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Action of nicking-closing enzyme on supercoiled and nonsupercoiled closed circular DNA: Formation of a Boltzmann distribution of topological isomers

(polynucleotide ligase/gel electrophoresis)

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ABSTRACT **Highly purified nicking-closing enzyme from mouse cells in 20-fold enzyme/substrate excess converts closed circular native PM2, ColE1, and Minicol DNA into limit product sets of DNAs. Each set has a mean degree of supercoiling of approximately zero. The individual species in the sets differ by $\Delta\tau = \pm 1, \pm 2$, etc., and the relative masses fit a Boltzmann distribution. It was also demonstrated that "nonsupercoiled" closed circular duplex molecules serve as substrates for the nicking-closing enzyme, and that a distribution of topological isomers is generated. Polynucleotide ligase, acting on nicked circular DNA, forms under the same conditions, the same set of closed DNAs. The latter enzyme freezes the population into sets of molecules otherwise in configurational equilibrium in solution.**

Nicking-closing (N-C) activities that alter the topological winding number (α) of closed circular DNA occur widely in nature (1–5). The topological winding number is the number of revolutions that one strand makes about the other if the molecule is constrained to lie in a plane. The activities have been demonstrated to be enzymatic with proteins from *Escherichia coli* (6), mouse (7), and human (8) cells in culture. The N-C enzyme from mouse LA9 cell nuclei, purified to homogeneity in good yield, is a major constituent of chromatin and accounts for about 1% of the total protein (H-P. Vosberg and J. Vinograd, unpublished work). It is similar to other eukaryotic N-C enzymes in its ability to relax both positive and negative superhelical turns. A probable *in vivo* role for the enzyme is to provide the transient swivels required for DNA replication. Such swivels may also be required in transcription, and in the condensation and decondensation of chromatin.

In this study we have examined the limit product of the action of N-C enzyme on several closed circular DNAs by gel electrophoresis. Under appropriate analytical conditions, the limit product separates into a set of species differing in topological winding number. Individual species, isolated from the set, regenerate the original distribution upon incubation with the N-C enzyme. We view the foregoing as the consequence of four necessary events: nicking of the DNA, relaxation, random rotation about the swivel, and closure (Fig. 1). A set of species is also found when *E. coli* polynucleotide ligase is used to close a nicked circular DNA (9, 10). It is shown here that distribution of products obtained with ligase is indistinguishable from that obtained with N-C enzyme when the incubation conditions are the same for both reactions.

The relative masses of the species, when plotted against α, fall on a Gaussian curve. Such a curve is anticipated for a Boltzmann distribution, when the energy of supercoiling is proportional to the square (11) of the degree of supercoiling. The similarity of the products obtained with two different enzyme systems and the further similarities of the values of the free energy of supercoiling obtained here and by nonenzymatic procedures strongly indicate that the Boltzmann distributions are characteristic of the thermally induced torsional fluctuations of free DNA uninfluenced by the presence of an enzyme.

Abbreviations: N-C, nicking-closing; EtdBr, ethidium bromide.

MATERIALS AND METHODS

Enzymes and DNA. *E. coli* polynucleotide ligase was a gift of Dr. H. Boyer. N-C enzyme was prepared from mouse LA9 cell nuclei (H-P. Vosberg and J. Vinograd, unpublished work). DNase I was purchased from Worthington. PM2 DNA was prepared according to (12). ColE1 DNA was prepared from bacterial strain JC411 (ColE1) (13). Minicol DNA was prepared from bacterial strain PVH51 supplied by Dr. H. Boyer (14).

