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Linking Number Anomalies in DNA under Conditions Close to Condensation†

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ABSTRACT: Changes in linking number and the apparent winding angle of pBR322 DNA have been evaluated in mixed ethanol–water solvents containing either Na or Mg as the major counterion contributing to the electrostatic shielding of the duplex. The average number of superhelical turns (τ) produced in the standard electrophoresis buffer (Tris–borate–EDTA, pH 8.0) by the transfer of DNA, relaxed in 200 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4, and 2 mM EDTA, pH 7, by calf thymus topoisomerase or ligated in 6.6 mM $MgCl_2$, 1 mM KCl, 1 mM ATP, 1 mM dithiothreitol, and 66 mM Tris, pH 7.6, by T4 ligase, was determined as a function of the EtOH concentration. At low enzyme concentrations, the τ values became increasingly more positive in the presence of both cations as the ethanol concentration increased, indicating that the duplex structure was overwound in the ethanol solvents. Winding angle changes between 0 and 20% ethanol, calculated from these values of τ, exhibited the same correlations with CD spectral properties as had been previously observed for 100% aqueous systems containing monovalent cations [Kilkuskie, R., Wood, N., Shinn, R., Ringquist, S., & Hanlon, S. (1988) *Biochemistry 27*, 4377-4386]. The results at higher concentrations of ethanol (25–30%), however, were anomalous for the Mg–ligase system. The anomalies increased with higher ethanol, ligase, or Mg concentration. Gel run under these conditions showed enhanced concentrations of slow-moving components, indicative of ligation of intermolecular associated DNA species. At a 10-fold higher level of ligase, ethanol appeared to unwind the duplex, confirming the results of Lee, Mizusawa, and Kakefuda [(1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2838-2842]. All of these anomalies occur under solvent conditions which are close to conditions which produce a heterogeneous dispersion of sedimenting species in ultracentrifugal experiments and compact rodlike structures, visualized by electron microscopy. The circular dichroism spectra at the onset of the formation of these structures show the characteristics of a chirally packed array of DNA duplexes. The reversal of the trend of the ethanol effect on linking number at higher enzyme and Mg(II) concentrations can be most easily explained by the promotion of the condensation phenomenon by either the ligase or a contaminating factor in the preparation. We suggest that the anomalies in the linking number and winding angle values are due to either ligation of chirally bent DNA species or a change in the helical period as the linear DNA adapts to the conformation required for collapse. At ethanol concentrations well below that required for DNA collapse, the average change in winding angle calculated from the relative linking number appears to be valid and independent of whether the relaxed DNA species was produced by the topoisomerase or the ligase. In the absence of condensation effects, CD changes are also a reliable index of small *average* winding angle changes in random-sequence B-form DNA in ethanol. Winding angle changes cannot, however, be reliably evaluated by gel methods at or close to conditions where chiral collapse or condensation of DNA occurs.

A number of observations conducted with random-sequence DNA have confirmed the correlation between winding angle changes and the circular dichroism (CD)[1] spectral properties. As long as an average B backbone geometry is mainained, an increase in the average winding angle of DNA in aqueous solutions is accompanied by a decrease in the rotational strength of the positive band above 260 nm in the CD spectrum (Johnson et al., 1981; Baase & Johnson, 1979; Chan et al., 1979; Kilkuskie et al., 1988). The conditions which produce changes in winding angle almost certainly produce other structural changes and have extraneous ion and solvent effects, but these seem to be coordinated with the winding angle changes. Thus, winding angle changes produced by the covalent attachment of a charged amine in the groove of the duplex have the same effect on rotational strength as do changes in the electrolyte content of the aqueous solvent environment (Kilkuskie et al., 1988).

Prior to undergoing the B → A transition in mixed aqueous–alcohol solvents, the CD spectra of a variety of DNAs

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[1] Abbreviations: EDTA, sodium ethylenediaminetetraacetate; EtOH, ethanol; CD, circular dichroism; bp, base pair(s); Lk, linking number; Wr, writhe; Tw, twist; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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generally undergo a decrease in the rotational strength of this long-wavelength positive band as the organic component increases. In light of the above observations, this would normally be interpreted as a reflection of an increase in winding angle. As part of a more general examination of winding angle changes in mixed aqueous–organic solvents, Lee et al. (1981) have investigated the changes in relative linking number of DNA ligated in mixed EtOH–H_2O solvents at compositions well below that required to induce the B → A transition. The term "relative linking number", as used by those authors, denotes differences in the population average value of Lk for the DNA sample ligated in a solvent of interest and that ligated in a standard state. This difference, $Lk - Lk_0$, or ΔLk, is equal to the number of supercoils, τ, produced by the transfer from the solvent of interest to the standard state (Wang et al., 1982). In their experiments, the values of τ became increasingly less positive as the concentration of ethanol increased. If the assumption is made that there are no differential bending or writhing effects in the ligated products in the mixed solvents, then the values of ΔLk, or τ, obtained in those studies reflect directly the solvent effects on the winding angle or helical period of the DNA. On the basis of this clearly specified assumption, Lee et al. (1981) have interpreted their results in terms of a decrease in the average winding angle of pBR322 DNA, relative to its value in aqueous solution, in all the organic solvents examined, including the EtOH–H_2O one, in the range of 0 to ca. 30% (v/v) of the organic component.

It would thus appear that the general correlation between the CD properties and the winding angle of B-form DNA has been violated in these cases when CD properties have been determined. There is, however, an important difference between the experiments of Lee et al. (1981) and the experiments conducted by others in aqueous and in mixed alcohol–aqueous solvents. Winding angle and CD spectral changes have generally been measured in solvents containing monovalent cations whereas the results of Lee et al. (1981) were obtained in the presence of 6.6 mM Mg(II), since this cation was required for ligase activity. Because small changes in winding angle can have a significant effect on interactions and structural changes which are dependent on the helical phase (Hochschild & Ptashne, 1986; Kitchin et al., 1986), we felt that it was important to investigate the role of the cation in changing the winding angle. We also sought to reconcile the apparent contradictions in the CD behavior in these mixed solvent systems, as the latter is a convenient method for assessing winding angle changes in solution (Kilkuskie et al., 1988). Finally, since DNA is known to condense in EtOH solvents in the presence of Mg(II) without undergoing a transformation to the A form (Gray et al., 1979), we sought to investigate the possibility that the condensation—or, possibly, some precondensation effect—was biasing the winding angle measurements.

Experimental Procedures

Isolation of Plasmid pBR322 DNA. Escherichia coli strain HB101 transformed with plasmid pBR322 was grown in LB medium containing 15 μg/mL tetracycline in 10-L fermentor batches. Plasmid DNA was amplified by the addition of 170 μg/mL chloramphenicol (Maniatis et al., 1982). The DNA was isolated by the procedures of Zasloff et al. (1978) and Maniatis et al. (1982). The preparation received extensive phenol and pancreatic RNase treatment and repeated EtOH precipitation in order to remove protein and RNA contaminants. Plasmid DNA was considered pure when its CD spectrum no longer exhibited an RNA component. (This turned out to be a more sensitive criterion than the absence of either gel electrophoresis evidence or slowly sedimenting UV-absorbing material in analytical ultracentrifugation experiments.) The yield of the highly purified pBR322 DNA was about 1.5 mg/L of fermentor culture. Supercoiled enriched pBR322 DNA was prepared from the purified stock solution by the acid–phenol extraction procedure of Zasloff et al. (1978). The preparation was judged to be free of small covalent insertions of RNA (Blair et al., 1972) by the failure to exhibit significant increases in nicked form II after treatment with RNase H or alkaline pHs. Linear pBR322 DNA was prepared from the purified enriched sample by digestion with *Eco*RI (Bethesda Research Laboratories) followed by phenol extraction, EtOH precipitation, and exhaustive dialysis against 20 mM NaCl–10 mM Tris, pH 8. Homogeneity of the linear preparation was checked by sedimentation velocity measurements as well as by electrophoresis, using *Eco*RI-cut λ DNA as size markers. In the latter experiments, 90–95% of the DNA moved with the mobility appropriate for a 4400 bp linear species. In the sedimentation velocity experiments, 95% of the DNA sedimented with an $s^0_{20,w}$ of 13.6 ± 0.1 S, equivalent to a molecular weight of $2.6E + 6$ (Eigner & Doty, 1965). The predicted molecular weight of pBR322, as the free acid, is $2.7E + 6$.

