text{free Et}}$ is related to the free ethidium concentration $c_{f,\nu}$ by the standard thermodynamic relationship:

$$\mu_{\text{free Et}} = \mu_{\text{free Et}}^0 + RT \ln(yc_{f,\nu}) \qquad (2)$$

where y is the activity coefficient.

In the limit that the sites are noninteracting:

$$\Delta\mu \equiv \mu_{\text{occ. site}} - \mu_{\text{free site}} = (\mu_{\text{occ. site}}^0 - \mu_{\text{free site}}^0) + RT \ln\left(\frac{\nu}{n - \nu}\right) \qquad (3)$$

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where n is the maximal number of sites. In the general case, $\Delta\mu$ is a much more complex function of ν:

$$\Delta\mu = f(\nu) \quad (4)$$

Substituting eq 2 and 4 into eq 1:

$$dG = [f(\nu) - \mu_{\text{free Et}}{}^{0} - RT \ln (\gamma c_{f,\nu})]N d\nu \quad (5)$$

For the same process with a superhelical DNA, there is an additional change in free energy due to the change in the number of superhelical turns resulting from the addition of $N d\nu$ moles of ethidium to the DNA. This free-energy term is:

$$dG_\tau = (dG_\tau/d\tau)d\tau = (dG_\tau/d\tau)(N\phi_e d\nu/360) \quad (6)$$

where τ is the number of superhelical turns and ϕ_e is the unwinding angle in degrees per bound ethidium. The total free-energy change for the superhelical DNA is:

$$dG^* = [f^*(\nu) - \mu_{\text{free Et}}{}^{0} - RT \ln (\gamma^* c_{f,\nu}{}^*)]N d\nu + \left(\frac{dG_\tau}{d\tau}\right)\frac{N\phi_e d\nu}{360} \quad (7)$$

The superscript asterisk denotes quantities for the superhelical DNA.

In a high salt medium, it is reasonable to assume that the activity coefficient of ethidium is a constant independent of concentration or:

$$\gamma = \gamma^* \quad (8)$$

We also make the important assumption that:

$$f(\nu) = f^*(\nu) \quad (9)$$

This assumption is essentially the same as saying that the *intrinsic* binding free energy is the same (at the same occupancy number ν) for the binding of an ethidium to a superhelical or a nonsuperhelical DNA.

Since both dG and dG^* are equal to zero at equilibrium, a combination of eq 5, 7, 8, and 9 gives:

$$\frac{dG_\tau}{d\tau} = \left(\frac{360}{\phi_e}\right) RT \ln (c_{f,\nu}{}^*/c_{f,\nu}) \quad (10)$$

Equation 10 states that the change in the free energy of superhelix formation per unit change in τ, at any ν (and therefore at any τ), is simply related to the free ethidium concentration in equilibrium with the superhelical and nonsuperhelical DNAs at the same ν. This provides a simple way of evaluating $(dG_\tau/d\tau)$, or, upon integration, ΔG_τ.

Since it is usually convenient to express binding isotherms as $\xi_\nu \equiv (\nu/c_{f,\nu})$ *vs.* ν plots, eq 10 can be written as eq 10a:

$$\frac{dG_\tau}{d\tau} = \left(\frac{360}{\phi_e}\right) RT \ln (\xi_\nu/\xi_\nu{}^*) = -\left(\frac{360}{\phi_e}\right) RT \ln (\xi_\nu{}^*/\xi_\nu) \quad (10a)$$

Materials and Methods

Chemicals. Ethidium bromide (Calbiochem) and optical grade cesium chloride (Harshaw) were dried *in vacuo* before use. The disodium salt of ethylenediaminetetraacetic acid (Kodak) was used in the preparation of a stock solution of Na_3EDTA by titrating with NaOH to pH 8.

Preparation of DNAs. PM2 phage was grown and purified by the procedures of Espejo and Canelo (1968). The phage was banded in a CsCl gradient and the purified phage was phenol extracted as described by Espejo *et al.* (1969). Over 95% of the DNA obtained is in the superhelical form, and the DNA was dialyzed into 0.100 M CsCl–0.01 M Na_3EDTA (pH 8.0).

Another preparation of PM2 DNA was further banded in CsCl–ethidium gradient (Radloff *et al.*, 1967) to remove the small amount of nicked DNA. Ethidium was removed by *n*-butyl alcohol extraction and chromatography on a Dowex cation exchanger column. The binding isotherms obtained with the two preparations are not significantly different.

The thymine-requiring lysogens, *Escherichia coli* N 100 (λdv 21 monomer) and N 100 (λdv 21 trimer) were kindly provided to us by Dr. D. E. Berg. A single colony of each containing the λdv episome was selected for inoculation of 100 ml of K medium containing 5 μg/ml of thymine. After the cells had grown to ~10^8 cells/ml, 0.5 mCi of [*methyl*-^{3}H]thymine or 0.1 mCi of [*methyl*-^{14}C]thymine (Schwarz/Mann) was added. Cells were harvested at a density of ~10^9 cells/ml and processed according to the method of Kellenberger-Gujer *et al.* (1975) to obtain the λdv DNA. The purified DNA, when sedimented in 3.0 M CsCl–0.1 M KOH–0.01 M Na_3EDTA at 20°, gave uncorrected sedimentation coefficients of 45 and 75 S for monomeric and trimeric λdv DNA, respectively.

Endonucleolytic Treatment of the Superhelical DNAs. Pancreatic DNase I (Worthington, DPFF 2 EK) was used to introduce ~1 single chain scission per DNA molecule in the presence of a saturating amount of ethidium bromide (Barzilai, 1973). The incubation mixture contained 10 mM Tris (pH 8.0), 2 mM $MgCl_2$, 1 mM Na_3EDTA, 0.1 mg/ml of bovine plasma albumin (Calbiochem, grade A), DNA, and 3 μg of ethidium/μg of DNA. Approximately 1 μg of the DNase was added to each milliliter of the incubation mixture. The precise amount of the enzyme was determined by trials with small amounts of the incubation mixture. Incubation was done for 15 min at 20°. Since more ethidium was bound to a nicked DNA relative to the superhelical form in the presence of excess ethidium, inhibition of DNase was more severe for DNA molecules which already had one single chain scission per molecule (Barzilai, 1973).