Relaxation of Closed Circular DNA. DNA (5–50 μg/ml) was incubated in 0.2 M NaCl, 0.01 M Tris·HCl, 0.1 mM EDTA at pH 7.4 with 5–20 units of N-C enzyme per μg of DNA for 24 hr at 37°. When relaxations were performed at other temperatures, a second addition of 10 units of enzyme per μg of DNA was made after 24 hr and the incubation was continued for a further 24 hr. Reactions were terminated by the addition of sodium dodecyl sulfate to a final concentration of 0.1%. For comparison of the enzymatically relaxed DNA with ligase closed DNA, both reactions were carried out in 0.2 M NaCl, 3 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 33 μM NAD, 0.2 mM EDTA, 0.1 mM spermidine, 20 μg/ml bovine

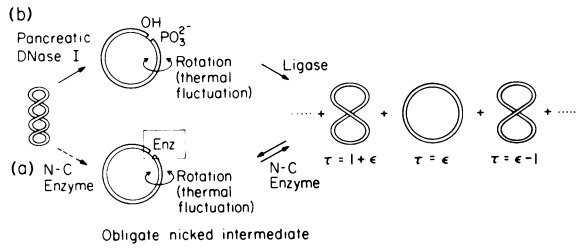

FIG. 1. The formation of a Boltzmann distribution of topological isomers of closed circular DNA. (*a*) The action of N-C enzyme on a closed circular DNA substrate. The diagram is not intended to specify the mechanism for N-C action. The obligate elementary steps include nicking, releasing preexisting supercoils, random rotation at the swivel, and closure. The quantity ϵ is the difference between the duplex winding number (β) of a nicked DNA and the duplex winding number of a similar hypothetical molecule with $\tau = 0$. The fractional turn ϵ is not illustrated in Fig. 1. (*b*) The closure of nicked circular DNA by polynucleotide ligase.

serum albumin, 30 mM Tris·HCl at pH 7.8 for 8 hr at 37°. Five micrograms of DNA and 140 units of N-C enzyme and/or 2×10^{-3} units of ligase were used in the reactions.

Electrophoresis. A vertical slab gel electrophoresis apparatus (Aquebogue) was used. Gels contained 1% agarose (Sea Kem), 40 mM Tris·acetate at pH 7.8, 0.5 mM EDTA. Magnesium acetate (5 mM) was added in most instances. Samples (5–100 μl) containing 200–400 ng of DNA were layered into the sample wells in a solution containing approximately 10% Ficoll 70 (Pharmacia) and 2 mM EDTA at pH 8. Up to 250 μg of DNA in 1 ml of the same solution was layered into the large sample well of a preparative gel. Two volts per cm were applied to the 8 mm preparative gels and 3 V/cm to the 4 mm analytical gels. Electrophoresis times were varied according to voltage gradient, molecular weight of the DNA, and temperature.

Determination of Relative Masses of DNA Species by Fluorescence Photography. Gels were stained in the dark overnight in 10 mM Tris·HCl, 2 mM EDTA, 2 μg/ml of ethidium bromide (EtdBr). The gels were illuminated from below with short wavelength ultraviolet light from a Transilluminator (Ultra Violet Products Inc.) and photographed on Kodak Plus X film. The films were traced on a Joyce-Loebl microdensitometer. Traces were evaluated with a Hewlett Packard 9864A Digitizer Platen and 9820A calculator. Relative fluorescence intensities (J_i) were evaluated and integrated over the band to obtain the relative mass with the equation

$$\sum_b J_i \Delta x_i = \sum_b (10^{D_i/\gamma} - 1)\Delta x_i \qquad [1]$$

where D_i is the optical density above background and γ is the slope of the characteristic curve of the film (γ was evaluated for each film). Where appropriate, the areas under each peak in the optical density traces were also evaluated. The areas give a good approximation to the relative concentrations of species providing $D/\gamma \ll 1$. Procedures for calculating ϵ and B (defined below) for the distributions were incorporated into the integration program. A full description of the experimental method will be presented elsewhere (D. E. Pulleyblank and J. Vinograd, unpublished work).

Extraction of DNA from Gels. Stained gels were placed on a mask with 3 mm slots parallel to the direction of the electrophoresis, and portions of the DNA bands were visualized by illumination from below. The gel was sliced so as to separate bands, and the sections that had received direct UV illumination were discarded. The remaining gel fragments were frozen and thawed three times and then centrifuged at 40,000 × g for 1 hr. The supernatant, containing approximately 30–50% of the DNA in the gel slice, was freed of ethidium by extraction with 1-butanol. The DNA was precipitated from the aqueous phase by the addition of 2 volumes of cold ethanol, followed by centrifugation in an SW 50.1 rotor at 35,000 rpm at 4° for 1 hr.