Sample Preparations. Samples of either linear or supercoiled enriched plasmid DNA in mixed EtOH–H_2O solvents were prepared by the dilution of the appropriate stock solution of pBR322 DNA into the ligation or relaxation solvents at 15 °C containing 0–30% (v/v) EtOH (Aaper Alcohol and Chemical Co.). Reaction was initiated by the addition of the appropriate enzyme at the completion of the set of dilutions for a given electrophoresis experiment (ca. 5 min). DNA concentrations in diluted samples containing EtOH were calculated from the known DNA concentration of the stock solutions and the dilution factor. EtOH concentrations are given in percent (v/v).

Both the relaxation and the ligation reactions were conducted with the above solutions in a thermostated bath at 15 °C for a period of 24 h. At the end of this period, the reactions were stopped by precipitating the products by the addition of NaCl and EtOH. They were then redissolved in loading buffer (10% glycerol, 0.1% SDS, and 0.01% bromophenol blue) for analysis by gel electrophoresis. The highly purified plasmid stock was relaxed in 200 mM NaCl, 2 mM EDTA, and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7, containing various concentrations of EtOH, using calf thymus topoisomerase at a concentration of 1–2 units/mL of reaction solvent. The topoisomerase preparation was isolated by the procedures of Pulleyblank and Morgan (1975) and Liu and Miller (1981), with modifications and assay conditions described by Kilkuskie et al. (1988). Ligated pBR322 DNA was prepared from the linear pBR322 DNA stock using T4 DNA ligase. (Commercial preparations obtained from Bethesda Research Laboratories, lots 43101, 51121, 52111, and 52112, were employed.) These preparations were found to be free of any topoisomerase I activity as demonstrated by the failure to relax intact form I pBR322 DNA over a 24-h period. The standard ligation buffer was 6.6 mM $MgCl_2$, 1 mM KCl, 1 mM ATP, 1 mM DTT, and 66 mM Tris, pH 7.6, plus various concentrations of EtOH. The bulk of the studies was done with an enzyme concentration of 1 unit of ligase/μg of DNA or 5 units/mL. However, additional studies were conducted in which the Mg(II) and enzyme concentrations were varied.

Analysis of Topoisomer Distribution. Topoisomers resulting from the ligation of linear DNA or the relaxation of form I DNA were resolved by electrophoresis on a 0.7% agarose gel

at room temperature (27 ± 1 °C) and 5 V/cm using a TBE buffer (90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA, pH 8) employed in the experiments of Lee et al. (1981). After being stained with ethidium bromide, the gels were photographed. The negatives were scanned with a Biomed Instruments densitometer (Model SL-2D) interfaced to an Apple IIE. The center of the Gaussian distribution of the topoisomers was evaluated from area measurements of the topoisomer bands, using either the Apple IIE computer data or digitized area data from an analysis of tracings of the densitometer patterns on a Talos Pad interfaced with a VAX 11/780. The change in winding angle ($\Delta\Psi$) in degrees per bp was evaluated from the measured average number of superhelical turns (τ) arising from the transfer to the electrophoresis buffer, using the Vinograd equation (Vinograd et al., 1965) with a sign change to reflect that the winding angle change is for the reverse process, i.e., the transfer *to* the reaction solvents. For our experiments, the equation has been recast as

$$\Delta\Psi = +(360\tau/N) \quad (1)$$

where $N = 4363$, the number of base pairs in pBR322 DNA. Correction of $\Delta\Psi$ to isothermal conditions employed the temperature coefficient, −0.012 deg/°C, of Depew and Wang (1975). After correction for the temperatures at which the gels were run, reproducibility between different gels was ±0.1 superhelical turn or ±0.01° in winding angle.

The sign of the supercoils produced by the transfer of the topoisomerase or ligation products from the reaction solvents to the electrophoresis buffer was determined by running the products in selected solvents in a second dimension in the presence of 0.6 μg/mL chloroquine, with ambient temperature carefully monitored. This also permitted a more accurate analysis of the number of superhelical turns (3.5) in the topoisomerase products in 0% EtOH in 1D experiments in which chloroquine (0.2 μg/mL) had been initially incorporated in the gels in order to displace the topoisomers from the linear and nicked form II. The numbers of superhelical turns for the topoisomerase products at higher concentrations of EtOH in these latter experiments were obtained by adding this value (+3.5) to the difference between the turns of the DNA relaxed in the solvent of interest and those of the DNA relaxed in 0% EtOH in the same experiment. This intercalator was not used in the ligase series, as the effect of the ligation buffer containing Mg(II) on the linking number of DNA was sufficient to displace the Gaussian distribution of topoisomers, even at 0% EtOH. The superhelical turns produced by transfer from this latter solvent were +4.9.

Spectroscopy. CD and absorbance spectra were obtained with a Cary Model 14 (Varian Instruments) as described previously (Chen et al., 1981) using a path length of 1–5 cm over the wavelength range of 210–400 nm. Unless otherwise specified, spectra were obtained at 27 °C. An extinction coefficient of 6600 M^{-1} cm^{-1} at 259 nm was used for calculating DNA concentration from the absorbance data in aqueous solvents. When appropriate, light-scattering corrections were made by using the method of Oster (1948). DNA concentration for all solution experiments was ca. 15 μM (in nucleotide residues).

Circular dichroism spectra were obtained with either a J-40A (Jasco Instruments) or a Cary Model 60 with CD attachment (Varian Instruments). Equivalence of instrumental response was ascertained as previously described by Chen et al. (1981). Except for the variable-temperature experiments, data were obtained at 27 °C. A path length of 5 cm was routinely employed, which permitted acquiring spectral data at the same concentrations used in the ligation and relaxation reactions. The spectra of linear DNA for the match of the winding angle data in the topoisomerase series were obtained in the topoisomerase reaction solvents. The spectra for the ligase series were obtained in solvent which lacked ATP and DTT but contained all other components of the reaction buffer. The data are reported as mean residue ellipticities in units of degrees centimeter squared per decimole of nucleotide.

Electron Microscopy. Electron micrographs were taken with a JEM 100CX (Jeolco Instruments) as described by Chen et al. (1981). DNA samples were diluted to 15 μM in the appropriate solvent and stored either at room temperature or in an ice bath until adsorption at temperatures between 23 to 27 °C onto carbon-coated glowed grids. The NaCl concentration of the Na–EtOH solutions was reduced to 20 mM in order to avoid excessive salt deposition on the grids. Polystyrene latex beads (96 nm) sprayed on the back of the grids after sample deposition served as a size marker. A few beads were sprayed on the sample surface in order to permit corrections for metal deposit. The DNA samples were lightly stained with 0.02% uranyl acetate and Pt-shadowed. Most of the measurements were made on rotatory-shadowed specimens, but a few were made on linearly shadowed samples for height estimates. Pictures were taken at a magnification of 33000×. Dimensions were read directly from the electron micrographs, using a toolmakers microscope (Nikon Shadowgraph).