Spectrophotometric Measurements. The DNA and ethidium bromide stock solutions were all made up in 0.100 M CsCl–0.01 M Na_3EDTA (pH 8.0). The concentrations of these solutions were determined spectrophotometrically. The molar absorptivity of PM2 DNA at 260 nm was taken as 6600 cm^2/mol of phosphate, based on that of calf thymus DNA which has approximately the same G + C content as PM2 DNA (Mahler *et al.*, 1964; Espejo *et al.*, 1969). The molar absorptivity of ethidium at 460 nm was taken as 4220 cm^2/mol (Wang, 1969).

All the ethidium binding experiments were done in 3.00 M CsCl–0.01 M Na_3EDTA by adding weighed amounts of solid CsCl to weighed amounts of DNA and ethidium stock solutions. The final concentration of DNA or ethidium was calculated from the known densities of 0.100 and 3.00 M CsCl solutions (1.013 and 1.378 g/cm^3, respectively).

Most of the spectrophotometrical measurements were done with a Cary 118 double-beam spectrophotometer. A Brinkman Lauda-thermostat was used to adjust the temperature of the cell chamber. The cell temperature was monitored by an electronic digital-reading Newport thermocouple set. The temperature was kept within ±0.1° of the desired temperature.

The base line was adjusted each time before spectrophotometric measurement with two matched cuvets containing

solvents. The cuvets had Teflon stoppers to prevent evaporation loss, which was found to be negligible by weighing the cuvets before and after measurements.

The solution in the reference cell was identical with that in the sample cell except no ethidium was added to the former. The difference in absorbance, ΔA, was used to calculate the free and bound ethidium concentrations, c_f and c_b, respectively, by the following equations:

$$c_f = \frac{\Delta A - \epsilon_b c_T}{\epsilon_f - \epsilon_b}$$

$$c_b = \frac{\epsilon_f c_T - \Delta A}{\epsilon_f - \epsilon_b}$$

where c_T is the total ethidium concentration in the sample cell and ϵ_f and ϵ_b are the molar absorptivities of free and bound ethidium, respectively. For the measurements done in the visible range we used $\epsilon_f = 4220$ and $\epsilon_b = 1315$ at 460 nm (Wang, 1969). For low binding ratio experiments (for ν less than 0.08), it is advantageous to do the spectrophotometric measurements in the ultraviolet (uv) region. Several measurements with ν in the range between 0.07 and 0.08 were performed in both uv and visible regions. From these measurements ϵ_b was calculated to be 1.63×10^4 at 287 nm. The molar absorptivity of the free ethidium at the same wavelength was 5.39×10^4.

The DNA concentrations were always in the neighborhood of 10^{-4} M (in nucleotides) and the total absorbance at 287 nm was never much higher than 1.5 for the uv measurements.

Corrections for the volume changes due to changes in temperature were made for all measurements. The molar absorptivity of the free ethidium at 287 nm changes negligibly at temperatures between 0 and 50°.

Analytical Ultracentrifugation. For the superhelical PM2 DNA, the amounts of nicked PM2 DNA before and after the spectrophotometric measurements were measured by band sedimentation (Vinograd *et al.*, 1963) in 3 M CsCl–0.1 M KOH–0.01 M EDTA. In no case was the amount of the nicked species greater than 4%.

In one set of experiments, the binding isotherms of ethidium to superhelical PM2 DNA from the mature phage and nicked PM2 DNA were determined by boundary sedimentation runs in a Model E (Spinco) analytical ultracentrifuge. Solutions of known DNA and ethidium concentrations were placed in the sample sectors of 30-mm double-sector cells. The reference sectors contained the solvent (3 M CsCl–0.01 M Na_3EDTA). The ultracentrifuge was equipped with a spherical reflecting mirror assembly (Spinco) to allow change of wavelength. Photoelectric tracings were taken at 287 nm, the maximum of the free ethidium spectrum. As the boundary of the DNA–ethidium complex moved downfield, the concentration of free ethidium could be obtained from the region free of DNA. The concentration of bound dye was then calculated from the known total ethidium concentration. In these measurements, the DNA concentration was selected to be in the range 10^{-5} to 10^{-4} M (in nucleotides) so that c_b and c_f were comparable in magnitude. Four cells were usually run simultaneously with at least one cell containing only ethidium to provide a calibration for the evaluation of c_f. These measurements gave both the binding isotherms and the functional dependence of the sedimentation coefficients of the native and nicked PM2 DNAs on ν, the number of ethidium bound per nucleotide.

Banding of ^{3}H-Labeled λdv Monomer and ^{14}C-Labeled λdv Trimer DNA in CsCl–Ethidium Gradient. ^{3}H-Labeled λdv monomer and ^{14}C-labeled λdv trimer DNA were first nicked by pancreatic DNase I, mixed, and then converted to the covalently closed form by ligase at 30° in the absence of ethidium (Wang, 1971). The reaction mixture was quenched by adding Na_3EDTA to 0.01 M, and extracted once with an equal volume of buffer saturated phenol. The DNA solution was dialyzed exhaustively against 0.1 M NaCl–0.01 M Na_3EDTA, and solid CsCl and an ethidium bromide stock solution were added to give a density of 1.56 g/cm^3 and a final ethidium concentration of approximately 100 μg/ml. A second solution was prepared by mixing the same amounts of 0.1 M NaCl, 0.01 M Na_3EDTA, solid CsCl, and ethidium bromide stock as the sample solution. The two solutions were centrifuged in a SW 50.1 rotor at 35K rpm and 15° for 50 hr. Fractions were collected by punching a hole at the bottom of each tube. Fractions from the sample tube were collected on filter paper circles, dried, and counted in a Beckmann LS 250 liquid scintillation counter. Fractions from the other tube were collected into test tubes for measurements of the absorbance at 287 nm and the refractive index. The free ethidium concentration of each fraction was calculated from its absorbance, and the density of each fraction was calculated from its refractive index (Bruner and Vinograd, 1965).

