Extracellular nucleases of Alteromonas espejiana BAL 31. IV. The single strand-specific deoxyriboendonuclease activity as a probe for regions of altered secondary structure in negatively and positively supercoiled closed circular DNA*

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ABSTRACT

The dependence of the initial rate of introduction of the first single-chain scission (initial nicking rate) into covalently closed circular phage PM2 DNA by the single strand-specific nuclease from Alteromonas espejiana BAL 31 upon the superhelix density (σ) of the DNA has been examined. The initial nicking rate decreases with decreasing numbers of negative superhelical turns (decreasing values of $-\sigma$), which behavior is characteristic of other single strand-specific nucleases as reported earlier. In contrast to earlier work, the initial nicking rates of closed circular DNAs by the action of the Alteromonas nuclease have been shown to be readily measurable at values of $-\sigma$ as low as 0.02. However, even at the elevated concentrations of enzyme and extended digestion periods required to cause nicking at an appreciable rate at near-zero values of σ, closed circular DNA containing very few superhelical turns (form I^0 DNA) is not cleaved at a detectable rate. When this DNA is rendered positively supercoiled by ethidium bromide (EtdBr), it is not affected by the nuclease until very high positive values of σ are attained, at which low rates of cleavage can be detected at elevated enzyme concentrations. The effects of EtdBr on the enzyme activity have been tested and are entirely insufficient to allow the interpretation of zero nicking rates as the result of inhibition of the nuclease activity by the dye. Positively supercoiled DNA is concluded not to contain regions having significant single-stranded character until values of σ are reached which are very much higher than the values of $-\sigma$ for which negatively supercoiled DNAs behave as if they contain unpaired or weakly paired bases.

INTRODUCTION

It has been amply demonstrated that molecules of negatively supercoiled closed circular duplex DNA (form I DNA) contain regions having altered secondary structure compared to that of nonsupercoiled molecules of the same DNA under the same conditions. The manner in which such altered regions are manifested in negatively supercoiled DNAs is consistent with the interpretation that these regions possess single-stranded character; *i.e.*, a fraction of the bases in a negatively supercoiled DNA molecule exist in unpaired or weakly hydrogen-bonded configurations

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for a greater fraction of the time than do the bases in the corresponding nonsupercoiled DNA. Evidence for such behavior includes the greatly enhanced reactivity of superhelical DNA, compared with that observed for the corresponding nonsupercoiled nicked circular (form II) DNA, with a variety of chemical reagents which react with certain bases in DNA.[1-6] Hydrogen-tritium exchange studies[7] have also yielded evidence for regions of single-stranded character in phage PM2 form I DNA, and several single strand-specific deoxyriboendonucleases have been shown to cleave supercoiled forms of DNA rapidly under conditions for which the corresponding forms of these DNAs, which are covalently continuous in both strands but contain very few superhelical turns, are cleaved at very low rates or not detectably.[8-13]

The dependence of the initial rate of introduction of the first single-strand scission (initial nicking rate) into closed circular DNAs by single strand-specific nucleases has been examined as a function of superhelix density at negative superhelix densities.[3,13] These experiments were done using samples of closed circular PM2 DNA which were artificially made so as to differ from one another only in the superhelix density (σ_0). For the single strand-specific nucleases from Neurospora crassa and from Mung bean, Wang[13] observed that the initial nicking rate was relatively slow at values of $-\sigma_0$ below about 0.08 and was only slightly dependent upon the value of $-\sigma_0$. However, for more highly negatively supercoiled DNAs, the initial nicking rate increased rapidly with increasing values of $-\sigma_0$. Woodworth-Gutai and Lebowitz[3] made similar observations with respect to the N. crassa enzyme, although they began to observe a substantial dependence of initial nicking rate upon $-\sigma_0$ near a value of 0.06. All superhelix densities reported here are corrected to reflect the redetermined value of the amount of unwinding of duplex DNA per intercalated ethidium moiety, which is now taken as 26° instead of the previously accepted value of 12° (refs. 14-16). The symbol σ_0 is used to represent the superhelix densities of DNAs in the absence of intercalating agents, while this symbol without the subscript represents the superhelix densities of DNAs in the presence of EtdBr.

Alteromonas espejiana BAL 31 (American Type Culture Collection 29659; see ref. to Chan et al. in title footnote) produces extracellularly a nuclease which is very highly specific for single-stranded DNA and for regions of altered helix structure produced in duplex DNA by

irradiation with ultraviolet light or by covalent reaction with certain carcinogenic or mutagenic agents.[12,17] The Alteromonas (As.) nuclease also cleaves negatively supercoiled DNA, but fails to cleave covalently closed circular DNA containing very few superhelical turns (form I^0 DNA) at a detectable rate under conditions for which 100% of supercoiled (form I) PM2 phage DNA would be cleaved almost instantly.[12,17]

In the present study, the action of the As. nuclease upon closed circular PM2 DNA has been examined as a function of σ for negatively supercoiled forms of this DNA, both in the presence and absence of ethidium bromide, and for forms of this DNA rendered positively supercoiled in the presence of ethidium bromide. The results indicate that nicking of closed circular DNA proceeds at readily detectable rates at much smaller values of $-\sigma$ than observed with the other nucleases under conditions for which form I^0 DNA is not significantly affected. Positively supercoiled DNA also is not cleaved by the As. nuclease until very high superhelix densities are reached, at which low rates of cleavage can be detected at elevated enzyme concentration and extended times of incubation. Evidence is presented in this study that the essentially zero nicking rates observed at all but very high positive values of σ are not the result of inhibition of the nuclease by the EtdBr which is present to render the DNA positively supercoiled. It is thus apparent that positively supercoiled DNA does not possess significant single-stranded character until values of σ are attained which are very much higher than the values of $-\sigma$ for negatively supercoiled DNA for which rapid cleavage is observed at much lower enzyme concentrations and periods of incubation. The present work also has implications as to the nature of the regions in supercoiled DNA which are sensitive to single strand-specific nucleases.

MATERIALS AND METHODS

Preparation of Enzymes. DNA ligase from E. coli was the preparation used in previous work from this laboratory.[18] Nicking-closing enzyme was obtained from mouse Ehrlich ascites carcinoma cells according to published procedures[19,20] as modified in this laboratory.[17]

The extracellular nuclease from As. BAL 31 (refs. 12, 17) was purified to homogeneity by an extension (Winston, T.P., Hodnett, J. L. and Gray, H.B., Jr., unpublished work) of the published procedure[12] and came from two fractions of the same preparation used in another study.[17]

Both of these fractions displayed the bulk of the protein in a single band, migrating at the position expected for the nuclease,[12] in polyacrylamide gel electrophoresis experiments in non-denaturing gels. The activity of the enzyme from one of these fractions, diluted 1000-fold in CAM buffer [0.1 M NaCl, 20 mM Tris-HCl, 5 mM each $CaCl_2$ and $MgSO_4$, 1 mM EDTA (pH 8.1)] and stored near 4°C, was unchanged with respect to the initial nicking rate for PM2 form I DNA over a six week period (see Results). Each series of experiments in this study was done with a single sample of nuclease and was carried out over a short enough period of time to preclude significant loss of enzyme activity.