Analytical Ultracentrifugation. Sedimentation coefficients were measured with a Beckman Model E analytical ultracentrifuge as described by Wolf and Hanlon (1975). The results are reported as the median sedimentation coefficients, with the standard corrections, but are uncorrected for preferential hydration of DNA in EtOH-containing solvents. Solutions in 30% EtOH were prepared in ice-cold solvents and maintained in an ice bath until loaded into the cell.

Results and Discussion

Relative Linking Number Determinations. For this study, we needed enzyme systems which, in principle, would produce covalently closed relaxed DNA molecules in mixed EtOH–H_2O solvents in the presence and absence of Mg(II). The experimental system of Lee et al. (1981) was used for the examination of the effects of Mg(II). This entailed linearizing the plasmid, pBR322 DNA, with *Eco*RI and then subsequently ligating the sample with T4 ligase in a set of solvents of increasing EtOH content (0–30% EtOH) containing a fixed concentration of Mg(II). For the creation of relaxed species in the absence of Mg(II), we used calf thymus topoisomerase I. This enzyme does not require Mg(II), exhibits maximal activity at moderate Na concentrations (200 mM), and is active with form I DNA at EtOH concentrations as high as 20%.

In this first series of experiments, we attempted to minimize any perturbations due to protein by keeping the level of added enzyme low and roughly comparable in the two types of experiments. After either ligation with T4 ligase (1–5 units/μg of DNA) or relaxation with the calf thymus topoisomerase (1–2 units/μg of DNA) in the mixed solvents, the samples were then transferred to the standard electrophoresis buffer and run out on agarose gels. The results of the electrophoresis experiments in the two solvent systems are shown in Figure 1A,B. In contrast to the results obtained by Lee et al. (1981), there is no evidence of a decrease in positive supercoiling as the concentration of EtOH increased in the ligation or the relaxation mixtures. The direction of the movement of the topoisomers suggested that the positive supercoils formed by the transfer of the ligated or relaxed products at 15 °C in the

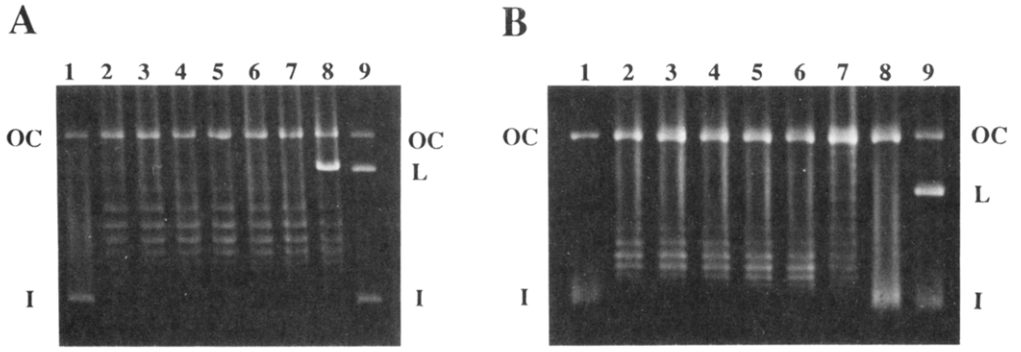

FIGURE 1: Gel electrophoresis patterns of pBR322 DNA in the standard electrophoresis buffer at 27 °C, transferred after ligation (A) or relaxation (B) in mixed EtOH–H_2O solvents at low enzyme concentrations. The lanes are numbered sequentially, from left to right, as 1–9. Lanes 1 and 9 in both figures represent the plasmid preparation. Lane 9 contains, additionally, the linear pBR322 DNA preparation added to the plasmid preparation prior to running the gel. Positions of the nicked open circular form II (OC), linear (L), and intact native form I (I) are marked accordingly. In panels A and B, lanes 2–8 contain the products resulting from the action of T4 ligase at 1 unit/μg of DNA (gel A) or calf thymus topoisomerase at 2 units/μg of DNA (gel B) in reaction solvents at 15 °C containing 0, 5, 10, 15, 20, 25, and 30% (v/v) EtOH, respectively. Gel B was made up in chloroquine (0.2 μg/mL) in order to displace the topoisomers from the nicked form II and linear species during electrophoresis. The ladder of topoisomers in lane 7 (25% EtOH) in gel B arises because of the effects of chloroquine on the incompletely relaxed pBR322 preparation. Enzyme activity ceased altogether at 30% EtOH (lane 8).

Mg(II) or Na reaction solvents to the electrophoresis buffer increased in positive magnitude as the concentration of EtOH increased. This was confirmed by running the products from selected solvents in a second dimension in chloroquine.

The supercoils (τ) produced in the above experiments are formed because of effects of both temperature and solvent on the DNA. Their magnitudes reflect differences between the linking number of the DNA ligated at 15 °C in a given EtOH solvent and that which would have resulted if the ligation had occurred in solvent with the ionic composition of the electrophoresis buffer at 27 °C. For convenience, we have chosen the latter for our standard state. These differences in Lk for this first set of experiments are plotted in Figure 2 in terms of the average values of τ(left-hand ordinate) as a function of the EtOH content for the Mg(II) (low ligase) and the Na solvents. [Because of a different choice of standard state, the left-hand ordinate in this figure and that shown in Lee et al. (1981) in Figure 3 of these authors differ by +3.] Invoking the same assumptions of Lee et al. (1981), we have used these data to calculate winding angle changes. Because we wish to compare spectral data gathered at 27 °C, we have additionally employed a thermal correction, using the coefficient of DePew and Wang (1975). These winding angle changes have been corrected to reflect the isothermal transfer *from* the electrophoresis buffer *to* the reaction solvent. These values of $\Delta\Psi$, indicated as $\Delta\Psi^*$ (relative to the electrophoresis buffer at 27 °C), corresponding to each of the τ values are given on the right-hand ordinate. The displacement of the 0 on this latter axis is due to the temperature correction of 0.144 deg/bp, which equals $-0.012°\ °C^{-1}\ (bp)^{-1}$ (−12 °C). Both the Mg(II) and the Na solvents in these experiments at low enzyme concentrations produce positive changes in winding angle, compared to the electrophoresis solvent. The overwinding increases in magnitude with increasing EtOH content. In contrast, to the results of Lee et al. (1981), the values of τ (or the equivalent ΔLk) never become negative in this set of experiments. For the same EtOH concentration, however, the slope is smaller for the Mg(II) solvents. In the 30% EtOH solvent in the latter experiments, we also observed the appearance of slower moving bands (discussed below) which travel with mobilities appropriate for linear dimers and higher multimers of pBR322.

The failure to reproduce the effects observed by Lee et al. (1981) in the ligase system was troublesome. In order to resolve this dilemma, we systematically examined our procedural differences. After we changed all of the possible variables (purity of preparation, ATP concentration, reaction time, enzyme content), only the change in the enzyme concentration in the ligation cocktail had a significant effect. This is demonstrated in Figure 3A, which shows the effects of variation over a 100-fold range in enzyme concentration on the supercoil population produced after ligation and transfer from the 30% EtOH solvent. As the level of enzyme concentration increases, the Gaussian center of the topoisomer bands shifts, and the magnitude of positive supercoiling decreases. This retardation is not attributable to the binding of protein, as the latter would be expected to result in a smear of topoisomer density and a retardation of the nicked relaxed and the linear forms. These effects are not observed either visually or in the densitometer tracings of this gel.