Results

Binding Isotherms and the Calculation of the Free Energy of Superhelix Formation. Two typical sets of binding curves, measured at 2.5 and 30°, are depicted in Figures 1a and 1b, respectively. For the nicked DNA, ξ to a good approximation is a linear function of ν (Waring, 1965; LePecq and Paoletti, 1967; Wang, 1969; Bauer and Vinograd, 1970; Gray *et al.*, 1971), and the least-squares line for the points is shown in each figure. For each datum point (ξ^*,ν) for the superhelical PM2 DNA, the corresponding point (ξ,ν) for the nicked PM2 DNA is read off the least-squares line. The quantity $\ln(\xi_\nu^*/\xi_\nu)$ is then calculated and plotted as a function of ν, as shown in Figures 2a and 2b.

Within experimental error, $\ln(\xi_\nu^*/\xi_\nu)$ is a linear function of ν, and can be expressed by eq 11:

$$\ln(\xi_\nu^*/\xi_\nu) = a_1(\nu - \nu_c) \tag{11}$$

The intercept of this line with the ν axis, at $\nu = \nu_c$, corresponds to the point at which the superhelical PM2 DNA is fully relaxed (*i.e.*, containing no superhelical turns), and therefore has the same affinity for ethidium as the nicked DNA with the same ν. The parameters a_1 and ν_c obtained at 2.5, 10, 20, 30, 40, and 50° are tabulated in Table I.

The number of superhelical turns τ and the superhelical density σ, defined as the number of superhelical turns per ten base pairs (Bauer and Vinograd, 1968), are related to $(\nu - \nu_c)$ by:

$$\tau = N\phi_e(\nu - \nu_c)/360 \tag{12}$$

and

$$\sigma = \phi_e(\nu - \nu_c)/18 \tag{13}$$

From eq 10a, 11, and 12:

$$\frac{dG_\tau}{d\tau} = -\left(\frac{360}{\phi_e}\right)RT \ln(\xi_\nu^*/\xi_\nu) = -\left(\frac{360}{\phi_e}\right)a_1RT(\nu - \nu_c) \tag{14a}$$

and

$$dG_\tau = -a_1NRT(\nu - \nu_c)d\nu \tag{14b}$$

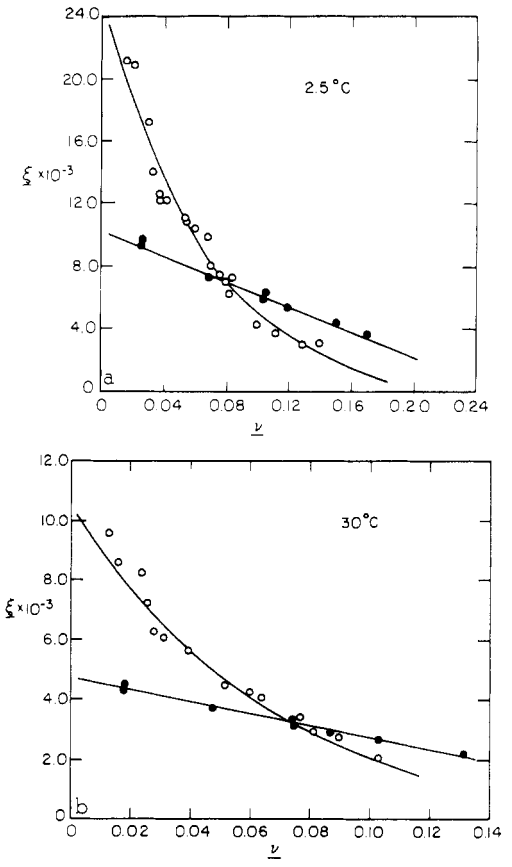

FIGURE 1: Scatchard plots of binding results at 2.5° (a) and 30° (b). The filled circles are data for the nicked PM2 DNA, and the line drawn is the least-squares line. The open circles are data for the superhelical PM2 DNA. The smooth curve drawn is calculated from $\xi_\nu^* = K(\nu_m - \nu)\exp[a_1(\nu - \nu_c)]$, where K and ν_m are obtained from the least squares line of the nicked DNA binding results, and a_1 is obtained as described in the text.

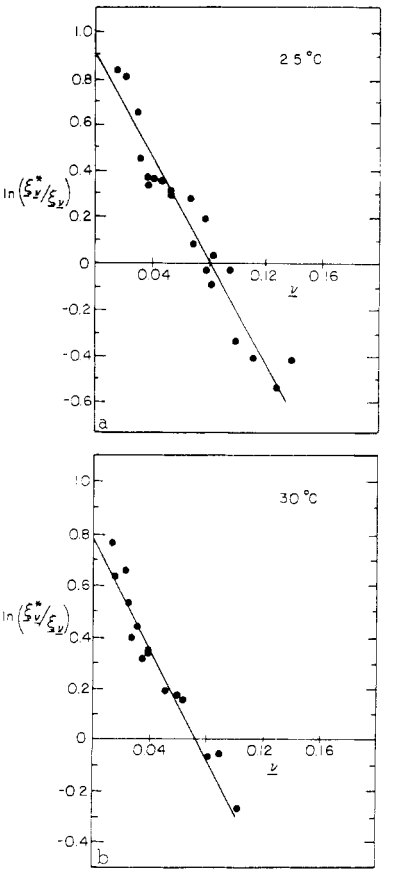

FIGURE 2: ln (ξ_ν^*/ξ_ν) as a function of ν. The line drawn is the least-squares line: (a) 2.5°; (b) 30°.