RESULTS

The Existence of Multiple Species of Closed Circular DNA in the Limit Product of N-C Enzyme Action. Keller and Wendel (4) were able to resolve a series of species in partially relaxed closed circular simian virus 40 DNA using agarose gel electrophoresis. Their assumption that adjacent, resolved species differ by a single superhelical turn has been used throughout the present study of DNAs with low numbers of superhelical turns. The validity of the assumption is considered in the *Discussion* section.

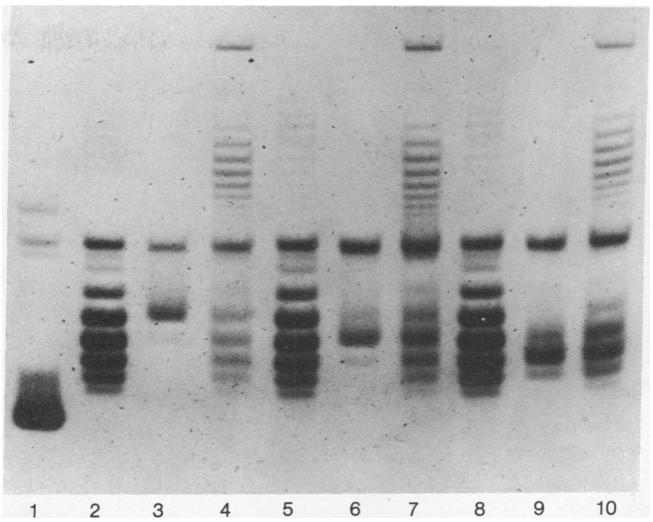

FIG. 2. Demonstration that non-supercoiled DNA is a substrate for the action of N-C enzyme and that N-C enzyme reacts with a homogeneously supercoiled species of closed circular DNA ($\tau \approx 0$) to form a thermal distribution of species. (*1*) Minicol I and II; (*2*, *5*, and *8*) limit products of the initial reaction of Minicol I with N-C enzyme; (*3*, *6*, and *9*) purified species with $\tau \approx +1, 0, -1$, respectively; (*4*, *7*, and *10*) limit products regenerated after reaction of purified species with N-C enzyme. Bands migrating slower than Minicol II in channels 4, 7, and 10 are sets of relaxed ColE1 DNA. Native ColE1 DNA was added to the reaction mixtures to monitor the activity of the enzyme.

In a study of the effects of electrophoresis conditions on the resolution of closed circular DNAs with differing values of α, it was found that the limit product of N-C enzyme action on closed circular DNA could be resolved into multiple species. Optimum resolution was achieved at 4° in the presence of 5 mM Mg^{++}. Under these electrophoresis conditions, the mean duplex winding number (β) is greater than during the relaxation reaction (15). The increase is compensated by the generation of $\Delta\tau$ negative superhelical turns in all members of the set according to the equation $\Delta\beta = -\Delta\tau$. Comigration of pairs of species that initially had equal numbers of positive and negative superhelical turns is eliminated, and the magnitudes of τ are such that the gel resolves species that differ by unit values of τ.

The Generation of a Set of Species from an Isolated Homogeneous Species. The thermal nature of the distribution observed in the limit products of the N-C enzyme action on closed circular DNA has been established with experiments such as shown in Fig. 2. The three dominant species of Minicol DNA present in the N-C enzyme limit products (channels 2, 5, and 8) were isolated separately from a preparative agarose gel and purified. These materials (channels 3, 6, and 9) were treated with the N-C enzyme under the conditions used to generate the original set of limit products. In each case a new set of products was generated (channels 4, 7, and 10) with the same distribution as the original set.