There are differences in this gel in the low mobility region which were not observed at lower ethanol concentrations and lower ligase concentrations. There is a gradual disappearance of DNA from the region of the gel where the supercoils are traveling as well as in the band marked D. This latter is

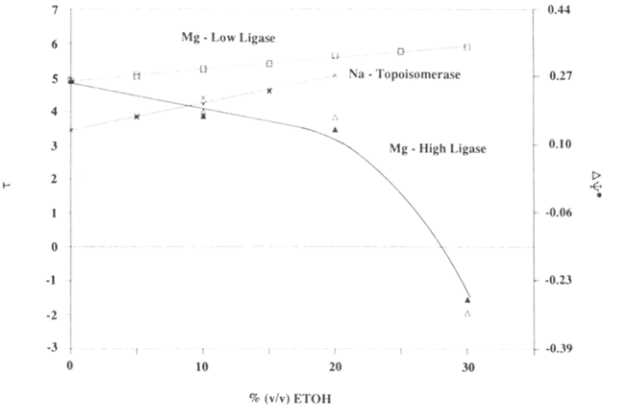

FIGURE 2: Changes in average number of superhelical turns (τ) and winding angle changes ($\Delta\Psi^*$) with increasing concentration of EtOH in ligation and relaxation solvents. Values of τ (turns per molecule), evaluated from the gel patterns shown in Figure 1 and in Figure 3B, are plotted on the left ordinate while the matching values of $\Delta\Psi^*$ (degrees per bp), reflecting an isothermal transfer from electrophoresis buffer to the ligation or relaxation solvent at 27 °C, are given on the right ordinate. Preparations are coded as follows: (×) topoisomerase relaxed; (□) ligated with T4 ligase at 1 unit/μg of DNA; (△) ligated with T4 ligase at 10 units/μg of DNA added after 5 min of equilibration at 15 °C; (▲) ligated with T4 ligase at 10 units/μg of DNA added after 24 h at 15 °C.

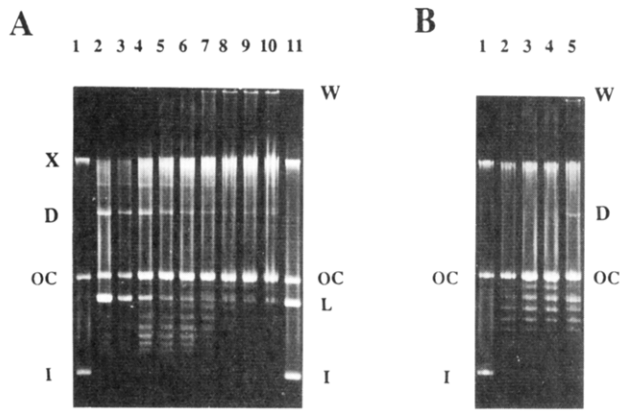

FIGURE 3: Gel electrophoresis patterns of pBR322 DNA in the standard electrophoresis buffer at 27 °C, produced by transfer from ligation buffers with variable concentrations of T4 ligase. (A) Ligation of pBR322 in 30% EtOH with 0.25, 0.5, 1, 2.5, 5, 10, 15, 20, and 25 units/μg of DNA in lanes 2–10, respectively. Lanes 1 and 11 are the plasmid preparation (no ligase added) with extra linear preparation added to lane 11. (B) Pattern obtained in variable EtOH concentrations using 10 units of ligase/μg of DNA. Lane 1 is the pattern produced by the plasmid preparation and contains no added ligase. Lanes 2–5 represent those of the products obtained by a ligation of linear DNA in 0, 10, 20, and 30% EtOH, respectively, with a prior equilibration of only 5 min at 15 °C before ligase addition. In both panels, positions of nicked form II (OC), linear monomer (L), and native form I (I) are indicated in the lower portions of the gels. Positions of the wells (W), an unknown component (X), and the presumptive linear pBR322 dimer (D) are indicated in the upper portion of the gels.

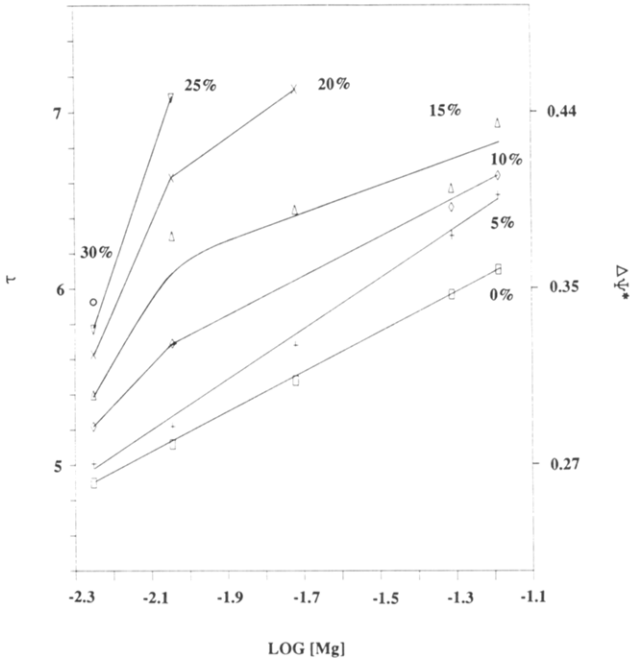

FIGURE 4: Effects of Mg(II) concentration in the ligation buffers of variable EtOH content on τ and $\Delta\Psi^*$. τ (turns per molecule) and $\Delta\Psi^*$ (degrees per bp) are defined as in Figure 2. The points represent Mg(II) concentrations of 0.0066, 0.010, 0.020, 0.050, and 0.066 M. The various EtOH concentrations are coded as follows: (□) 0%; (+) 5%; (◇) 10%; (△) 15%; (×) 20%; (▽) 25%; (○) 30%. Missing points reflect the fact that the ligase reaction did not occur at those Mg(II) concentrations in the indicated solvent.

moving with the mobility expected for an 8700 bp linear fragment and is almost certainly the linear dimer. The fainter band above is probably the linear trimer. These two bands are always observed in 25–30% EtOH, even at the low ligase levels. This decrease in intensity of the supercoils and the dimer band is paralleled by an increase in the density of the lower mobility band marked X in the upper portion of the gel and an increasing although small amount of DNA left behind in the wells (W). The identity of the X species is not known. It is usually present in the pBR322 preparations but can be removed by repeated acid–phenol extraction, which suggests that it is nicked concatemers or nicked form II multimers. It is absent in the linear preparations prepared from the acid–phenol-extracted pBR322 preparation. At low ligase concentrations, it is generally not observed in samples ligated at EtOH concentrations below 25%. Its identity in the ligated sample, therefore, is probably attributable to ligated multimeric linear species of PBR322 DNA. The position at which it travels in the gel is equivalent to 20 000 bp and over, and species higher than the linear trimer would be expected to travel in that region. The linear multimers, if present, would not show as discrete bands since the gel is nondiscriminatory at these molecular weights. On the basis of this interpretation, the results would suggest that there is an increasing extent of intermolecular association as the ligase concentration is increased.