Upon integration of eq 14b:

$$\Delta G_\tau = \int_0^\tau dG_\tau = -\int_{\nu_c}^{\nu} a_1 NRT(\nu - \nu_c)d\nu$$

$$= -\frac{a_1 NRT}{2}(\nu - \nu_c)^2 \qquad (14c)$$

In the absence of ethidium, *i.e.*, at $\nu = 0$, the free energy of superhelix formation for the superhelical DNA is:

$$\Delta G_{\tau,\nu=0} = -\frac{a_1 NRT\nu_c^2}{2} \qquad (14d)$$

Defining the quantity $(2/N)\Delta G_{\tau,\nu=0}$, the free energy of superhelix formation per base pair, as $\Delta g_{\tau,\nu=0}$:

$$\Delta g_{\tau,\nu=0} = -a_1 RT\nu_c^2 \qquad (14e)$$

Enthalpic and Entropic Contributions to ΔG_τ. The quantity $\Delta G_{\tau,\nu=0}$ for superhelical PM2 DNA from the mature phage, which is the excess free energy of the DNA relative to the same DNA without superhelical turns, is also tabulated in Table I for the six temperatures from 2.5° to 50°. In principle, since $(\partial\Delta G/\partial T)_P = -\Delta S$, both the entropic and enthalpic contributions can be evaluated. One might conclude from the tabulated $\Delta G_{\tau,\nu=0}$ values that the unfavorable free energy is a result of an unfavorable (positive) enthalpic term, since $\Delta G_{\tau,\nu=0}$ decreases with temperature, indicating a favorable (positive) entropic term. While it is certain that the formation of a superhelix is associated with a negative *configurational* entropy change (Jacobson, 1969; Laiken, 1973), the total entropy change could be positive, due to solvation change for example.

Table I[a]

T (°C)	$-a_1$	ν_c	$\Delta G_{\tau,\nu=0}$ (kcal/mol)
2.5	11.2 ± 0.9	0.082 ± 0.006	406
10	11.2 ± 0.7	0.076 ± 0.004	360
20	11.2 ± 0.6	0.076 ± 0.003	370
30	10.7 ± 0.8	0.074 ± 0.004	346
40	10.0 ± 1.0	0.075 ± 0.004	346
50	9.8 ± 1.4	0.068 ± 0.004	288

[a] Parameters obtained by the least-squares fitting of the ln (ξ^*/ξ) *vs.* ν plots at six different temperatures. See text for the definitions of a_1 and ν_c. $\Delta G_{\tau,\nu=0}$ is the free energy of superhelix formation of PM2 DNA in the absence of ethidium.

It does not seem appropriate, however, to calculate the enthalpic and entropic terms from the temperature dependence of $\Delta G_{\tau,\nu=0}$. This is because for a given superhelical DNA the number of superhelical turns is dependent on temperature (Wang, 1969; Upholt *et al.*, 1971). An increase in temperature lowers the number of negative superhelical turns, as can be seen from the general decrease in the ν_c values tabulated in Table I. Since lowering the absolute number of superhelical turns is favored thermodynamically, this effect would contriubte to $\Delta G_{\tau,\nu=0}$. A more appropriate analysis of the data would be to plot the tempera-

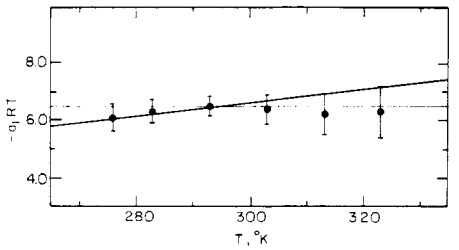

FIGURE 3: The quantity $-a_1RT$ as a function of temperature. The horizontal line represents the case that there is no entropic contribution to the free energy of superhelix formation. The other line represents the case that there is no enthalpic contribution to the free energy of superhelix formation. Both lines are arbitrarily drawn through the 20° datum point.

ture dependence of the coefficient a_1RT, which is a measure of the free energy of superhelix formation for a DNA with a *fixed* number of superhelical turns. This is shown in Figure 3.

Since the experimental errors in these measurements are substantial, it is difficult to assess the relative contributions of the enthalpic and entropic terms. For the limiting case that the entropic term is zero, a horizontal line is to be drawn. In the other limit that the enthalpic term is zero, the slope of the line should be a_1R. Both lines are shown in the figure. It appears that both the enthalpic and entropic terms contribute to the free energy of superhelix formation, but more refined data are necessary before a firm conclusion can be drawn.

Effect of the Molecular Length of DNA. As discussed by others (Bauer and Vinograd, 1970; Davidson, 1972), $\Delta g_{\tau,\nu=0}$ is expected to be insensitive to the molecular length of the DNA. In other words, the constant a_1, though obtained for PM2 DNA, is expected to be applicable to other DNAs of different length. This expectation is substantiated by two experiments.

In the first set of experiments, binding isotherms were obtained spectrophotometrically for linear λb2b5c DNA (25.8×10^6 daltons) and superhelical λb2b5c DNA with $\nu_c = 0.035$. The a_1 value calculated from these isotherms is -11 at both 0 and 20°, in agreement with the results for PM2 DNA (6.5×10^6 daltons; Kriegstein and Hogness, 1974).

Since λb2b5c DNA and PM2 DNA differ in other respects as well as in molecular length, a second experiment was done by banding ^{3}H-labeled monomeric λdv DNA and ^{14}C-labeled trimeric λdv DNA in CsCl in the presence of excess ethidium, as described in the section on Materials and Methods. Since covalent closure of the differentially labeled λdv DNAs by ligase was done under identical conditions in the absence of ethidium, the two samples have identical superhelical densities in the absence of the dye (Wang, 1969). Thus, in the presence of excess ethidium, any difference in the coefficient a_1 for the two DNAs would result in a difference in ν, and therefore a difference in their positions in the density gradient. The results of this experiment are shown in Figure 4. The positions of the two covalently closed DNAs are found not to be significantly different.

The smallest difference detectable for the coefficient a_1 for the two DNAs can be estimated as follows. It can be shown that:

$$\xi_m^*/\xi_t^* = \exp\{a_{1,m}(\nu_m - \nu_c) - a_{1,t}(\nu_t - \nu_c)\}$$

where the subscripts m and t denote the quantities for the monomer and trimer λdv, respectively. Since the two bands do not resolve, $\xi_m^*/\xi_t^* \approx 1$ and therefore the exponent in the above equation is approximately zero. Thus:

$$\frac{a_{1,m}}{a_{1,t}} \approx \frac{\nu_t - \nu_c}{\nu_m - \nu_c} \text{ or } \frac{a_{1,m} - a_{1,t}}{a_{1,t}} \approx \frac{\nu_t - \nu_m}{\nu_m - \nu_c}$$