The above results lead to the following conclusions. (*i*) The species found in the original reaction mixture are the limit products of the reaction and are not generated by incomplete relaxation of the supercoiled substrate. Therefore, under the conditions of reaction, the materials in channels 3, 6, and 9 contained approximately +1, 0, and −1 superhelical turns, respectively. (*ii*) Species are generated with higher and lower superhelical winding numbers than the substrates for the rereaction. Rotation about the swivel must, therefore, be driven by thermal fluctuations. The creation of a multi-

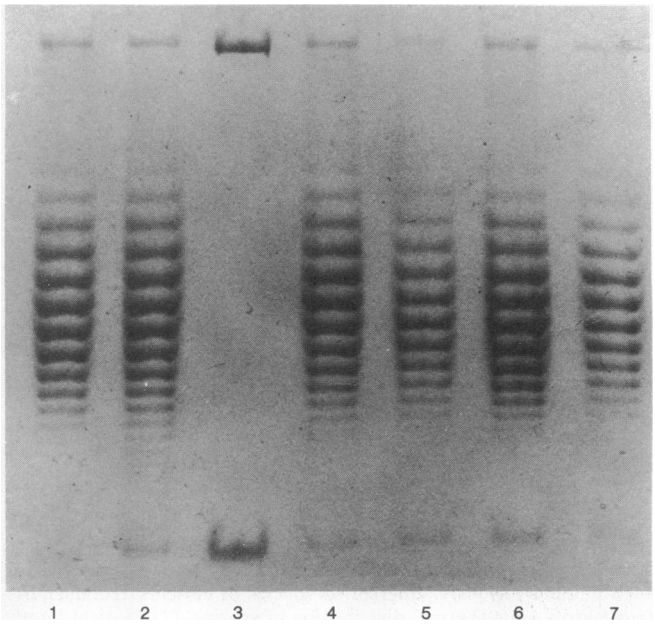

FIG. 3. The limit product of the N-C enzyme is indistinguishable from the ligase-closed product. (*1*) PM2 II + Ligase; (*2*) PM2 I + N-C Enzyme; (*3*) I, II; (*4*) mixture of products in (*1*) and (*2*); (*5*) PM2 I, II + N-C Enzyme+Ligase; (*6*) PM2 II + N-C Enzyme+Ligase; (*7*) ligase closed PM2 + N-C Enzyme. Reactions were carried out in 0.2 M NaCl, 3 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 33 μM NAD, 0.2 mM EDTA, 0.1 mM spermidine, 20 μg/ml of bovine serum albumin, 30 mM Tris·HCl at pH 7.8 for 8 hr at 37°; 5 μg of DNA, 140 units of N-C Enzyme, 2×10^{-3} units of ligase were used in the reactions. Nicked and linear DNA were removed by EtdBr–CsCl buoyant centrifugation. PM2 I (10 ng) was added to each channel.