Preparation of PM2 DNAs. The modified[17] procedure of Richardson[21] was used to obtain PM2 form I DNA. Nicked circular (form II) PM2 DNA was produced by the limited action of bovine pancreatic DNase upon form I DNA.[22] Samples of negatively supercoiled PM2 DNA differing in superhelix density were obtained from the action of *E. coli* DNA ligase upon form II DNA in reaction mixtures containing different concentrations of EtdBr.[23-25] PM2 form I^0 DNA was produced directly from form I DNA by the action of the nicking-closing enzyme from mouse tumor cells as described[17] except that incubations with the extract containing the nicking-closing enzyme activity were carried out for up to 14 hours. Separation of closed circular DNA from other forms of DNA was accomplished by centrifugation in buoyant CsCl density gradients containing saturating levels of propidium diiodide;[24] the intercalating dye was removed by chromatography on Dowex resin[26] with the resin pretreated as noted.[27] All samples of DNA were dialyzed extensively against BE buffer [0.1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA (pH 8.1)].

Determination of Superhelix Densities. The dye-buoyant density gradient method originally described by Gray *et al.*[25] was used to measure the superhelix densities of the closed circular PM2 DNAs. Samples were centrifuged for at least 48 hours at 20°C at 40 Krpm in a Beckman SW50.1 rotor, using starting CsCl solution densities of 1.55 and 1.58 g/ml for EtdBr and propidium diiodide, respectively. Several closed circular DNAs were usually centrifuged in a single gradient. Propidium was used only where necessary to achieve separation of closed circular DNAs of similar values of σ_0. Viral PM2 form I DNA was used as the reference DNA and all separations were measured from the position of the PM2 form II DNA present in all the centrifuge tubes. The tubes were photographed under ultraviolet illumination using an apparatus similar

to that described by Watson *et al.*,[28] and the photographic negatives were subjected to measurement according to Gray *et al.*[25]

The use of a closed circular DNA other than the form I DNA of SV40 virus as the reference DNA necessitates modification[29,30] of the original equation[25] describing the relationship between the relative buoyant separation and the superhelix density. Upholt[29] and Burke and Bauer[30] have independently modified the relationship between relative buoyant separation and σ_0 to allow for the use of DNAs other than SV40 viral form I DNA as the reference. If the superhelix densities of SV40 form I DNA and PM2 form I DNA (based on the assumption of a 12° unwinding of the duplex by a single ethidium moiety) are taken to be -0.039 and -0.053, respectively,[25] Eq. (6b) of Gray *et al.*[25] for DNA banded in EtdBr-CsCl gradients can be written

$$\sigma_0^{unk} = -0.053 + 0.101\,(\Omega_c - 1) \qquad (1)$$

where Ω_c is the relative buoyant separation[25] defined as

$$\Omega_c = f^{-1}(\bar{r}/\bar{r}^*)(\Delta r/\Delta r^*)$$

in which f^{-1} is a correction factor that is unity if all the DNAs in question have the same G+C content, as is the case here, $\bar{r}$ is the actual distance from the axis of rotation of the positions midway between the bands of open and closed circular forms of DNA, and Δr represents the separation, in actual distance in the centrifuge tube, between the bands of open and closed circular DNA. The asterisk denotes the value corresponding to the reference form II - form I DNA pair, the closed circular member of which must have the same value of σ_0 as viral PM2 form I DNA if the constants in Eq. (1) are used. For DNA banded in propidium diiodide-CsCl gradients, Eq. (8b) of ref. 25 becomes

$$\sigma_0^{unk} = -0.053 + 0.08(\Omega_c - 1) \qquad (2)$$

if viral PM2 form I DNA is used as the reference material.

As the above superhelix densities correspond to the 12° ethidium intercalation angle, multiplication by the factor 26°/12° = 2.17 is required to convert these values to those based on a 26° unwinding angle.

The values of σ_0 obtained by the above procedure correspond to those which would be observed in 2.83 M CsCl at 20°C. In order to correct these values to those which would be observed under the temperature and solvent conditions of this work (below), published estimates of the effects upon σ_0 of changing counterions from Cs^+ to Na^+ (ref. 23) and then decreasing the concentration of Na^+ from 2.8 M to 0.1 M (ref.

31) were used. These data yielded a value of 0.018 to be added to the values of σ_0 obtained from the buoyant separation procedure. This yields a value of σ_0 = -0.100 for viral PM2 form I DNA in 0.1 M NaCl at 20°C.

The required correction factor was also obtained from sedimentation-dye titration and viscometric-dye titration data for PM2 form I DNA in BE buffer containing 50% (v/v) D_2O at 20°C (ref. 32). These data yield a value of σ_0 = -0.099 for PM2 form I DNA in this solvent, indicating a correction factor in excellent agreement with that calculated above. Finally, the value of σ_0 for PM2 form I DNA obtained from boundary sedimentation-dye titration under the solvent conditions of this work (below) is -0.100, which again indicates the same corrèction factor and shows that the magnesium and calcium ion present in this solvent does not detectably affect the superhelix density of PM2 DNA. Hence, the correction factor of 0.018 was used.

Analytical Ultracentrifugation. Analytical band sedimentation[33] was done as described [18,34] using as solvents the neutral and alkaline CsCl solutions having the compositions given elsewhere.[35]

Boundary sedimentation velocity-dye titration experiments were performed on PM2 form II, form I and form I^0 DNAs in order to measure the sedimentation coefficient (s) as a function of the molar ratio of ethidium bound per DNA nucleotide (ν). The latter quantity is calculated from the measurement of the concentration of EtdBr centripetal to the sedimenting boundary and the known total concentrations of dye and DNA nucleotides.[14,36] The superhelix density of the closed circular DNA at a given dye concentration (σ) is then calculated from

$$\sigma = 1.45(\nu-\nu_c) \quad (3)$$

where ν_c is the value of ν corresponding to the complete relaxation (σ = 0) of the closed circular DNA in question.

These experiments were performed at 20°C and 30 Krpm with the monochromator adjusted to 285 nm (ref. 14) in CAM buffer. A calibration curve was constructed by centrifuging solutions of EtdBr of known concentrations in CAM buffer. This curve displayed good linearity between observed absorbance and dye concentration. The photoelectric scanning system was calibrated during each experiment using the "stairstep" calibration procedure so that the observed absorbances could be corrected for slight variations in the pen displacement corresponding to a given absorbance.[36] Concentrations of EtdBr and DNA were calculated on

the basis of reciprocal extinctions of 79.2 μg/ml-$A_{1\ cm}^{485\ nm}$ and 50 μg/ml-$A_{1\ cm}^{260\ nm}$, respectively, in BE buffer. The concentration of DNA was constant at 24.9 μg/ml in all the boundary sedimentation experiments.

Thermal convection in the boundary sedimentation experiments was obviated by the use of a modified rotor temperature indicator and control system[37] as described.[38]

Values of ν were also measured for the binding of EtdBr to PM2 form I^0 DNA at higher dye concentrations, at which the ultraviolet absorbance due to the EtdBr would have precluded the use of the boundary sedimentation method, by pelleting the DNA-EtdBr complex from solution in a preparative ultracentrifuge and assaying the concentration of dye in the supernatant photometrically at 485 nm. The conditions of buffer, initial DNA concentration, and temperature were as above. Solutions 0.7 ml in volume were centrifuged at 44 Krpm for 17 hours in a Beckman SW50.1 rotor, using adaptors which permit the use of 5 mm x 42 mm cellulose nitrate tubes. In order to compensate partially for minor effects apparently caused by redistribution of the dye in the centrifugal field, solutions containing EtdBr at each of the starting dye concentrations used in these experiments, but containing no DNA, were centrifuged under the same conditions. The small differences in the absorbances of these solutions from their absorbances before centrifugation were used to correct the absorbances of the DNA-containing solutions for the above effect. A Cary 118C spectrophotometer was used and care was taken to remove the same portion of each supernatant solution from the liquid column.