Since these two DNAs were converted to the covalently closed form at 30° in the ligase reaction medium, in a concentrated CsCl solution at neutral pH and 20° ν_c is expected to be 0.009 (Wang, 1969). In the presence of excess ethidium, from the results of Paoletti *et al.* (1971), ν is ~0.10. The detection limit in $\nu_t - \nu_m$ can be estimated as follows. The difference in ν between the covalently closed and the nicked DNA bands is ~0.10, assuming that ν for nicked DNA is ~0.20. These species are separated by 14 fractions. If a difference of less than one-half of a fraction is not de-

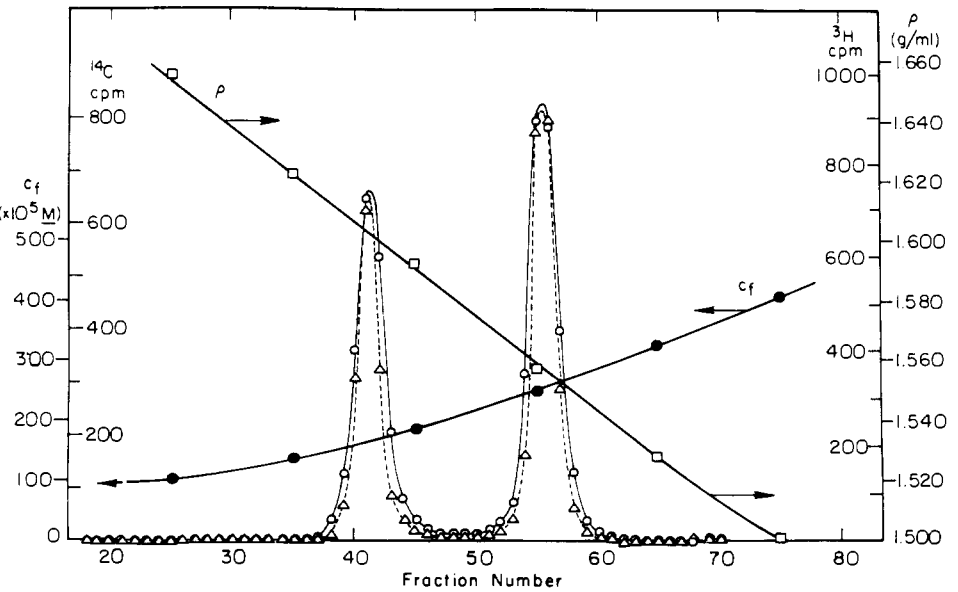

FIGURE 4: Banding patterns of ligase-closed ^{3}H-labeled λdv monomer DNA and ^{14}C-labeled λdv trimer DNA in a CsCl density gradient containing excess ethidium: (○) ^{3}H, cpm; (△) ^{14}C, cpm; (□) free ethidium concentration; (●) compositional density gradient. The experimental conditions were described under Materials and Methods.

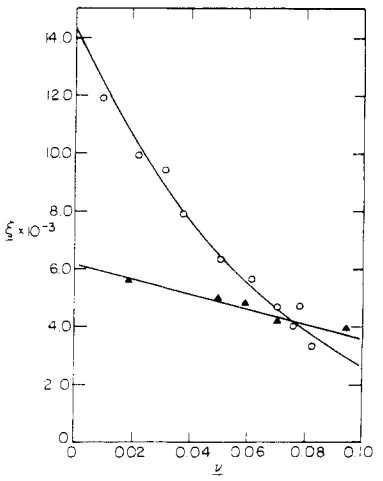

FIGURE 5: The Scatchard plot of binding data, at 20° and in 3 M CsCl–0.01 M Na_3EDTA (pH 8), obtained by the boundary sedimentation technique described under Materials and Methods: (○) data for superhelical PM2 DNA from the mature phage; (▲) data for nicked PM2 DNA. The curves drawn were obtained from independent spectrophotometric measurements at 20°, as described in the text and in the legend to Figure 1. The agreement is very good.

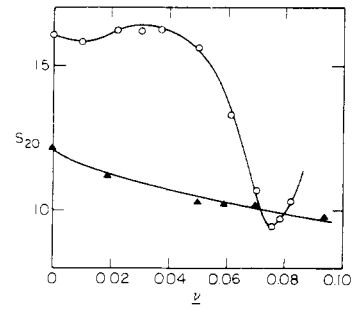

FIGURE 6: The dependence of the sedimentation coefficients s of superhelical PM2 DNA (○) and nicked PM2 DNA (▲) on ν, the number of bound ethidium per nucleotide. Both s and ν were calculated directly from the boundary sedimentation experiments as described in the text. Sedimentation measurements were done in 3 M CsCl–0.01 M Na_3EDTA (pH 8), at 20° and 31,460 rpm.

tectable, the smallest detectable difference in $\nu_t - \nu_m$ is ~0.005. Thus $(\nu_t - \nu_m)/(\nu_m - \nu_c)$ is 0.06. Therefore the difference in $a_{1,m}$ and $a_{1,t}$ is no more than 6%.

Binding Isotherms from Boundary Sedimentation Measurements. To test the precision of the binding isotherms obtained by spectrophotometry, the binding isotherms for nicked and superhelical PM2 DNA were also determined at 20° by the boundary sedimentation method described in the Materials and Methods section. This technique has the advantage that no information on the molar absorptivity of bound dye is needed. The data points are depicted in Figure 5. The binding isotherms drawn in the figure were obtained from spectrophotometric measurements. As can be seen, the agreement is excellent. This indicates that the spectroscopic parameters used in the calculation of the binding isotherms are correct. Furthermore, the assumption made in previous works (Wang, 1969), that the hydrostatic pressure gradient generated during centrifugation has no effect on the binding of ethidium by DNA, is valid.

The sedimentation measurements also give directly the ν dependence of both the sedimentation coefficient of the superhelical PM2 DNA, s_I, and that of the nicked PM2 DNA, s_{II}. These are depicted in Figure 6. The ν value at the minimum of the s_I *vs.* ν curve, 0.075, is in good agreement with the ν_c value obtained from the binding isotherms, 0.076 (Table I and Figure 6).