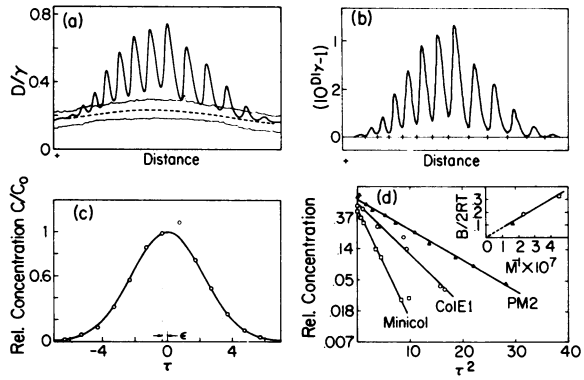

FIG. 4. Processing of data for the relative masses in each set. The Boltzmann distribution. (*a*) Absorbance trace of a photograph of an ethidium stained gel of relaxed PM2 DNA with background traces from both sides of the sample channel. Sample background (- - -) was calculated by averaging the background traces. The ordinate is plotted in units of D/γ. (*b*) Plot of fluorescence intensity against distance, generated from the optical density trace shown in 4a. The curve was calculated by the equation $J = 10^{D/\gamma}-1$, where D is the optical density above background. The peaks were integrated between the indicated limits, to determine the relative masses of the species present. (*c*) The concentration of species present in solution are normalized by C_0, the concentration of a theoretical species lacking supercoils. The curve through the points is the calculated Gaussian curve with the best least squares parameters, 0.105 and 0.33 for $B/2RT$ and ϵ, respectively. The point lying above the curve at $\tau = 0.67$ was not used in the least squares fit because of contamination of this species by linear PM2 DNA. (*d*) The natural logarithm of the relative masses of species in the limit products of three closed DNAs treated with N-C enzyme, plotted against the square of the superhelical winding number. The intercepts of the traces have been displaced for clarity of presentation.

ple set from a single species represents an increase in the entropy of the system. (*iii*) Supercoiling of the closed circular substrate is *not* a requirement for the action of the mouse N-C enzyme. Linear and nicked circular DNAs can therefore, with confidence, be considered substrates for the enzyme.

Sets of Species Formed with N-C Enzyme and Closed Circular DNA Are Indistinguishable from Those Formed with Ligase and Nicked Circular DNA. Ligase closure of nicked circular DNA also leads to a set of species with different topological winding numbers (10). The distribution of species was compared with the distribution of N-C enzyme products (Fig. 3). Since environmental conditions affect the duplex winding number, and hence the position of the center of the distribution (see below), it was necessary to perform the reactions under conditions that were as similar as possible. The observed distributions were indistinguishable (channels 1 and 2). The addition of a second enzyme and/or substrate to a reaction mixture caused no detectable change in the distributions (channels 4, 5, 6, and 7), and ruled out the possibility that the correspondence between the distributions in channels 1 and 2 was due to adventitious effects of protein binding. The N-C enzyme did not close the ligase substrate PM2 II (data not shown).

We conclude that the forms of the distributions generated by each of the two systems are the same, with respect to both the center of the distribution and, with less accuracy, to the relative concentrations of the species present. The comparison of products generated by the two enzymatic systems corroborate conclusion (*i*) of the previous section.

The Boltzmann Distribution. A set of molecules at thermal equilibrium contains a distribution of states with respect to all degrees of freedom available to the molecules. The number of molecules in a given state (N_i) within the set (N_t) is related to the energy of the state (E_i) by the Boltzmann equation

$$N_i/N_t = A \exp(-E_i/RT) \quad [2]$$

where A is a normalization factor; R and T have their usual meanings. A closed circular DNA with a given value of α cannot come to thermal equilibrium with respect to α, because of the requirement for breakage and reformation of a covalent bond in the phosphodiester backbone. The N-C enzyme catalyzes these reactions, and allows the system to come to equilibrium.

The molar free energy associated with supercoiling of closed circular DNA (G_τ) has been determined for superhelical simian virus 40 DNA (11) and PM2 DNA (15) and is related to the number of superhelical turns by the equation

$$G_\tau = (B\tau^2/2) \quad [3]$$

where B is a constant. The superhelix density, $\sigma = 20\ \tau/N$ where N is the number of nucleotides in the DNA is substituted into [3]

$$G_\tau = B(N\sigma/20)^2/2 \quad [4]$$

We observe, providing G_τ is proportional to N when σ is constant, that B must be inversely proportional to N.

Upon incorporating Eq. [3], the Boltzmann Eq. [2] becomes

$$N_i/N_t = A \exp(-B\tau^2/2RT) \quad [5]$$

The equation is Gaussian, and a plot of ln N_i against τ^2 should be linear. We have defined ϵ ($-0.5 < \epsilon \leq 0.5$) as the difference between the duplex winding number β of a

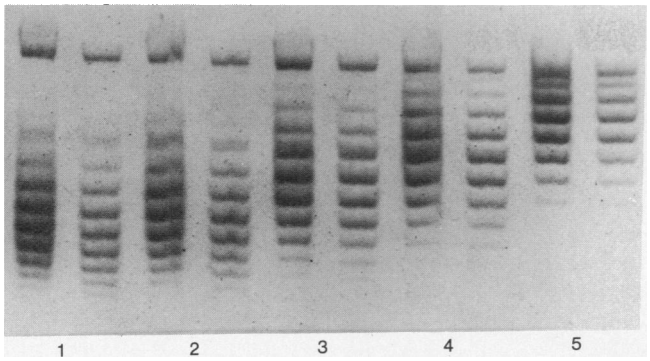

FIG. 5. The effect of the incubation temperature on the position of limit products in a slab-gel electrophoresis experiment. The paired samples contained 100 and 200 ng of DNA. The incubation temperatures were 41.5°, 37.2°, 29.6°, 22.5°, and 13.4° in (*1*) to (*5*).