Calculation of Parameters of Binding of EtdBr to DNA. The values of ν obtained for PM2 form II DNA in the boundary sedimentation experiments were used to calculate the intrinsic binding constant $\underline{K}$ and the maximum level of dye binding ν_m from the Scatchard[39] equation

$$\nu/c_f = K(\nu_m - \nu) \qquad (4)$$

where c_f represents the molar concentration of unbound EtdBr in equilibrium with bound dye. Since the total concentration of dye is simply

$$c_t = c_f + N_t\nu \qquad (5)$$

where N_t is the known molar concentration of DNA nucleotides, sufficient information is available to plot ν/c_f vs. ν and obtain values for $\underline{K}$ and ν_m. The five data points obtained fell on a straight line and yielded the values (linear least squares) of 1.78 ± 0.14 x 10^5 liters/mole and 0.24 ± 0.02 for $\underline{K}$ and ν_m, respectively. These values apply to nicked

circular and linear duplex DNA in CAM buffer at 20°C.

Ultracentrifuge Assay of As. Nuclease. The time course of the conversion of closed circular PM2 DNA to a mixture of form II and form III DNAs was monitored by band sedimentation in the alkaline solvent of aliquots of the reaction mixtures as described.[12] PM2 DNA in BE buffer, EtdBr in BE buffer, and BE buffer comprised 0.7 of the 140 µl volume of the reaction mixture, which were mixed with 0.2 volume of a buffer containing 0.1 M NaCl, 22.5 mM each of $CaCl_2$ and $MgSO_4$, 20 mM Tris-HCl, 1 mM EDTA (pH 8.1) after which 0.1 volume of *As*. nuclease solution in CAM buffer was added. The salt and buffer composition of the final digestion mixture was thus that of CAM buffer. The initial compositions of reaction mixtures were identical to those of the solutions centrifuged in the boundary sedimentation experiments except for the presence of the nuclease. From four to seven 20 µl aliquots were taken from each reaction mixture at appropriate times of incubation at 20°C (depending upon the initial nicking rate) and mixed with sufficient 0.25 or 0.5 M EDTA solution (pH near 8.1) to stop the reaction.[12] These solutions were assayed directly by band sedimentation. Initial nicking rates were estimated from the initial slopes of the curves of log(% covalently closed DNA) *vs*. time.[13]

Problems have been occasionally encountered, in this and in other laboratories, with divalent metal cation-dependent nicking of closed circular DNA in the absence of the addition of any agents known to introduce breaks into duplex DNA. In order to monitor possible adventitious nicking, experiments were routinely carried out in which CAM buffer was substituted for the nuclease and incubation at 20°C was continued for at least 2 hours. Nicking of closed circular DNA in the solutions subjected to boundary sedimentation was monitored by alkaline band sedimentation of the DNA recovered after each boundary sedimentation experiment.

The rate of conversion of PM2 form II DNA to form III DNA[12,17] was measured as a function of the concentration of EtdBr in reaction mixtures similar to those used in the case of closed circular DNA. Aliquots were assayed by band sedimentation in the neutral solvent.[12] As much higher concentrations of *As*. nuclease are required to effect this conversion at a reasonable rate compared to those used in the case of highly supercoiled DNA, the double-strand exonuclease activity of this nuclease[12] is able to cause appreciable conversion of the form III

DNA molecules to non-sedimenting material during the incubations. The amounts of material in the sedimenting bands corresponding to linear duplex PM2 DNA thus do not correspond to the amounts of form II DNA cleaved in the reaction. Accordingly, a constant amount of phage $\lambda b_2 b_5 c$ DNA was added to each aliquot to use as a reference against which the decrease in the percentage of form II DNA with time of digestion could be calculated.[17]

It has been shown that lesions induced in PM2 form I^0 DNA by ultraviolet irradiation can serve as substrate sites for the As. nuclease.[17] PM2 form I^0 DNA, irradiated with ultraviolet light for 15 seconds as described,[17] was incubated with As. nuclease in CAM buffer at 20°C in the presence of various concentrations of EtdBr in order to test the effect of the intercalating dye upon this activity. The elevated concentrations of enzyme required to achieve cleavage at readily measureable rates again necessitated the use of the $\lambda b_2 b_5 c$ reference DNA. These reaction mixture aliquots were assayed in the alkaline solvent for band sedimentation.

Photometric Assay of As. Nuclease. A photometric assay based on that described by Vogt[40] using denatured calf thymus DNA as substrate has been used to determine the activity of preparations of the As. nuclease.[12] The unit of activity described by Vogt[40] for the S_1 nuclease has been adopted as the unit of activity of the As. nuclease. Measurements of activity of the As. nuclease are done in the medium described by Gray *et al.*[12] at 30°C.

The effect of EtdBr upon the activity of the As. nuclease with single-stranded DNA as the substrate was tested using the photometric assay as described[12] except that the reactions were carried out in CAM buffer at 20°C. Control reaction mixtures (no nuclease) contained EtdBr at the same concentration as in the nuclease-containing samples.

RESULTS

Effect of EtdBr Upon the Activities of the As. Nuclease. Some of the results of this study would be open to question if the As. nuclease were strongly inhibited by EtdBr. The effect of this dye upon the activity of the nuclease against several types of substrates has accordingly been examined.

The effect of EtdBr up to a concentration of 40 µg/ml on the activity of As. nuclease with alkali-denatured calf thymus DNA as the substrate is shown in Figure 1. It was important to monitor this activity

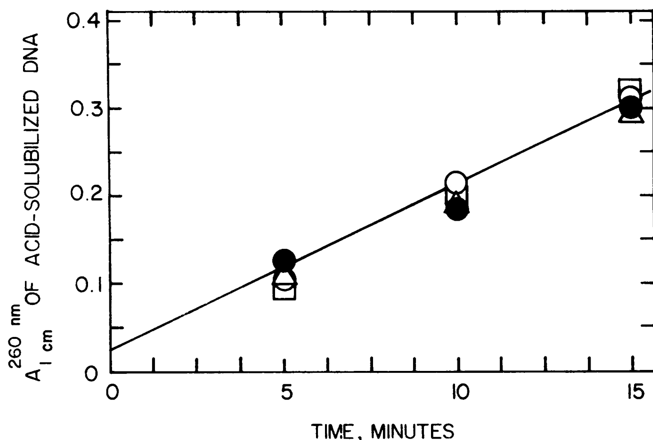

Figure 1. The effect of EtdBr upon the activity of As. nuclease against single-stranded calf thymus DNA. Concentrations of EtdBr (μg/ml) were: ○, 0; □, 10; Δ, 30; ●, 40. The line represents the linear least squares curve fit to the data. Enzyme assay and reaction conditions are given in Materials and Methods. Approximately 4.5 units/ml of As. nuclease were used in the reaction mixtures.

as a control for the possible activity of the enzyme upon supercoiled closed circular DNA in the presence of EtdBr, since it is presumably regions in negatively supercoiled DNA which behave as if single-stranded or weakly hydrogen-bonded that give rise to the sensitivity of such DNAs to single strand-specific nucleases. There is clearly no significant effect of EtdBr upon the rate at which single-stranded DNA is rendered acid-soluble by the As. nuclease.