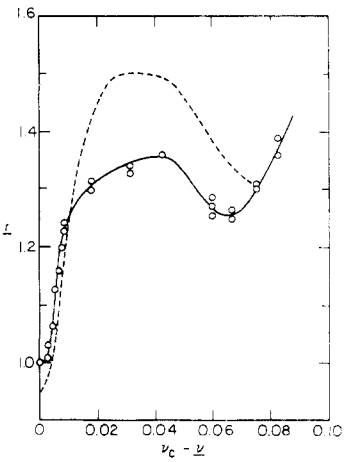

FIGURE 7: The relative sedimentation rate of a covalently closed PM2 DNA as a function of the degree of superhelicity. Open symbols (○), for pure DNA, *i.e.*, the ratio r is defined as s_I/s_{II}, where s_I is the sedimentation coefficient of a covalently closed PM2 DNA (with a degree of superhelicity represented by ν_c), and s_{II} is the sedimentation coefficient of nicked PM2 DNA. Both s_I and s_{II} were measured in 3 M CsCl–0.01 M Na_3EDTA at 20°. Data are taken from Wang (1975a), with small corrections for samples with $\nu_c > 0.01$ so that ν_c values of the samples are relative to $\nu_c = 0.075$ for the DNA from the mature phage. Dotted curve, for the DNA–ethidium complex. Data obtained from Figure 6 for PM2 DNA ($\nu_c = 0.075$) with varying amounts of bound ethidium, ν. The ratio r is defined as $s_I(\nu)/s_{II}(\nu)$, *i.e.*, the ratio of the sedimentation coefficients of the covalently closed and nicked DNA with the same amounts of bound ethidium.

In Figure 7, the ratio $r = s_I/s_{II}$ is depicted as a function of $(\nu_c - \nu)$, which is a measure of the superhelical density σ [see eq 13]. For a family of PM2 DNAs with different superhelical densities, the ratio s_I/s_{II} in the absence of ethidium ($\nu = 0$) has been measured previously (Wang, 1974a). These results are also shown in Figure 7 for a comparison. The experimental error in r is about ±0.02. While in the region of low degree of superhelicity the difference between the two curves is relatively small; in the region of high degree of superhelicity the difference is large. Examination of data in Figures 5 and 7 indicates that the difference in the shapes of the two curves in Figure 7 (in the region of high superhelicity) appears to be primarily due to the significant decrease in s_{II} as ν increases. Thus, while there is a local minimum at $(\nu_c - \nu) \approx 0.065$ ($\nu \approx 0.01$) for the $s_I(\nu)$ *vs.*ν curve (Figure 6) as well as for the curve obtained from superhelical PM2 DNAs in the absence of ethidium, such a minimum is absent when $s_I(\nu)/s_{II}(\nu)$ is plotted against $\nu_c - \nu$ (Figure 7).

These results illustrate the complexity of the sedimentation properties of a superhelical DNA. A twisted DNA and a DNA–ethidium complex of the same degree of superhelicity do not have the same r value. One should therefore be very cautious in interpreting changes in hydrodynamic properties of a highly superhelical DNA by the binding of ligands. An increase or decrease in the sedimentation coefficient is not necessarily an indication of a change in the degree of superhelicity.

Discussion

Free Energy of Superhelix Formation per Base Pair. As shown in the Results section, the quantity $\Delta g_{\tau,\nu=0}$ can be calculated directly from the ethidium binding curves. The calculation does not involve any assumption on the mode of

ethidium binding (whether the binding of ethidium is represented by the independent sites model or any other model does not matter), nor does it require any knowledge of the unwinding angle ϕ_e per ethidium bound. While values of $\Delta g_{\tau,\nu=0} = -a_1 RT\nu_c^2$ were obtained mainly by analyzing binding isotherms for PM2 DNA, the lack of significant molecular length dependence, at least for DNAs with molecular weights higher than 4×10^6, indicates that the values obtained are applicable to other DNAs as well.

For SV 40 DNA in ~ 5.8 M CsCl at 25°, the treatment of Bauer and Vinograd gives the free energy of superhelix formation as 18 cal/mol of base pair. From the data tabulated in Table I, a_1 at 25° is interpolated to be −11.0. Taking $\nu_c = 0.045$ for SV 40 DNA (Bauer and Vinograd, 1968), the present analysis gives $\Delta g_{\tau,\nu=0} = 13$ cal/base pair for SV 40 DNA, which is about a factor of 1.5 lower than the value obtained by Bauer and Vinograd (1970).

This difference is not a result of the different theoretical treatment employed in the analysis of the binding isotherms. The treatment of Bauer and Vinograd indicates that the intrinsic binding constants for the nicked and the superhelical DNAs are essentially the same, which is a basic assumption of our treatment. When our analysis is applied to the binding isotherms of SV 40 DNA reported by Bauer and Vinograd, the free energy of superhelix formation calculated is essentially in agreement with their result. The most likely source for the discrepancy is an error in the binding isotherms. Since the binding isotherms reported by both groups give the correct crossover points (ν_c values), the error would have to be a systematic one. The binding isotherms reported by Bauer and Vinograd were calculated from the measured buoyant densities by a four-components thermodynamic theory. It is plausible that a certain assumption (or assumptions) in the theory may introduce a significant error in the calculation of ν from the buoyant density (Bauer and Vinograd, 1970). Our spectrophotometric measurements might also be subject to a systematic error, such as an error in the molar absorptivity of bound ethidium. The agreement between the binding isotherms obtained from the spectrophotometric measurements and those obtained from boundary sedimentation measurements indicates, however, that the spectroscopic parameters we used are correct, and that the binding isotherms we obtained are reliable.

There is another possible source for the discrepancy. As can be seen from eq 10, the differential free energy of superhelix formation, $dG_\tau/d\tau$, at any point $\nu = \nu_c$, is for the DNA–ethidium complex with ν bound ethidium per nucleotide. Strictly speaking, it is applicable to pure DNA only for the extrapolated point $\nu = 0$. Thus to obtain ΔG_τ rigorously one would have to measure lim $(\nu \rightarrow 0)$ $(dG_\tau/d\tau)$ for a family of superhelical DNAs with different degrees of superhelicity, and then to perform the proper integration. The ΔG_τ calculated from eq 14 is therefore an approximation. Any difference in molecular parameters (related to the bending and twisting of the DNA) between the DNA and the DNA–ethidium complex is expected to make the ΔG_τ calculated from eq 14 different from the true ΔG_τ for pure DNA. Thus, for two DNAs of different ν_c values, the ΔG_τ values calculated for the two from ethidium binding isotherms are not strictly comparable.