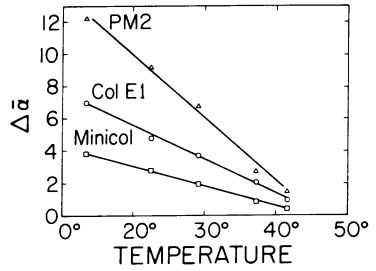

FIG. 6. The temperature dependence of the center of the Boltzmann distribution. Each distribution was analyzed by the least squares procedure to obtain the value of ϵ, and hence the position of the center of the distribution. A corresponding band was used in each set as a reference; the number of turns including partial turns (ϵ) to the center of each distribution was measured. The center of the distribution corresponds to $\bar{\alpha}$ as defined in the *Discussion*.

nicked DNA and the duplex winding number of a similar hypothetical closed molecule with $\tau = 0$ in the same environment. The number of superhelical turns in a member of the distribution is defined by the equation $\tau = I + \epsilon$ where I is an integer.

The relative concentrations of the species present in the limit products of N-C enzyme action on Minicol, ColE1, and PM2 DNAs were determined from traces of photographs of ethidium stained gels (Fig. 4a and b). The relative concentrations were fitted by a least squares procedure to a Gaussian equation to determine the best value for ϵ and $B/2RT$ (Fig. 4c). The natural logarithm of the relative concentrations are plotted against τ^2 (Fig. 4d). The slopes of the lines ($B/2RT$) are inversely proportional to the molecular weight of the DNA (Fig. 4d, insert); this is in agreement with the prediction made above. The values of $B/2RT$ measured at 37° for PM2, ColE1, and Minicol DNA were 0.10, 0.17, and 0.32, respectively. The reproducibility was ±10% for measurement of 16 channels in two gels for ColE1 DNA, and measurements of 12 channels in two gels for PM2 and Minicol DNAs. Note that the number of species observed is approximately proportional to the square root of the molecular weight of the DNA (Fig. 4d).

The Thermal Unwinding of DNA. The value of the equilibrium duplex winding number (β) is sensitive to small changes in the environment of the DNA. Analysis of the distributions obtained after N-C enzyme action on closed circular DNA give accurate values for ϵ. Changes in β can be measured by counting the number of turns including partial turns (ϵ) between the center of a sample distribution and the center of a reference distribution. In general ϵ can be estimated to within ±0.1 duplex turn; this corresponds to a limit of accuracy for PM2 DNA of about ±0.2 turn in the 10^3 duplex turns.

Temperature and protein binding are examples of factors that affect β and $\bar{\alpha}$ (see *Discussion* for the definition of $\bar{\alpha}$). Here closed circular PM2, ColE1, and Minicol DNA samples were relaxed at different temperatures (Fig. 5). Each distribution was analyzed by the least squares procedure to obtain the value of ϵ, and hence the position of the center of the distribution. One of the species in the distributions was used as an arbitrary marker, and the number of helical turns to the center of each distribution was calculated and plotted against the temperature of reaction (Fig. 6). The temperature dependence of the rotation angle was $-1.4 \pm 0.1 \times 10^{-2}$ °/C°, base pair.

Quantitation of the mass distributions in Fig. 5 showed that the values of $B/2RT$ were constant to within ±10%, which indicates that the entropy of supercoiling is the major component of the superhelix free energy.