In Figure 3B, we have displayed the results of an experiment conducted over the entire range of ETOH concentrations at a ligase concentration equivalent to that employed in the experiments of Lee et al. (1981) (10 units/μg of DNA or 50 units/mL reaction volume). The upper portion of the gels also shows the slow-moving bands which become increasingly more pronounced as the ethanol concentration in the ligation mixture increases. The values of τ and $\Delta\Psi^*$, evaluated from the data in the lower portion of the gel, are plotted as the open triangles in Figure 2. The closed triangles represent the results of a duplicate experiment in which the linear DNA preparation was allowed to incubate in the reaction solvent for a 24-h period prior to enzyme addition. The latter was the time normally allotted for the reaction at the lower ligase concentration. Although we allowed a similar reaction period for the ligation at the higher enzyme concentration, the actual reaction is over very rapidly under these conditions. The similarity in the two sets of data eliminates the possibility that the difference in the results obtained at high and low concentration of ligase was caused by a slow transformation of a metastable secondary or tertiary structure of the linear DNA during the 24-h reaction period in the reaction solvent. The data from both sets of experiments at the higher ligase concentration are much closer to those which Lee et al. (1981) obtained.

Another type of anomaly in relative linking number behavior was observed in the ethanol solvents containing variable concentrations of Mg(II) at lower ligase levels used in the experiments shown in Figure 1. Anderson and Bauer (1978) have found that winding angle changes, relative to a standard electrophoresis buffer at 20 °C, are a linear function of the log of the monovalent cation concentration. We have examined the effects of variations in Mg(II) concentration on the supercoil population ligated in the standard series of EtOH concentrations. When τ and $\Delta\Psi^*$ evaluated from the gels are plotted against the log of the Mg(II) concentration, the results shown in Figure 4 were obtained. Linear behavior was observed in the 100% aqueous solvent as well as the low concentration of EtOH (5%) over the range of Mg(II) concentrations (6.6–66 mM) covered. As the concentration of EtOH increased, however, the behavior became increasingly nonlinear. At the higher Mg(II) concentrations, the superhelical turns or winding angle changes were smaller than would have been predicted from the slope characteristics at the lower Mg(II) concentrations. Furthermore, the ligase ceased to function at some of the higher Mg(II) concentrations, and the only species seen on the gel in those concentrations was the

linear form. (Failure to observe activity is indicated by absence of points for that EtOH concentration.) In fact, there was no activity in 30% EtOH above 6.6 mM Mg(II). Since the enzyme was active at the higher Mg(II) concentrations in the lower EtOH concentrations, the inhibition was not due to Mg(II) itself. For those conditions where activity was observed, the nonlinear behavior of winding angle change was always accompanied by increases in the density in the lower mobility bands at the top of the gel.

Circular Dichroism Spectroscopy. The CD spectra of the highly purified linear preparation of DNA were obtained in the same solvents used for the ligation and the relaxation, with the exception that ATP and DTT were not included in the ligation solvents. Ligation in the presence and absence of added ATP (1 mM) in aqueous solutions containing 6.6 mM Mg(II) revealed a difference in τ of 0.085 which translates to an increase of 0.007° in the 6.6 mM solvent lacking ATP.[2] This is due to the stoichiometric binding of Mg(II) by the 1 mM ATP present in the ligation mixture, thus reducing the free Mg(II) concentration to 5.6 mM. This winding angle difference could be confirmed by using the regression characteristics of the data shown in Figure 4 for 0% EtOH. Although this value is within our random experimental error of 0.01°, it is a systematic error. The winding angle changes in all of the Mg(II) solutions have correspondingly been compensated for this 1 mM decrease in effective concentration of Mg(II).

The CD spectra of linear pBR322 DNA at either 15 or 27 °C in the ethanol solvents were conservative B-type spectra, with the exception of those obtained in 28–30% EtOH at 27 °C in the ligase buffer (see discussion below). The long-wavelength positive band is more or less symmetrical about 275 nm, and hence the value of $[\theta]_{275}$ can be used as a measure of the rotational strength of this band. A plot of these values against $\Delta\Psi^*$ evaluated from the first set of experiments at low enzyme concentrations is shown in Figure 5A as points. The curved solid line is the result of a nonlinear regression analysis, taken from Kilkuskie et al. (1988), for the monovalent cation effects in 100% aqueous solutions over the entire winding angle range examined in that study (0–0.8°). The solid straight line is a linear approximation of the low ligase (omitting the 25–30% EtOH points) and topoisomerase data over the winding angle range of 0–0.4°. With the exception of the Mg–EtOH data at the higher EtOH concentrations (25–30%), the data points differ insignificantly from the regression characteristics established from the previous studies of the effects of monovalent cations in aqueous systems. When all but the data points in the Mg–25–30% EtOH solvents are used, the value of the slope, $d[\theta]_{275}/d[EtOH]$, of the linear approximation in the $\Delta\Psi$ range of 0.0–0.4° is -4.7×10^3 deg cm^2 dmol^{-1} deg^{-1} (bp)$^{-1}$. This value is identical with that found previously for both PM-2 DNA (Kilkuskie et al., 1988) and calf thymus DNA (Chan et al., 1979) in the same winding angle range. These results demonstrate that the transformations of pBR322 DNA by the divalent Mg(II) in aqueous solvents and at low concentrations of EtOH and ligase, as well as that due to the interaction of Na in the mixed EtOH–H_2O solvent, are very similar and also parallel the transformation of other species of DNA effected by monovalent cations in 100% aqueous solvents. In contrast, Figure 5B shows that incorporation of the data from the experiments conducted with