It was also necessary to examine the effect of EtdBr upon the activity of As. nuclease against what is essentially a duplex DNA substrate, because the supercoiled closed circular DNA substrates are very largely duplex in character. One type of such substrate which can be present in duplex DNA is a single-strand scission (nick), which gives rise to cleavage by the As. nuclease of the initially intact strand in the vicinity of the nick. In the case of form II DNA, such cleavage clearly results in the formation of linear duplex (form III) molecules.[12,17] The effect of the dye upon the kinetics of conversion of PM2 form II DNA to form III DNA by the nuclease are presented in Figure 2. The dye decreases the initial rate of conversion between the two forms of DNA by a factor of about 0.5 between zero concentration of EtdBr (ν = 0) and a 20 μg/ml concentration (ν = 0.21) of this interca-

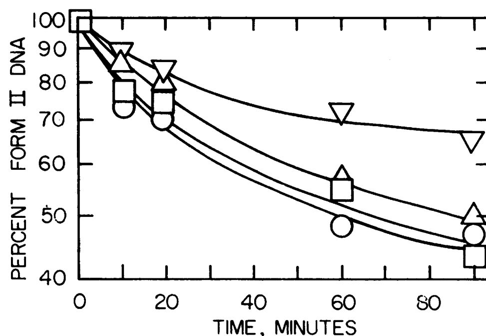

Figure 2. The effect of EtdBr upon the rate of conversion of PM2 form II DNA to form III DNA by the As. nuclease. Reaction mixtures in CAM buffer at 20°C contained 24.9 μg/ml of DNA, approximately 225 units/ml of nuclease, and EtdBr at the following concentrations: (μg/ml):○, 0; □, 1; Δ, 2.5; ∇, 20. The values of ν corresponding to these concentrations of EtdBr were calculated using the values of $\bar{K}$ and ν_m obtained as described in Materials and Methods and are 0, 0.026, 0.068, and 0.206, respectively. The assay for the conversion of form II DNA is described in Materials and Methods. The point at zero time of incubation is taken to represent 100% starting substrate in this figure and in the other figures in which percent starting substrate is plotted as a function of incubation time.

lating agent. The highest dye concentration used in the studies presented below at which no cleavage of the closed circular DNA took place was 10 μg/ml and corresponded to a value of ν near 0.12. Since EtdBr present at a concentration higher than 10 μg/ml and at a molar binding ratio higher than 0.12 did not prevent the attack of the nuclease upon a duplex substrate, it is unlikely that the failure of the nuclease to cleave closed circular DNA in the presence of EtdBr, where observed, is due to the inhibition of the As. nuclease by the intercalating agent.

PM2 form I^0 DNA irradiated with ultraviolet light becomes susceptible to cleavage by the As. nuclease, presumably due to the presence of pyrimidine dimer photoproducts.[17] Untreated PM2 form I^0 DNA is not cleaved by the enzyme. Thus, it was also possible to test the effect of EtdBr upon the action of the nuclease against a predominantly duplex, closed circular substrate which contains lesions known to serve as substrate sites for the enzyme in the absence of EtdBr. PM2 form I^0 DNA was irradiated (Materials and Methods) and was then exposed to the As. nuclease in the absence of EtdBr and in the presence of various concentrations of EtdBr (Figure 3). The data show that the initial rate of

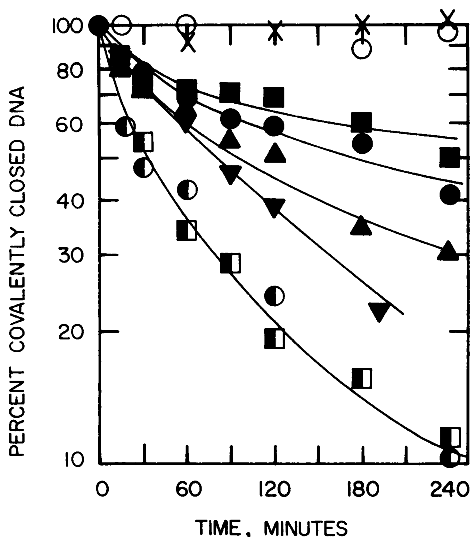

Figure 3. The effect of EtdBr upon the activity of the As. nuclease against untreated and ultraviolet-irradiated PM2 form I^0 DNAs. Reaction mixtures in CAM buffer at 20°C contained 24.9 μg/ml of DNA and EtdBr and nuclease at the concentrations indicated. PM2 form I^0 DNA, irradiated with ultraviolet light (Materials and Methods) was incubated with approximately 225 units/ml of As. nuclease in the presence of EtdBr at the following concentrations (μg/ml): ●, 0; ■, 7; ▲, 10; ▼, 20; ◐, 40; ◧, 100. X, untreated form I^0 DNA incubated in the presence of 100 μg/ml EtdBr with CAM buffer substituted for enzyme solution. O, untreated PM2 form I^0 DNA incubated with approximately 225 units/ml of nuclease in the absence of EtdBr. The assay for the nicking of closed circular DNA is described in Materials and Methods.

cleavage of the irradiated form I^0 DNA is significantly greater in the absence of dye than in the presence of 7 μg/ml of the intercalating agent, but then increases monotonically with increasing EtdBr concentration up to 40 μg/ml. Increasing the concentration of dye in the reaction mixtures to 100 μg/ml, the highest concentration used in these studies, does not markedly alter the initial nicking rate from that observed at 40 μg/ml (bottom two curves in Figure 3). A series of control experiments at 100 μg/ml of EtdBr, in which CAM buffer was substituted for the nuclease solution, showed that the EtdBr stock solution contained no agents which could effect the nicking of non-irradiated PM2 form I^0 DNA. Similar experiments were done with the irradiated form I^0 DNA in the presence of 100 μg/ml of EtdBr, and with both non-irradiated and irradiated form I^0 DNAs in the absence of the

dye, with no detectable nicking of these DNAs (data not shown).

These results show that EtdBr at low concentrations does decrease the rate at which ultraviolet-irradiated form I^0 DNA is cleaved, but that at higher concentrations the rate at which nuclease-sensitive sites are cleaved again increases. This is consistent with the results of experiments in which untreated PM2 form I^0 DNA is incubated with nuclease in the presence of EtdBr as will be shown below. Since, as mentioned earlier, non-irradiated PM2 form I^0 DNA is not attacked by the *As*. nuclease in the presence of EtdBr at concentrations of 10 µg/ml and below, the results of Figure 3, which indicate that irradiated form I^0 DNA is attacked at all concentrations of EtdBr by the nuclease at the same enzyme concentration, may be taken as further strong evidence that any failure of closed circular DNA to be cleaved in the presence of EtdBr cannot be attributed to inhibition of the nuclease by EtdBr.