DISCUSSION

The assumption that each resolved species differs from its neighbors by a single turn in its topological winding number is supported by two arguments. It is the simplest explanation for the observation of discrete bands. Other proposals, for example, that adjacent species differ by two turns in the topological winding number, appear artificial and especially unlikely in view of the similarity of the results obtained here with N-C enzyme and polynucleotide ligase. If a shift in the center of the distribution by a single band is equivalent to a 360° change in the winding of the duplex, we calculate the thermal unwinding angle of duplex DNA to be $-1.4 \pm 0.1 \times 10^{-2}$ °/C°, base pair. This value corresponds well with $-1 \pm 0.2 \times 10^{-2}$ °/C°, base pair calculated from the results of Wang (16) by using 26° instead of 12° for the unwinding angle (ϕ) of ethidium when bound to DNA (17, 18).

The Free Energy of Supercoiling. The free energy of supercoiling has been determined in the past by studying the relative binding affinities of a closed circular DNA and its nicked circular counterpart for the unwinding ligand, ethidium (11, 15). To compare the more direct results presented here with those obtained previously, it is necessary to know ϕ. Provided that the DNA is negatively supercoiled, a term ν_c is defined as the molar ratio of bound ethidium to DNA phosphate when all superhelical turns have been removed. If ϕ is expressed in degrees $\tau = N\ \phi\nu_c/360$, where N is the number of nucleotides in the DNA. Eq. [3] can then be written

$$G_\tau = (B/2)(N\phi\nu_c/360)^2 \qquad [6]$$

As noted previously, B is inversely proportional to the molecular weight of the DNA. We define a molecular weight independent term $b \equiv BN/2$ to compare the present results with those obtained previously by others. Eq. [3] assumes the form $G_\tau = b\tau^2/N$ where G is in cal mol^{-1} when RT is in cal mol^{-1}. The value of b/RT calculated for the three DNAs in the present study is $2.06 \pm 0.14 \times 10^3$. We have reevaluated the coefficient of Eq. [**24**] of Bauer and Vinograd (11), with the more recent value of 26° for ϕ, and obtained 1.78×10^3. With the value of a_1 as defined and determined by Wang (ref. 15, cf. Eq. [**14e**] and Eq. [**6**] above), we obtain a value for b/RT of 1.05×10^3. The correspondence between these values is satisfying in view of the widely differing experimental methods and the present uncertainties about the cor-

rect value of ϕ (18). It should be pointed out that the correspondences may be the result of compensating errors, in particular since the salt concentration was 0.2 M instead of 3.0 and 5 M in the previous studies.

Superhelix Density Heterogeneity and the Relationships among the Winding Numbers. We define a time dependent variable β_t as the number of duplex turns in a nicked circular DNA molecule. The equilibrium duplex winding number β is defined as the time-averaged or ensemble-averaged value of β_t. Since the system of closed circular molecules generated by the closure of a nicked circle is not homogeneous with respect to α, we define a new term $\bar{\alpha}$ as the median of the Gaussian curve that fits the Boltzmann distribution in α (Fig. 4c). The term $\bar{\alpha}$ is equal to the value of β at the time of ring closure, and approximates the average value of α. In addition, the term $\bar{\tau}$ is defined by $\bar{\tau} = \bar{\alpha} - \beta$. Under the conditions of ring closure, $\bar{\tau} = 0$, whereas individual molecules have values of $\tau = I + \epsilon$, where I is integral. The equation $\bar{\tau} = \bar{\alpha} - \beta$ replaces, in the case of a Gaussian distribution, the previous equation, $\tau = \alpha - \beta$, derived for a single species.

Mechanism of Action of N-C Enzyme. The results presented here do not allow us to distinguish between alternatives for one aspect of the N-C enzyme action on "nonsupercoiled" DNA: the single turn mechanism in which rotation at the swivel is limited to one turn during a nicking-closing event, and the multiturn mechanism in which several rotations can occur during the nicking-closing event. The direction of rotation in both cases would be biased by the free energy of supercoiling. Comparable alternative explanations have been considered for the appearance of intermediates during the relaxation of highly supercoiled DNA (4, 7).

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