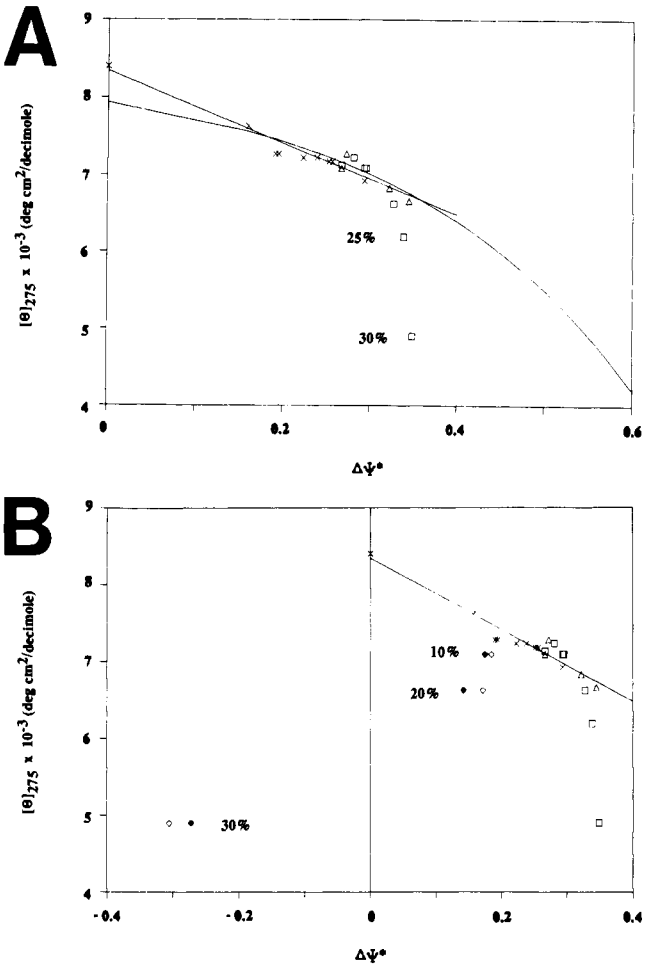

FIGURE 5: Correlation of the changes in rotational strength of the long-wavelength CD band with winding angle changes produced by mixed EtOH–H_2O solvents. The value of the mean residue ellipticity at 275 nm ($[\theta]_{275}$) on the ordinate is plotted against the winding angle change per base pair for the isothermal transfer ($\Delta\Psi^*$) of the individual sample. (A) Winding angles measured at low enzyme concentrations. Points are coded as follows: (×) topoisomerase series at variable concentrations of EtOH from 0 to 20%; (△) ligation products (1 unit of ligase/μg of DNA) at 0% EtOH, ligated in variable concentrations of Mg(II) from 6.6 to 66 mM; (□) ligation products (1 unit of ligase/μg of DNA) obtained from the standard ligation buffer [6.6 mM Mg(II) with variable concentrations of EtOH from 0 to 30%]. The straight line represents the linear regression of data over a $\Delta\Psi^*$ range of 0–0.4°. The nonlinear curve represents the regression analysis of data for the effects of monovalent cations, taken from previous studies of Kilkuskie et al. (1988). The two ligation products which deviate markedly from this latter curve represent the data from 25% and 30% EtOH. (B) All winding angle determinations. Samples are coded as in (A), with addition of the winding angle data obtained at high ligase concentrations, with ligase added within 5 min (◇) and 24 h (◆) after incubation in the ligase solvent at 15 °C. The solid straight line represents the linear regression described in panel A. Numbers close to the points in both panel represent the ethanol concentrations (percent v/v).

the higher ligase concentration in such a plot yields a pattern which is clearly deviant.

Even at low ligase concentrations, however, the 25% and 30% EtOH points are markedly divergent. Using the nonlinear regression analysis taken from Kilkuskie et al. (1988), we can estimate the values of $\Delta\Psi^*$ appropriate for the mean residue ellipticity values of these two solutions as 0.43° and 0.55°, respectively. This means that the product ligated in 30% EtOH should have exhibited an average τ of ca. +8 rather than the value of +6 (displayed in Figure 2) upon transfer to the electrophoresis buffer. A similar calculation for the data from the experiments conducted with the higher ligase con-

[2] Although the enzyme requires ATP for activity (Weiss et al., 1968), enough ATP seems to be carried over as a contaminant in these commercial preparations so that some circular ligated products are formed if ATP is not added.

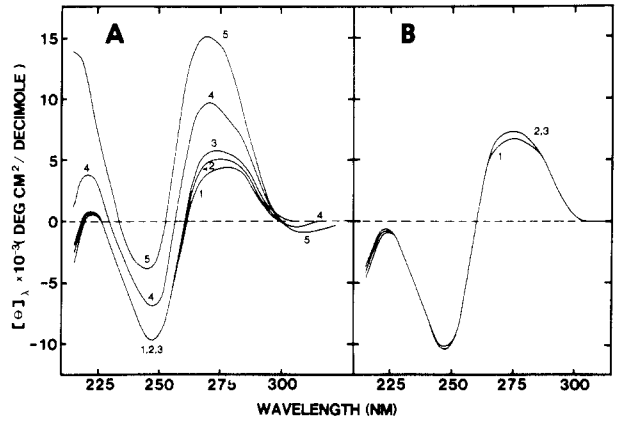

FIGURE 6: Effects of temperature on the CD spectra of linear pBR322 DNA in ligation (A) and relaxation (B) solvents containing 28% EtOH. (A) Spectra 1–5 were taken in ligation buffer at 15, 20, 27, 30, and 40 °C, respectively. (B) Spectra 1–3 were obtained in relaxation buffer at 15, 27, and 40 °C, respectively.

centration (10 units/μg of DNA) in 30% EtOH indicates that the population average of that sample is deficient by +10 superhelical turns.

The mean residue ellipticity at 275 in the Mg–30% EtOH solvent had to be obtained at 15 °C and corrected, as described below, to 27 °C because of a condensation phenomenon which occurred at the higher temperature. This effect in Mg–EtOH has been reported by Gray et al. (1979), using other techniques, for both linear and supercoiled species of DNA, and we have also observed it in calf thymus DNA (Ringquist et al., 1985). We have found that this phenomenon is highly cooperative and temperature-driven. The process for linear pBR322 DNA occurs at 27 °C in 28% EtOH in the ligation solvent (without ATP and DTT included). The effects on the CD spectrum are illustrated in Figure 6A. Maintenance of the solution at 15 °C will show no time-dependent changes in the CD properties or significant amounts of light scattering above 350 nm in the absorption spectra. When the temperature is raised, the CD spectra begin to transform in the manner shown. Spectra 3–5 are time dependent, and maintenance of the solution at 27 °C will result in a slow change in the CD properties, yielding a family of spectra resembling spectrum 5. Further increases in EtOH content or time at 27 °C will lead to a spectrum of shape similar to spectrum 5 but exhibiting a 5-fold increase in the rotational strength of the positive band and light-scattering artifacts in both the CD and the isotropic absorbance spectra.

All the CD spectra described above have some of the characteristics of an A-form spectrum, insofar as the positive band has increased in intensity and shifted to 260 nm. They lack the telltale negative signals below 230 nm, however, characteristic of the CD spectra of the A form. They arise because of the interaction of circularly polarized light with chirally packed duplex structures with the properties described by Maestre and Reich (1980). Further evidence against the presence of an A secondary structure comes from X-ray diffraction experiments of Gray et al. (1979). These workers found that condensates of calf thymus DNA formed between 30 and 60% EtOH in the presence of Mg(II) clearly exhibit B-type geometry.

As Figure 6B demonstrates, the DNA in the solvent containing 28% EtOH and 200 mM Na exhibited no equivalent temperature effects on the spectral properties over this same thermal range. Temperature-driven transformations of the CD spectra in the Na solvent as well as in the lower EtOH concentrations in the Mg(II) system were mainly evident as

Table I: Sedimentation Properties of Linear pBR322 DNA in Na and Mg Solvents

solvents			% DNA remaining	
cation content + buffer[a]	% EtOH	temp (°C)	at speed,[b] ±5%	$s^0_{20,w}$[c] (±0.2 S)
100 mM NaCl	0	23.4	100	13.6
20 mM NaCl	30	22.9	100	17.4
6.6 mM $MgCl_2$	0	22.9	104	13.8
6.6 mM $MgCl_2$	30	23.6	91	17.2 (80%), 24 (20%)
6.6 mM $MgCl_2$	30	24.5	57	18.1 (80%), 135 ± 3 (20%)
6.6 mM $MgCl_2$	31	24.5	37	16.4 ± 1.0 + polydisperse leading component

[a] Buffer for the 20 and 6.6 mM $MgCl_2$ solvent was 66 mM Tris, pH 7.6. That for the 100 mM NaCl solvent was 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0. [b] 50 740 rpm. [c] Median sedimentation coefficients of DNA species left at speed. Uncorrected for preferential hydration.

a slight increase in the rotational strength of the positive band above 260 nm without associated changes in the other bands at 245 and 220 nm. As has been pointed out in the introduction, these changes are usually diagnostic of a decrease in winding angle. If the slope of the linear approximation in Figure 5 was used to assay the small change in winding angle for these small changes in $[\theta]_{275}$ in this and other duplicate experiments, the magnitude of the average changes observed in the Na–EtOH solvents was consistent with a temperature coefficient of −0.01 deg/°C. This agrees very well with the value of DePew and Wang (1975) and indicates that the temperature coefficient of winding angle changes in the mixed EtOH solvent is not anomalous under noncondensing conditions.