Dependence of the Initial Nicking Rate by As. Nuclease upon the Superhelix Density. The initial rate of loss of closed circular PM2 DNA was measured as a function of σ_0 at 20°C. The open plot symbols of Figure 4 represent experiments with samples of PM2 DNA produced so as to have various superhelix densities. These digests were carried out in the absence of EtdBr. A very rough comparison may be made of the rate at which strand breaks are introduced into a supercoiled DNA (*e.g.*, viral PM2 form I DNA) and into a single-stranded DNA. A value of K_m and values of V_m as a function of the number of enzyme units/ml have been determined for the *As*. nuclease-catalyzed hydrolysis of the single-stranded circular DNA of coliphage øX174 at 30°C (Winston, T. P., Hodnett, J. L. and Gray, H. B., Jr., unpublished results). Since the reaction mixtures were treated with alkaline phosphatase and the extent of the reaction monitored by the release of acid-soluble phosphate, it is possible to estimate the number of single-strand scissions per DNA nucleotide per unit time from these data. The number of breaks per DNA nucleotide per unit time in closed circular DNA may be estimated from the initial nicking rate through the Poisson formula and the number of nucleotides per molecule of PM2 DNA. At the enzyme and DNA nucleotide concentration of Fig. 4, it is calculated that roughly 350 single-strand scissions would be made in single-stranded DNA for every scission in viral PM2 form I DNA. This ratio is only a crude estimate because the reaction temperatures and compositions of the solvents are different for the two sets of measurements (CAM buffer at 20°C in the present

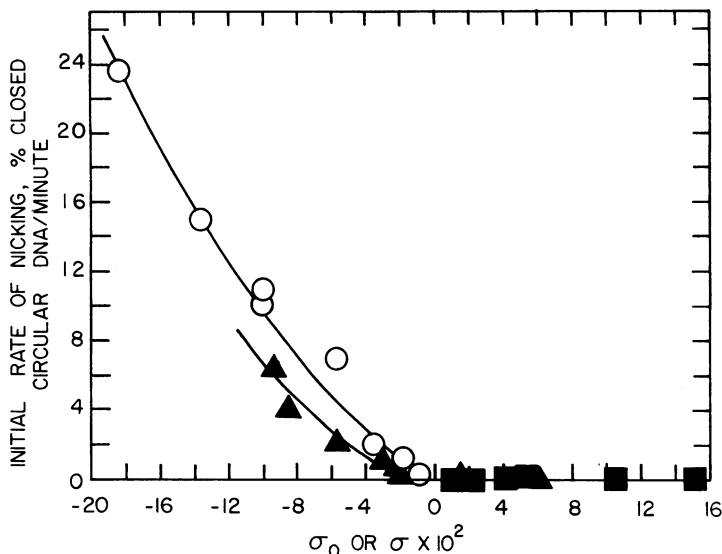

Figure 4. Dependence of the initial nicking rate of closed circular PM2 DNA upon superhelix density. Reaction mixtures in CAM buffer at 20°C contained 24.9 μg/ml of DNA and nuclease at a concentration of approximately 0.45 units/ml. Open symbols, reaction mixtures contained no EtdBr; filled symbols, reaction mixtures contained EtdBr to yield the superhelix densities indicated as calculated from boundary sedimentation-dye titration data as described in Materials and Methods. ▲, PM2 form I DNA; ■, PM2 form I^0 DNA.

case and the solvent described by Gray et al.,[12] which contains 0.6 M NaCl, at 30°C in the case of the data for single-stranded DNA). Using another set of estimates for the kinetic parameters of hydrolysis of ϕX174 phage DNA obtained by photometric monitoring of the reaction, also at 30°C in the solvent of Gray et al.,[12] the ratio of scissions in single-stranded DNA to scissions in PM2 form I DNA which would be made by a fixed concentration of As. nuclease per unit time is estimated at approximately 650. Hence, PM2 form I DNA in CAM buffer at 20°C behaves as if it contains the equivalent of very roughly one base in a single-stranded configuration for every 250 base pairs.

The data represented by the open plot symbols in Figure 4 appear to indicate that nicking of closed circular DNA takes place at a finite rate even at very small negative values of σ_0. However, for the three points closest to $\sigma_0 = 0$, the apparent initial nicking rates are so small as to raise some doubt as to whether these are indeed non-zero

rates of conversion. In order to demonstrate more convincingly that the nuclease is active against closed circular DNA at negative superhelix densities near $\sigma_0 = 0$, aliquots of the four negatively supercoiled samples nearest zero superhelix density (including PM2 form I^0 DNA, $\sigma_0 = -0.013$) were subjected to the action of the As. nuclease at a concentration roughly 500 times that used in the set of experiments with all the negatively supercoiled DNAs. Readily measureable initial nicking rates were obtained for all forms but PM2 form I^0 DNA (Figure 5). The data for PM2 form I^0 DNA represent a different set of experiments than that represented by the open circular plot symbols in Figure 3. The initial nicking rates obtained from Figure 5, and from the data represented by open circular plot symbols in Figure 3, are represented by the open plot symbols in Figure 6. Clearly, PM2 form I^0 DNA is not a substrate for the As. nuclease even at the highly elevated enzyme concentration and extended times of incubation of these experiments, in agreement with earlier results,[17] whereas negatively supercoiled PM2 DNA of $-\sigma_0$ near 0.02 and greater is cleaved. The extremely high specificity of the As. nuclease in this regard is thus clearly

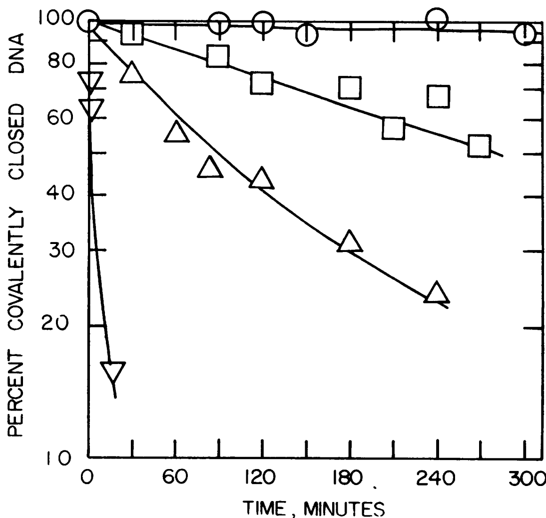

Figure 5. Percentage of closed circular DNA remaining plotted logarithmically as a function of time of incubation with As. nuclease in the absence of EtdBr. Reaction mixtures in CAM buffer at 20°C contained 24.9 μg/ml of DNA and approximately 225 units/ml of enzyme. The superhelix densities (CAM buffer, 20°C) of the DNAs are: ○, -0.013; □, -0.017; Δ, -0.034; ∇, -0.053.

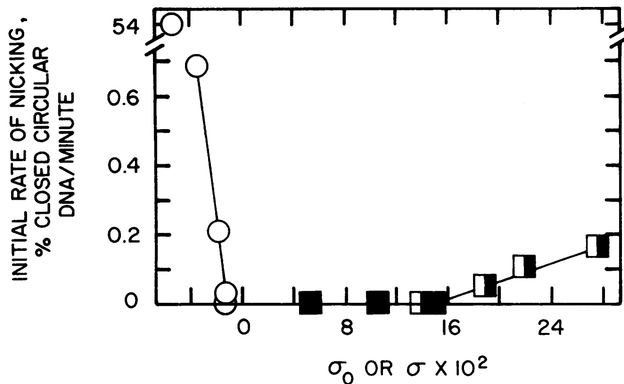

Figure 6. Dependence of the initial nicking rate of closed circular PM2 DNA upon superhelix density. Reaction mixtures were as in the legend to Figure 5. Open symbols, reactions were done with DNAs of Figure 5 and in the absence of EtdBr; filled and half-filled symbols, reactions were done with PM2 form I^0 DNA in the presence of EtdBr to yield the superhelix densities indicated. Values of σ were calculated from boundary sedimentation-dye titration data (filled symbols) or from the concentration of EtdBr remaining in the supernatant after ultracentrifugation of DNA solutions containing EtdBr (half-filled symbols). Note break in ordinate scale.

demonstrated.