The difference in sedimentation property for the superhelical DNA and the superhelical DNA–ethidium complex of the same degree of superhelicity is suggestive that the factor discussed in the above paragraph might be of some importance. Our measurements with superhelical λb2b5c DNA ($\nu_c = 0.035$) and PM2 DNA ($\nu_c = 0.076$), however, give essentially the same a_1 value, indicating that the difference in ν_c for SV 40 DNA and PM2 DNA is not accountable for the discrepancy in the ΔG_τ values calculated for the two DNAs.

Dependence of the Affinity of a Ligand on the Degree of Superhelicity. If the binding of a ligand to a DNA causes an unwinding (or winding) of the DNA helix by an angle ϕ, the affinity of the ligand to a covalently closed DNA is dependent on the degree of superhelicity of the DNA. Consider the free-energy change for the reaction:

$$\text{L–C*}(\nu_c = 0) + \text{C*}(\nu_c) \rightleftharpoons \text{L–C*}(\nu_c) + \text{C*}(\nu_c = 0)$$

where C* denotes a covalently closed DNA, L-C* denotes the ligand–DNA complex, and the degree of superhelicity of a DNA is expressed by the ν_c value given in parentheses. Under conditions such that the structure of the binding sites is the same for the two covalently closed DNAs, which differ only in the degree of superhelicity, the free-energy change ΔG for the reaction is just the change in the free energy of superhelix formation ΔG_τ. Assuming that the binding of L changes the degree of superhelicity of each DNA but little, the dissociation of L from an untwisted DNA, C* ($\nu_c = 0$), is not associated with a free energy of superhelix formation. The binding of L to the superhelical DNA C* (ν_c), however, is associated with a free energy of superhelix formation ΔG_τ such that:

$$\Delta G_\tau \approx \left(\frac{dG_\tau}{d\tau}\right)\left(\frac{\phi}{360}\right) = \left(\frac{\phi}{\phi_e}\right) a_1 RT\nu_c \qquad (15)$$

If K_a^0 and $K_a^{\nu_c}$ are the association constants for the binding of the ligand to the two DNAs C* ($\nu_c = 0$) and C* (ν_c),

$$K_a^{\nu_c}/K_a^0 = \exp(-\Delta G_\tau/RT) = \exp[-a_1\nu_c(\phi/\phi_e)] \qquad (16)$$

Thus knowing the unwinding angle of the ligand relative to that of ethidium, ϕ/ϕ_e, the ratio of the binding constants $K_a^{\nu_c}/K_a^0$ can be calculated (Davidson, 1972). For example, for *E. coli* RNA polymerase, $\phi/\phi_e \approx 10$ (Saucier and Wang, 1972), and therefore $K_a^{\nu_c}/K_a^0$ is calculated to be 3 and 190, respectively, for superhelical DNAs with $\nu_c = 0.01$ and 0.05. This large increase in K_a with increasing ν_c is reflected by the dependence of the transcriptional template activity of a covalently closed DNA on its degree of superhelicity (Botchan *et al.*, 1973; Wang, 1974a).

Conversely, if the ratio $K_a^{\nu_c}/K_a^0$ is measured experimentally, the unwinding angle ϕ, relative to that of ethidium, ϕ_e, can be calculated. An example is the recent results on the binding of *lac* repressor to λplac DNAs of varying degree of superhelicity. The unwinding angle per bound *lac* repressor is calculated to be ~3 times that of ethidium (Wang *et al.*, 1974). To obtain the unwinding angle ϕ, however, requires knowing ϕ_e. While up to now ϕ_e has been taken as 12° from the model building results of Fuller and Waring (1964), recent results of this laboratory indicate that $\phi_e = 26°$ (Wang, 1974b).

Stability of a Base Pair in a Superhelical DNA. For a negative superhelical DNA with a certain degree of superhelicity (indicated by a certain ν_c value), assuming that the denaturation of a base pair on the average unwinds the DNA helix by 0.1 turn, the average free-energy gain due to the disruption of one base pair can be estimated from:

$$\Delta G_{\tau,D} \approx \frac{dG_\tau}{d\tau} \times 0.1 = \left(\frac{36}{\phi_e}\right) a_1 RT\nu_c \qquad (17)$$

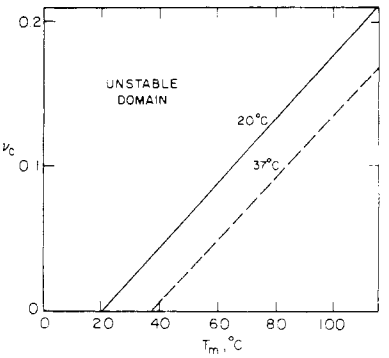

FIGURE 8: The stability of base pairing in a superhelical DNA. See text for details.

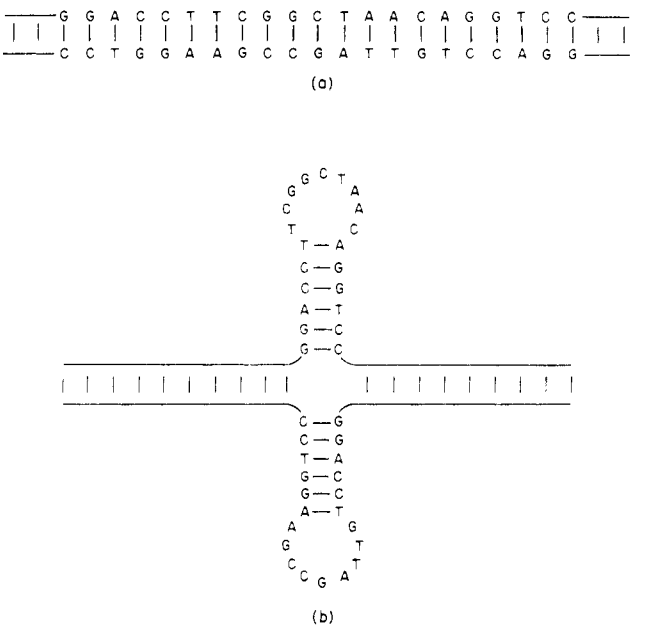

FIGURE 9: (a) An arbitrary sequence with two hexameric oligonucleotide sequences related to each other by a twofold rotational symmetry. (b) A hair-pinned structure. The stability of such a hair-pinned structure in a negative superhelical DNA is discussed in the text.