We correspondingly used this coefficient together with the slope of the linear approximation [−4.7 deg cm^2 dmol^{-1} deg^{-1} (bp)$^{-1}$] based on the data in Figure 5 to correct the value of $[\theta]_{275}$ of DNA in the Mg–30% EtOH solvent from the measured value at 15 °C to that appropriate for 27 °C. The corrected value, included in Figure 5, was only slightly lower than the measured value obtained in 28% EtOH at zero time, after rapidly changing the temperature from 15 to 27 °C.

Molecular Size Dispersion. In an attempt to obtain some structural and packing information on the condensates, we first undertook a set of sedimentation studies. Because of the limitations of the instrumentation, these experiments had to be conducted with solutions of DNA at concentrations 5–7-fold higher than used in the enzyme and spectral studies. The data shown in Table I revealed that species with sedimentation coefficients substantially higher than monomeric linear pBR322 DNA were formed in 30–31% EtOH just at the condensing temperature, which is 24.5 °C in the Mg–30% EtOH solvent. These effects were not observed in a Na solvent at comparable ionic strength and were only marginally obvious in the Mg–30% EtOH solvent at a temperature just below the condensing temperature. The ratio (1.4) of the $s^0_{20,w}$ for the major and minor components in the latter solution is approximately that which can be calculated from the Eigner and Doty (1965) relationship for the ratio of two linear double-stranded DNAs which differ in molecular weight by a factor of 2. The boundary shape of this minor component, however, is markedly polydisperse. Although it probably contains the linear dimer, there are clearly other sedimenting species, as well.

The increases in $s^0_{20,w}$ for the monomer species (13.6 to 17.2 S) in going from 0 to 30% EtOH in both the Na and the Mg solvents below the condensation temperature are excessively

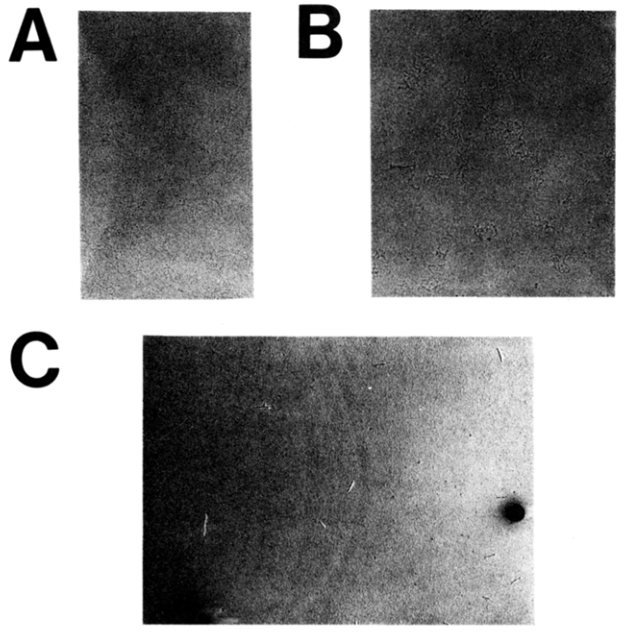

FIGURE 7: Electron micrographs of linear pBR322 deposited from 30% (v/v) EtOH solvents containing Na or Mg. (A) 20 mM NaCl/66 mM Tris, pH 7.6, at 25 °C. (B) 6.6 mM $MgCl_2$/66 mM Tris, pH 7.6, maintained at 0 °C until adsorption to grid. (C) 6.6 mM $MgCl_2$/66 mM Tris, pH 7.6, warmed to 25 °C prior to adsorption to grid. Samples in (A) and (B) were rotary shadowed, while that in (C) was linearly shadowed. The polystyrene bead size marker is 96 nm in diameter.

high for preferential solvation effects. These changes were also observed in calf thymus DNA in similar solvent sets, with no abrupt transitions noted in the 0–30% EtOH range. Since the latter sample does not have "sticky ends", as does the linear pBR322 DNA, the increase in $s^0_{20,w}$ for the monomer species in either the Na or the Mg solvent is unlikely to be due to end to end aggregation. The changes are more consistent with a contraction of the DNA in the EtOH solvents. Although there are other species present in the Mg–30% EtOH solvent, the similarity of values for the *monomer* suggests that the contracture of this species is similar in the presence of the two cations.

As the temperature increased to and above the condensation point, there was excessive loss of DNA from the solution below 15 000 rpm, making it difficult to analyze the minor rapidly sedimenting components left at speed. In general, the boundary formed by the species left at speed appeared to consist of at least two slower moving components together with minor fast-moving polydisperse components. Average values of the sedimentation coefficients for slow and fast components have been entered in Table I, and the fractional amounts of sedimenting species are only approximate.

In order to examine the size dispersion of DNA at concentrations equivalent to that employed in the topoisomerase and ligase studies, we turned to an electron microscopic examination of the linearized samples in various EtOH solvents. These included Mg–EtOH solvents below and above the condensation concentration of EtOH and the Na–EtOH solvents at comparable ionic strengths. Most of these efforts yielded only qualitative results since temperature and other variables were difficult to control. In addition, the concentration of 15 μM was too high for some solvents and less than adequate for others. Nevertheless, the observations reported below were consistent with the other experimental data and supported the formation of end to end linear aggregates under condensing conditions in EtOH.

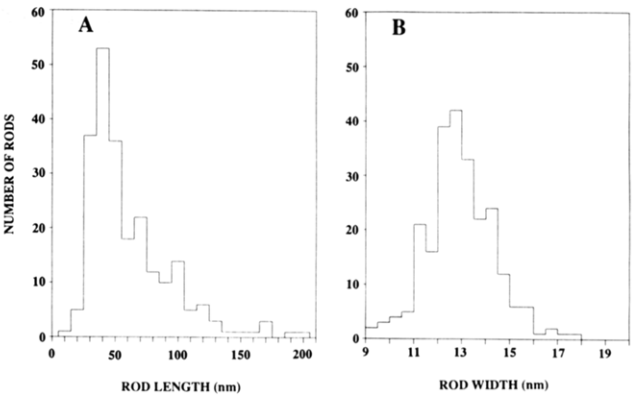

FIGURE 8: Characteristic dimensions of the rod structures seen in electron micrographs of linear pBR322 DNA at room temperature (25–27 °C) in ligation solvent containing 30% EtOH. (A) Length histogram. Average length for rods between 0 and 65 nm is 42 ± 11 nm. (B) Width histogram. Average taken over entire distribution is 13 ● 1 nm. 240 structures were counted in both panels.

Up to 30% EtOH, the linear Na samples exhibited more or less a dispersed appearance, although the samples appeared to be more contracted than at 0% EtOH. Above that concentration, significant contraction of the linear species became apparent with suggestions of toroidal supercoiled forms appearing at 60% and a few rods and toruses at 80%. Figure 7A shows the appearance of a sample deposited from 20 mM NaCl–30% EtOH. Although the DNA appears to be tangled, there does not seem to be any consistent pattern. The samples of linear DNA in EtOH and 6.6 mM Mg at 10 and 20% EtOH were also contracted, compared to 0% EtOH. Between 20 and 30%, the contraction increased, and there was a great deal of variability in the pattern. As the EtOH concentration increased to 30%, rodlike structures began to appear mixed in with the contracted structures. If the samples were maintained in an ice bath until deposition, a greater proportion of contracted structures, having the appearance of those displayed in Figure 7B, were observed. The rods increased in frequency with higher ethanol content or temperature. Figure 7C shows samples deposited at room temperature from the Mg–30% EtOH solvent. Under these conditions, the reproducibility of rod formation was quite high with no other structures apparent.