Although the concentration of As. nuclease in the experiments represented in Figures 5 and 6 is roughly 500 times that used in the experiments of Figure 4, there is nowhere near a 500-fold increase, for a given DNA, in the initial nicking rates of Figure 6 over those of the other set of data. This is presumably due, in the case of the most negatively supercoiled DNA, to the very rapid initial nicking rate at the elevated nuclease concentration (Figure 5), which was far too large to measure accurately. The apparent rates of nicking for the DNAs at the other three superhelix densities for which digests were done at both nuclease concentrations are not in the expected ratio, probably because of the considerable inaccuracy in estimating initial nicking rates in reactions for which only a few percent of the closed circular molecules are cleaved, as was the case at the lower nuclease concentration.

The filled symbols in Figure 4, and the filled and half-filled symbols in Figure 6, represent experiments done in the presence of various concentrations of EtdBr under the same conditions of solvent, temperature, and enzyme concentration as those represented by the open symbols in each figure. Boundary sedimentation experiments were

performed on samples identical to each of those represented by filled symbols in Figures 4 and 6 except that CAM buffer was substituted for the CAM buffer solution of As. nuclease. Hence, the value of σ for each sample represented by a filled plot symbol in Figures 4 and 6 is known (Materials and Methods). When the concentration of EtdBr is sufficiently low that PM2 form I DNA is still negatively supercoiled, this DNA is cleaved at slower rates than those observed when the dye is not present. This is in accord with the results presented above showing that EtdBr decreases the rate of cleavage by As. nuclease of essentially duplex substrates, but clearly does not stop the reaction. The two points (open symbols) corresponding to viral PM2 form I DNA (σ_0 = -0.10) were obtained at the outset, respectively, of the experiments done in the absence of EtdBr and those in which various concentrations of EtdBr were used with PM2 form I DNA. This shows that the activity of the highly dilute nuclease preparation did not change noticeably over the approximately six weeks between the initiations of the two sets of experiments.

For PM2 form I^0 DNA at all concentrations of EtdBr, and for form I DNA at concentrations of dye sufficient to render the superhelix density of this DNA approximately -0.02 and greater, the initial nicking rate is indistinguishable from zero at the relatively low nuclease concentration used in connection with the data of Figure 4. Form I^0 DNA also is not cleaved in the presence of EtdBr at the much higher concentration of enzyme used to obtain the data of Figure 6 up to and including a dye concentration of 10 μg/ml, which concentration is represented by the filled and half-filled plot symbols near σ = 0.15 in that figure. At still higher concentrations of EtdBr, low rates of nicking were observed (remaining half-filled plot symbols of Figure 6). In order to more fully document these findings, the kinetic data used to obtain the points represented by the half-filled plot symbols of Figure are shown in Figure 7. Linear least-squares analysis of all the data points of each curve of Figure 7 was used to estimate the nicking rates. The data point representing the highest value of σ in Figure 6 corresponds to a value of 0.20 for the molar binding ratio. Since the maximum value of ν under these conditions was determined to be 0.24 ± 0.02 (Materials and Methods), the point at the highest value of σ in Figure 6 corresponds to near-saturation binding of the dye.

Figure 8 shows the ratio of sedimentation coefficient of closed

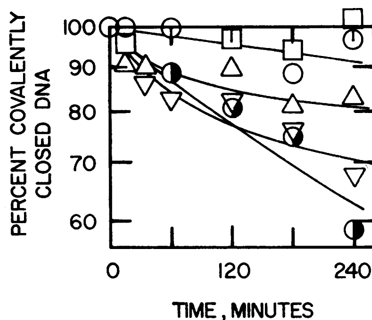

Figure 7. Percentage of closed circular PM2 form I^0 DNA remaining plotted logarithmically as a function of time of incubation with As. nuclease in the presence of various concentrations of EtdBr. Reaction mixtures were as in the legend to Figure 6. EtdBr concentrations (μg/ml) were as follows: ○, 0; □, 10; Δ, 20; ∇, 40; ◑, 100. Data corresponding to zero EtdBr concentration is the same as in Figure 3.

circular PM2 DNA to that of the nicked circular form as a function of σ. The correlation between hydrodynamic behavior and susceptibility of closed circular PM2 DNA to cleavage by the As. nuclease may be ascertained by comparison of Figures 4 and 8. The highest value of σ represented in Figure 8 corresponds to an EtdBr concentration of 10 μg/ml, the highest dye concentration examined in these studies for which no cleavage of PM2 form I^0 DNA was observed, even at an elevated enzyme concentration (Figures 6 and 7). It proved impractical to obtain values of s at higher concentrations of EtdBr (Materials and Methods).

The local maximum and minimum at positive superhelix densities in Figure 8 have been evidenced previously[32,41] and are confirmed in this work. This behavior must reflect a property of the closed circular DNA, since the value of s for form II DNA simply decreases monotonically with increasing values of ν. Such non-monotonic dependence of s upon superhelix density for negatively supercoiled DNA has been well-documented[13,23,25,31,42] and is present whether the curve is generated by an EtdBr titration of an initially highly negatively supercoiled DNA, or whether a series of closed circular DNAs made artificially from a given species of DNA is examined directly in the absence of dye. The initial nicking rate of closed circular DNA by single strand-specific nucleases is not correlated with the local minimum and maximum in the hydrodynamic properties at negative superhelix densities in that no non-monotonic behavior is evidenced for this rate. Rather, this rate

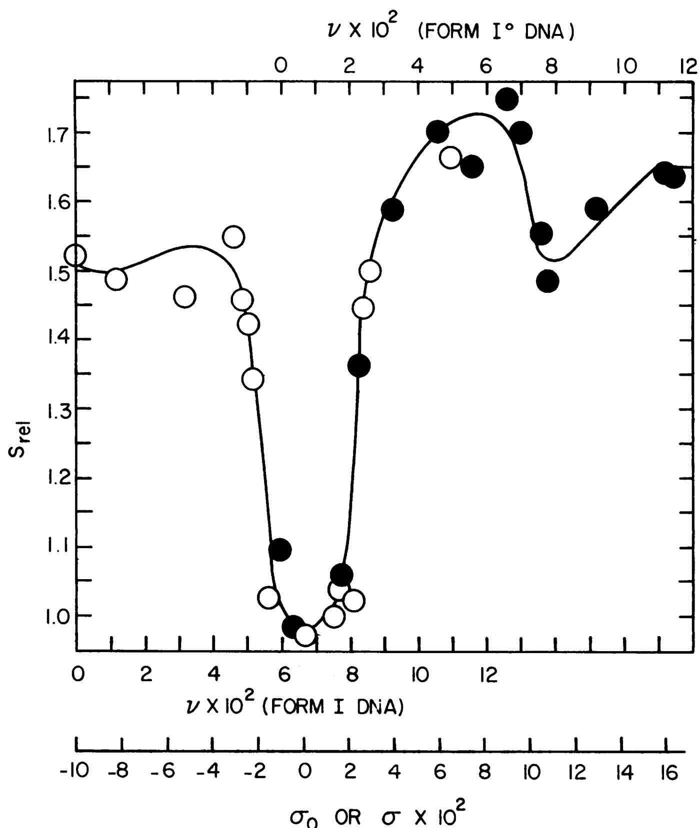

Figure 8. Ratio of the sedimentation coefficient of PM2 form I or form I^0 DNA to that of PM2 form II DNA at a given value of ν as a function of ν (upper two abcissae) and superhelix density (lower abcissa). Boundary sedimentation was carried out (CAM buffer, 20°C) as described in Materials and Methods. The values of $\underline{s}$ for form II DNA corresponding to the values of ν at which values of $\bar{s}$ were measured for the closed circular DNAs were obtained from a plot of $\bar{s}$ for form II DNA as a function of ν, which yielded a straight line. Values of σ, σ_0, and ν were obtained as described in Materials and Methods. ○, PM2 form I DNA; ●, PM2 form I^0 DNA.