If this free-energy gain is much greater than the free energy needed for the denaturation of a base pair, the superhelical DNA is expected to contain denatured loops.

The standard free energy of denaturation, per base pair at temperature T, can be estimated from:

$$\Delta G_D = \Delta S_D(T_m - T) \qquad (18)$$

where T_m is the melting temperature and ΔS_D is the entropy change for the denaturation of a base pair, which is ~20 eu (Shiao and Sturtevant, 1973).

The condition $\Delta G_{\tau,D} + \Delta G_D = 0$ at a given temperature T can be represented by a straight line in a ν_c *vs.* T_m plot. In Figure 8, two representative lines are shown for $T = 20$ and 37°, respectively, taking the a_1 values from Table I and $\phi_e = 26°$ (Wang, 1974b). The region to the left of each line represents conditions at which $\Delta G_{\tau,D} + \Delta G_D < 0$ at the temperature specified by the line. In this region, the DNA is likely to contain a segment or segments of unpaired bases.

As an example, consider a segment composed of only AT base pairs in a superhelical DNA. In a typical enzyme reaction medium containing a few millimoles/liter of Mg^{2+} or a medium containing a few tenth moles/liter of Na^+ or K^+, the T_m for this segment is ~70°. From Figure 8 then, for a negative superhelical DNA with a ν_c value greater than 0.11, this pure A · T segment is expected to be unstable with respect to denaturation. Under the same conditions a segment composed of pure G · C pairs is expected to be stable in a negative superhelical DNA with a ν_c as high as 0.2.

In the above discussion, the additional unfavorable free energy for the formation of a denatured loop has not been considered. If the size of the loop is small, the loop formation free energy is an appreciable fraction of the total free-energy change. This tends to make the estimated ν_c value above which unpaired regions may form a lower estimate. Once a loop is formed, however, eq 17 may no longer give the correct quantitative result, as the presence of the loop may considerably modify the properties of the DNA molecule with respect to bending and torsional strains. Thus the usefulness of Figure 8 is primarily for estimating the limiting value in ν_c above which unpairing may occur.

Since all naturally occurring superhelical DNAs examined so far have ν_c values less than 0.08, they are not expected to contain an appreciable number of unpaired bases at room temperature in a medium containing a few tenth moles/liter of Na^+ or a few millimoles/liter of Mg^{2+}. This is in agreement with the results of Wang (1974a). For a highly superhelical PM2 DNA prepared *in vitro*, with a ν_c value of 0.15, the buoyant density of this DNA in neutral CsCl at 20° is higher than that of an untwisted PM2 DNA by ~2 mg/ml (Wang, 1974b). This is indicative that such a sample might contain a significant region of unpaired bases.

Hairpin Formation. Segments of base sequences with a twofold rotational symmetry are often found in DNA. Such a symmetry satisfied a condition for the formation of intrastrand base pairs. The formation of intrastrand base pairs at the expense of disrupting interstrand base pairs is not favored thermodynamically in an ordinary DNA. For a negatively superhelical DNA, however, the disruption of interstrand base pairs causes a reduction of the number of the negative superhelical turns, since it reduces the number of turns the two complementary strands make around each other. Since any reduction in the number of superhelical turns is associated with a favorable free energy of superhelix formation, the formation of hairpins from sequences related by a twofold rotational symmetry is more favorable in a negatively twisted DNA than an untwisted DNA.

As an example, an arbitrary sequence is shown in Figure 9a. An estimate can be made for the free-energy change from the structure in a, to the hair-pinned structure in b. This transition requires the net reduction of five G · C pairs and four A · T pairs. Under conditions such that the average free energy for the disruption of an A · T pair is 1 kcal and that for a G · C pair is 2 kcal, this amounts to 14 kcal. The transition from a to b also involves the formation of two hair-pin loops each consisting of nine bases. It can be estimated that the formation of these two loops requires ~10 kcal (Scheffler *et al.*, 1970). There is an additional unfavorable free-energy change due to the generation of two junctions between the two hair-pinned stems and the long DNA helix. We make a rough guess of 5 kcal for this term. The total free-energy change for the conversion of the structure shown in a to the hair-pinned structure shown in b is therefore ~30 kcal. For a negatively superhelical DNA, the conversion from a to b involves the disruption of 21 interstrand pairs and therefore a reduction of ~2.1 superhelical turns. The corresponding favorable free-energy change is ~2.1 ($dG_\tau/d\tau$). From eq 14a, taking ϕ_e as 26°, it follows that for a negative superhelical DNA with $\nu_c > 0.16$, the

negative (favorable) free-energy change for the loss of ~2.1 turns is greater than 30 kcal. In other words, for a negative superhelical DNA with a ν_c value greater than 0.16, the hair-pinned structure shown in Figure 9b is expected to be thermodynamically stable. In superhelical DNAs from natural sources, with $\nu_c < 0.08$, structure b is not expected to form.

Similar estimates can be made for other sequences. Qualitatively, formation of long hairpins (from long sequences with twofold rotational symmetry) with short single-standed loops is favored in a negative superhelical DNA. There is no evidence at present whether such structures are present in naturally occurring superhelical DNAs. For the base sequence of the *lac* operator, recent results (Wang *et al.*, 1974) argue against the formation of such hair-pinned structures by the binding of the repressor protein. The suggestion of Beerman and Lebowitz (1973) and Lebowitz *et al.* (1974) that hair-pinned structures are present in superhelical ϕX RF and PM2 DNAs seems to be based on observations which, upon careful examination, cannot be explained by such a suggestion. This has been discussed elsewhere (Wang, 1974a).

If the base sequence of a DNA segment in question is known, the thermodynamic data provide a basis for estimating the degree of superhelicity necessary to stabilize such structures. The proper DNA substrates containing such structures can therefore be prepared. This might be useful in testing hypotheses related to the possible roles of such structures.

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