The distribution of lengths and widths of these rods is given in Figure 8A,B. The length distribution is typical of that expected from a random end to end polymerization of a major monomer species of ca. 40 nm. The width distribution, in contrast, was more Gaussian in shape, and there was no striking inverse correlation of length with width. This suggests that the rod structures form a homologous series. Such a series could arise as a result of the end to end docking of preformed monomer rods or, alternatively, by the folding of linear oligomers formed by the associations of their complementary ends. Since the products formed by the ligase at 15 °C in 30% EtOH always include significant amounts of linear dimers and trimers, this suggests that precursor multimers are available for rod formation in this solvent, thus favoring the latter explanation.

The length of the major rod species is 42 nm, with a standard deviation of 11 nm. The average width is 13 ± 1 nm, measured directly. If adjustment for the Pt deposit is made, this value is closer to 12 nm, which is also consistent with height determinations on linearly shadowed preparations (11 ± 1 nm). Using these values together with a partial specific volume of 0.55 and a molecular weight of the Mg(II) salt of pBR322 DNA of 2.8×10^6, we found that the dimensions of the major rod species can accommodate the

packing of one molecule of DNA per rod but would be uncomfortably close for two. The packing of the DNA molecule within the rod is not known for certain, but some arrangements can be eliminated by the constraints imposed by the observed geometry. A simple first-order toroiodal coil of either chiral sense is not feasible since it would require that the successive turns of B-form DNA be separated by a center to center distance of only ca. 1 nm, a value which is improbably small. However, higher order toroidally wound coils of the type described by Lang (1973) and Lang et al. (1976) are possible.

Although a simple antiparallel arrangement with one 180° chain reversal can be ruled out, a variety of antiparallel arrangements of the duplex with multiple chain reversals can be fitted comfortably in the dimensions of the monomer rod. These structures can be either nonchiral or chirally interwound or plectonemic arrangements of either sense. Since the CD spectra indicate the formation of chirally packed duplexes under condensing conditions, the arrangement of the duplex in these structures is almost certainly chiral. Furthermore, if the observations of Maestre and Reich (1982) on twisted films of DNA can be extrapolated to solution, the net chiral sense of these structures is right handed.

Conclusions

The behavior of the linking number (with concomitant calculations of winding angle changes in the ethanol solvents), relative to the standard aqueous electrophoresis buffer, does not become anomalous until conditions close to condensation are achieved. Although full-blown condensation effects are not in evidence, it is reasonable to conclude that some precondensation phenomenon is changing the linking number and biasing the winding angle estimate. Gel patterns indicate the existence of enhanced association between the linear pBR322 species, and hydrodynamic and electron microscopic observations suggest contracture as well as association.

There are two major types of precondensation phenomena which can result in the type of bias seen in these studies. Both involve a contraction or bending of the DNA duplex. In one instance, this bending is chiral and reflects the initial conformation appropriate for folding into the rod structures seen in the condensation process. Alternatively, the DNA molecule, as it assumes the conformation appropriate for packing in the condensates, may bend in a nonchiral manner, but the formation of these bends necessitates base pair melting or untwisting

The latter phenomenon, base pair untwisting, will directly account for the fact that the winding angle in some of the Mg–EtOH solvents is less than expected. Since it need involve only a very small fraction of the base pairs, it would have minimal effect on the CD measurements. Smooth chiral bending, on the other hand, does not generally require a deviation in twist, and the values of $[\theta]_{275}$ would be those appropriate for the average value of the twist in the solvents in question. The calculated winding angle change for those solvents, however, would be in error. The origin of the error resides in the failure of the assumption of a net writhe of 0 for the intramolecular ligated species in the ethanol solvent in which the precondensation phenomenon is occurring. In this situation, the linking number in those solvents would reflect both twist and writhe. In such cases, the values of ΔLk, or τ, in the electrophoresis experiments cannot be used as a direct reflection of winding angle changes caused by the transfer from those ethanol solvents to the standard electrophoresis buffer.

The precondensation structures inferred from the probable packing arrangements of the rods described in the electron microscope experiments are compatible with either option. The results of the CD spectral studies at the onset of condensation reveal, however, that the structures formed by condensation are chiral. It is reasonable, therefore, to conclude that the precondensation structures must also be chiral. The deficiencies in linking number under conditions close to condensation require that these precondensation structures be either left-handed toroidal coils or right-handed interwound supercoils. The sedimentation data indicate that these must be relatively loose coils, in contrast to the more contracted structures which appear at the onset of collapse. Indeed, there is probably a dynamic interconversion of the two coiled forms in the EtOH solvent, in equilibrium with the straight-chain form. Their formation may be favored by the formation of noncovalently closed duplexes whose "sticky ends" show increasing association in the Mg–EtOH solvent as the ethanol or Mg concentration increases.

The differences observed in the effects of a given Mg–EtOH solvent on winding angle at the "low" vs the "high" ligase concentrations can be rationalized in a number of different ways. The most straightforward explanation is that either the ligase or a contaminant of the ligase preparation facilitates the precondensation process. At the higher ligase concentrations, even the EtOH solvents below 30% showed evidence of linear dimer forms. We think this is diagnostic of increased association of DNA duplexes in these solutions which always seems to precede the condensation process.

Previous investigations of the changes in winding angle effected by changes in the monovalent cation content have been rationalized in terms of the lowering of the electrostatic free energy of the DNA by ion condensation (Manning, 1978; Anderson & Bauer, 1978) or site binding of cations to the DNA helix (Kilkuskie et al., 1988). The effects of EtOH on the winding angle both in the Na and in the Mg(II) solvents, where normal behavior is observed, can be similarly explained. By lowering the bulk dielectric constant and decreasing the water activity, EtOH will effectively increase the linear charge density, thus promoting increased condensation of counterions in the vicinity of the DNA. The same changes will also lead to increased immobilization of ions, such as Mg(II), which have a partial site bound character.

As the site binding and ion condensation increase with decreasing dielectric constant in these EtOH solvents, the electrostatic neutralization finally reaches the point at which DNA condensation or collapse occurs (Wilson & Bloomfield, 1979). The crucial neutralization value of 0.9 (Wilson & Bloomfield, 1979) in the Mg–EtOH solvent is surprisingly sensitive to temperature. The sign of the temperature dependence would suggest that the condensation event is entropically driven since the enthalpy change is positive. The origin of the positive entropy increase upon collapse of the DNA is not known for certain. A likely source, however, is the release of a solvation shell as the duplex packs into the collapsed structures.

What is certain from these studies is that the mixed EtOH solvent under noncondensing conditions does not promote a decrease in the winding angle of the duplex B form. Rather, the results indicate that the *average* winding angle of the duplex increases and the DNA becomes overwound, as the changes in the CD spectra would suggest. Furthermore, the relationship between rotational strength of the long-wavelength positive band and the average winding angle seems to be the same as that observed in the aqueous solvents previously examined. CD spectral changes can thus be used to assess small winding angle changes in the duplex B structure under noncondensing conditions where direct determination is not fea-

sible. One might use this approach, for instance, to evaluate winding angle changes at high superhelical densities. By the same token, direct determinations by gel methods, even at low superhelical densities, under conditions where condensation or precondensation effects may be operative will yield invalid results.

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Registry No. EtOH, 64-17-5; Mg, 7439-95-4; Na, 7440-23-5.

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