decreases monotonically with increasing σ in this and in previous work.[3,13] The present study also shows no correlation between nicking rate and σ at positive superhelix densities, since the nicking rates are indistinguishable from zero at all values of σ or σ_0 from approximately -0.02 up to at least 0.15, by which value the local minimum has been passed (Figure 8). The nicking rate for PM2 form I^0 DNA at positive

superhelix densities was not distinguishable from zero in these experiments until sufficient dye was bound to yield $\sigma = 0.19$ (Figure 6).

DISCUSSION

It has been established that the duplex structure of a closed DNA containing negative superhelical turns is destabilized, relative to a nonsupercoiled duplex, as the free energy of formation of superhelical turns is positive.[42,43] The enhanced reactivity, compared to that of nonsupercoiled DNAs, of negatively supercoiled DNAs with chemical reagents[1-6] and single strand-specific nucleases[8-13] has been assumed to be a result of the destabilization of the DNA helix in negatively supercoiled structures. Since the unwinding of the duplex removes negative superhelical turns, a slight unwinding of the duplex in negatively supercoiled DNA is thermodynamically favored compared to such unwinding in nonsupercoiled duplexes. This is consistent with the idea that the enhanced reactivity of negatively supercoiled DNA is due to an overall slight unwinding of such duplexes, which gives rise to unpaired or weakly hydrogen-bonded bases in these molecules. Since any process which unwinds the duplex in positively supercoiled molecules will increase the number of superhelical turns, such unwinding, compared to that in nonsupercoiled DNA, is energetically unfavored. On this basis, it would not be expected that chemical reagents or enzymes that fail to react appreciably with nonsupercoiled DNA would react with positively supercoiled DNA.

The nature of the regions in negatively supercoiled DNA which display single-stranded character is not known. Such regions possibly could be described by an increase, over the value associated with nonsupercoiled duplexes, in the equilibrium constant corresponding to the ratio of transiently unpaired to paired bases in certain portions (presumably enriched in A·T base pairs) of the molecule. Models in which regions of local disruption of the duplex exist stably in negatively supercoiled DNA molecules have been proposed and are discussed below in connection with the present findings.

Wang[13] has suggested that the sites in supercoiled DNA which are sensitive to cleavage by single strand-specific nucleases, or to reaction with chemicals specific for non-duplex structure in DNA, are regions in which the helix bends sharply back upon itself. These regions, in this view, are characterized by local disruptions of the DNA helix and might correspond to the ends of the superhelical branches

evidenced in electron microscopic examinations of highly negatively supercoiled DNAs.[31,44] Such sites require only a slight unwinding of the duplex; moreover, their existence could be strongly energetically favored by sharp bending of the helix so that the thermodynamics of supercoiling might not preclude their presence in highly positively supercoiled DNA.

Some of the observations which led to the above interpretation were that the rate of cleavage of supercoiled PM2 DNA with the single strand-specific nucleases used in Wang's[13] study was relatively low and not strongly dependent on σ_0 until a value of σ_0 was reached (near -0.08) that corresponds to the region of superhelix density in which the molecules begin to appear tightly twisted in the electron microscope. This region also corresponds to that in which the sedimentation coefficient begins to decrease as the value of $-\sigma_0$ is increased from zero. The decrease in sedimentation coefficient has been interpreted as corresponding to the formation of tightly twisted molecules.[31]

In contrast, the data of the present study show that closed circular PM2 DNA at any value of $-\sigma_0$ greater than approximately 0.02 is definitely cleaved by the As. nuclease under conditions for which PM2 form I^0 DNA ($-\sigma_0 = 0.013$) is not affected. These findings indicate that regions susceptible to attack by an enzyme which is highly specific for non-duplex structure in DNA are present in negatively supercoiled DNA at values of $-\sigma_0$ which are as much as 0.06 units below those for which tightly twisted structures begin to appear in the electron microscope.[31] The hydrodynamic behavior of negatively supercoiled DNA also clearly depends monotonically upon superhelix density at values of σ at which the nuclease begins to display activity (Figure 8). The results of this work thus strongly suggest that tightly twisted superhelical structures, giving rise to sharp bends in the duplex, are not required for the presence of a detectable level of non-duplex character in these DNAs. If such sharp bends were required, it would be very difficult to account for the cleavage of DNA of low values of $-\sigma_0$ by the As. nuclease, the rate of which cleavage depends strongly upon σ_0, when essentially non-supercoiled closed circular DNA is unaffected under the same incubation conditions. The "tight bending" model thus does not appear to account for the observed single-stranded character of negatively supercoiled PM2 DNA, as manifested by the cleavage of this DNA by the As. nuclease, over the entire range of σ_0 for which sensitivity to the enzyme is

observed. Such a model can, however, account for the cleavage of very highly positively supercoiled DNA as noted below.

Another model has been advanced by Lebowitz and his co-workers[2,3,5] in which the regions of single-stranded character are associated with "cruciform" structures. In this model, negative superhelical turns are removed from the molecule, without the need for unpairing approximately ten bases for every superturn removed, by maintaining most of the base pairs through intrastrand base pairing. The intrastrand pairing requires that palindromic (inverted repeated) base sequences be present at the sites of the cruciform structures. The loops necessarily present at one end of each region of intrastrand pairing contain several unpaired bases and represent the initial sites of reaction of the DNA with chemical reagents or enzymes specific for non-duplex structure in DNA.

This model, however, may be convincingly ruled out as accounting for the cleavage by the As. nuclease of closed circular PM2 DNA at the lower values of $-\sigma_0$ at which cleavage was shown to occur. Hsieh and Wang[42] have obtained an expression for the free energy changes associated with supercoiling and have compared these changes with those estimated to accompany the formation of a cruciform structure of arbitrarily chosen size and palindromic base sequence. They concluded that the formation of such structures is unlikely even for DNAs containing substantially more titratable superhelical turns than viral PM2 form I DNA. Although there may be sufficient uncertainty in some of the numerical estimates to allow some room for debate as to the presence of cruciform structures in DNAs as highly supercoiled as viral PM2 form I DNA, it is certain that such structures cannot exist stably in some of the DNAs shown in this study to be cleaved by the As. nuclease. For example, PM2 closed circular DNA of $\sigma_0 = -0.03$, which corresponds to the value $\nu_c = 0.021$, would readily be attacked by the nuclease (Fig. 5). Calculation in Eq. (14a) of Hsieh and Wang[42] using their empirically determined value of a corresponding to 20°C, a value of zero for ν, the above value for ν_c, and the value of 26° for the amount by which duplex DNA is unwound by the intercalation of a single molecule of EtdBr, yields the value of -1.9 kcal/mole for the free energy change accompanying the removal of one titratable superhelical turn. The cruciform structure chosen by the above authors would have removed 2.1 superhelical turns and was estimated to require 30 kcal/mole

for its formation independently of its effects upon supercoiling. According to the above estimate, a free energy gain of approximately 4.0 kcal/mole would correspond to the unwinding of 2.1 superhelical turns. Thus, even if the estimated and measured parameters were so seriously in error as to result in an overestimate of the free energy of formation of the cruciform structure by a factor of as much as seven, the formation of a stable cruciform structure would not take place in a molecule which is nonetheless readily susceptible to cleavage by an enzyme which is highly specific for non-duplex structure in DNA. In addition, the substantial unwinding of the duplex associated with the formation of such structures strongly mitigates against their being present in positively supercoiled DNA of any superhelix density and renders it very unlikely that such structures could account for the sensitivity of very highly positively supercoiled DNA to the nuclease.

The _As_. nuclease does not cleave positively supercoiled DNA at a detectable rate until sufficient EtdBr is bound to reach positive values of σ which are far greater than the values of $-\sigma$ for which cleavage of negatively supercoiled DNA occurs at readily observable rates. At approximately 500 times the nuclease concentration causing the nicking of over 50% of PM2 form I DNA in 5 minutes, positively supercoiled DNAs at values of σ at least up to 50% greater than the value of $-\sigma_0$ for PM2 form I DNA are unaffected in a 240 minute incubation. These results are most readily interpreted as indicating that the regions having single-stranded character in negatively supercoiled DNA at low values of $-\sigma$ do not have counterparts in the positively supercoiled structures until these are very highly positively supercoiled. Any alternative interpretation which attributes the lack of activity of the nuclease against positively supercoiled DNA to effects due to the EtdBr, such as the protection from the nuclease of regions of unpaired or weakly hydrogen-bonded bases by bound dye, is rendered untenable by the fact that positively supercoiled DNA is definitely cleaved, albeit at a relatively low rate, at very high superhelix densities by the nuclease at the highest concentration employed in this study.

Further evidence that any effects due to EtdBr cannot be responsible for the absence of activity of the nuclease against positively supercoiled DNA over the range $-0.02 < \sigma < 0.15$ is present in the studies with the predominantly duplex form II and ultraviolet-irradiated form I^0 substrates. These experiments have shown that EtdBr decreases

the rate at which the nuclease attacks lesions such as single-strand breaks and photoproducts from ultraviolet irradiation, but that this inhibition is by no means great enough to prevent the observation of enzyme activity. It should be noted that the data of Figure 3, representing the activity of the As. nuclease against irradiated form I^0 DNA at various concentrations of dye, are entirely consistent with the data of Figure 7, representing similar experiments using non-irradiated form I^0 DNA. At the dye concentrations for which detectable activity against the non-irradiated closed circular DNA was seen, the rate of cleavage of the irradiated samples increased markedly over the values characteristic of the lower dye concentrations. Such a result is expected, as the irradiated DNA presumably contains both types of sites (those arising from ultraviolet radiation-induced damage and those present due to extremely high positive supercoiling) which serve as substrate sites for the nuclease.

In view of the above, and because neither the tight bending model nor the cruciform model appear to account for the observed cleavage of closed circular PM2 DNA by the As. nuclease at low values of $-\sigma_0$, we are led to favor a rather unstructured model in which regions in the DNA that contain bases which are unpaired or weakly hydrogen-bonded to an extent greater than that in form I^0 DNA provide substrate sites for the nuclease at values of $-\sigma_0$ below those for which tightly twisted structures appear. As the formation of such regions requires a slight unwinding of the duplex, the probability of their presence, relative to that in non-supercoiled DNA, is greater or lesser in negatively and positively supercoiled DNA, respectively, on the thermodynamic basis discussed above. Hence, DNA which is positively supercoiled, at least at values of σ below those for which tightly twisted structures would begin to appear, would be expected to display less single-stranded character than form I^0 DNA and thus would not be cleaved by the As. nuclease.

Although a correlation between superhelix density and the appearance of tightly twisted structures has not been made in the case of positively supercoiled DNA, it has been shown that such DNA can display structures similar to those of highly negatively supercoiled DNA. PM2 form I DNA, mounted for electron microscopy in the presence of EtdBr using the aqueous Kleinschmidt technique, appears highly twisted, with several superhelical branches per molecule, in the presence of 100

μg/ml of EtdBr. Progressively less twisted structures are observed at EtdBr concentrations of 50 and 25 μg/ml.[45] Thus, it is reasonable to suggest that sharp bends in the duplex, representing sites at which base pairs become disrupted in Wang's model,[13] could serve as additional substrate sites for the *As.* nuclease in the case of highly negatively supercoiled DNA (*i.e.*, at values of $-\sigma_0$ for which superhelix density-dependent cleavage with the *N. crassa* and Mung bean nucleases is observed) and as the only substrate sites in the case of very highly positively supercoiled DNA, against which a low level of nuclease activity was evidenced in this study.

It is possible that the cleavage of negatively supercoiled DNA with the Mung bean and *N. crassa* nucleases[3,13] does require the formation of structures such as sharp bends in the DNA helix, which are present only at the values of $-\sigma_0$ above which the initial nicking rate with these enzymes depends strongly upon superhelix density. If the value of σ_0 for which such structures begin to appear is near -0.08, as suggested from Wang's data,[13] then it is not surprising that the value of σ for which the positively supercoiled structures become sufficiently tightly twisted to give rise to substrate sites for the nuclease is somewhat greater than 0.08 (this value is greater than 0.15 in these experiments). This is because even the slight unwinding of the duplex required for the formation of nuclease-sensitive sites is expected to be thermodynamically unfavored at positive superhelix densities, so that such sites would be expected to occur at a lower value of $-\sigma$ for negatively supercoiled DNA than that of σ for positively supercoiled DNA. It is suggested that the pronounced twisting of the molecules at very high positive superhelix densities produces bends in the molecule which are so sharp as to overcome the unfavorable energetics and cause sufficient unstacking of bases to provide substrate sites for the nuclease. A factor which would favor attack at such sites in negatively supercoiled DNA over those in positively supercoiled DNA would exist if the binding of the nuclease requires a slight unwinding of the DNA.

It is evident that the *As.* nuclease recognizes non-duplex structure in negatively supercoiled DNA at values of $-\sigma_0$ well below those for which the other nucleases recognize such structure under their conditions of use in the earlier work.[3,13] This is found to be the case even though the incubations in this study were carried out at 20°C compared to 37°C for the studies with the other enzymes. Tests with the Mung

bean and N. crassa nucleases for superhelix density-dependent cleavage at values of $-\sigma_0$ below those for which such cleavage was observed in previous work would require elevated enzyme concentrations and extended periods of incubation. Experiments of this kind would be severely hampered because of the significant superhelix density-independent nicking shown by these enzymes (or by contaminating activities) at the lower values of $-\sigma_0$ (ref. 13) and because of the acidic pH optimum for the Mung bean enzyme,[46] which could result in acid-catalyzed depurination or other damage to the DNA in extended incubations. The As. nuclease displays very high specificity with respect to its failure to attack form I^0 DNA or positively supercoiled DNA until very high values of σ are reached. The enzyme is not effectively inhibited by EtdBr at concentrations corresponding to near-saturation binding to duplex DNA and is active near neutral pH (ref. 12). The above clearly constitute definite advantages for the use of the As. nuclease in studies of this type.

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