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Application of the Method of Phage T4 DNA Ligase-catalyzed Ring-closure to the Study of DNA Structure

II. NaCl-dependence of DNA Flexibility and Helical Repeat

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In this work, we demonstrate that it is possible to determine the molar cyclization factor j_M from single ligation reactions in which both circular and linear dimer DNA species are formed concurrently from linear monomers. This approach represents a significant improvement over previous methods, in which j_M is evaluated from the ratio of the rate constants for two separate processes; namely (1) the cyclization of linear DNA and (2) the association of two linear molecules to form linear dimers. Determination of j_M for a 366 base-pair molecule yields $5{\cdot}8 \times 10^{-8}$ M, in close agreement with the value of $5{\cdot}6 \times 10^{-8}$ M determined by Shore *et al.* for the same molecule.

Using the current approach for the determination of j_M, we have investigated the dependence on NaCl concentration (0 to 162 mM-NaCl, 1 mM-$MgCl_2$) of both the lateral and torsional flexibilities of DNA. The principal observation is that both quantities are essentially constant over the above range of NaCl concentrations, with the persistence length $P \approx 450$ (± 15) Å, and the torsional elastic constant $C \approx 2{\cdot}0$ ($\pm 0{\cdot}2$) $\times 10^{-19}$ erg cm. These observations are in accord with the previous theoretical prediction that P becomes essentially independent of NaCl concentration above 10 to 20 mM. We have examined the dependence of the helical repeat of DNA on NaCl concentration over the above range, and have found the value of 10·44 base-pairs per turn to be essentially constant over that range. This last result suggests that earlier studies have overestimated the dependence of DNA helical twist on salt concentration.

1. Introduction

An understanding of the forces that must be overcome in bending or twisting DNA is essential for full comprehension of such diverse phenomena as the packaging of DNA into viral particles (Earnshaw & Harrison, 1977; Riemer & Bloomfield, 1978; Widom & Baldwin, 1983), organization of DNA into chromatin (Klug *et al.*, 1980; Richmond *et al.*, 1984), induced curvature of DNA by site-specific proteins (Wu & Crothers, 1984; Kotlarz *et al.*, 1986; Matthew & Ohlendorf, 1985; Brennan *et al.*, 1986; Dripps & Wartell, 1987), intrinsic, sequence-dependent curvature of DNA (Hagerman, 1985; Koo *et al.*, 1986) and various other regulatory and recombinational events (Johnson *et al.*, 1986; Johnson & Simon, 1987).

It has been demonstrated (Shore *et al.*, 1981; Shore & Baldwin, 1983*a*,*b*; Horowitz & Wang, 1984) that measurements of the rates of formation of DNA circles, catalyzed by phage T4 DNA ligase, can be used as a sensitive approach for the determination of both torsional and bending flexibilities of DNA as well as its helical repeat. In particular, Shore *et al.* (1981) demonstrated that the molar cyclization factor j_M, defined previously as the ratio of the equilibrium constants for (non-ligated) circle and linear dimer formation, could be defined in terms of the ratio of the rate constants for formation of those two species in a ligation reaction. Others (Ulanovsky *et al.*, 1986; Kotlarz *et al.*, 1986) have utilized the basic principle of ligase-catalyzed ring-closure in order to demonstrate either protein-induced or sequence-dependent curvature of DNA; however, in no instance have those latter studies taken into account the effects of torsional alignment

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(i.e. helical repeat) for the specific sequences being studied. Shore & Baldwin (1983*a*) demonstrated that a difference of one-half turn between two approximately 250 bp† DNA molecules can result in a difference in j_M of more than an order of magnitude. Such a difference could be realized with two distinct 250 bp molecules if their helical repeats differ by only 0·02 bp per turn. Hence, failure to consider small variations in helical repeat can lead to drastic misinterpretation of measured differences in j_M values. Consequently, in order to be of use as a quantitative tool, the ring-closure methodology must take into account the effects of fractional twist on each series of DNA molecules being studied.

The work presented here therefore focuses on two main areas in order to permit the application of ring-closure methodologies to the quantitative examination of DNA structure and flexibility: (1) the development of a simplified approach for the measurement of the kinetics of phage T4 ligase-catalyzed DNA circle formation, whence values for the molar cyclization factor j_M are obtained; and (2) the design of a series of closely related DNA molecules to permit the accurate inclusion of torsional effects on the rates of circle formation. The current kinetic approach takes advantage of the fact that, for sufficiently early times in the ligation reaction, the ratio of the amounts of monomer circle and linear dimer formed is a direct reflection of j_M. Thus, all of the information required for the determination of j_M can be obtained from a single ligation reaction. The design of the DNA molecules to be used for ring-closure measurements incorporates two important features; (1) a phasing sequence located in one half of each member of the set and differing in 2 bp increments among members of the set, and (2) a collection of centrally located restriction sites to facilitate the insertion of additional sequences of interest; for example, in conjunction with the study of DNA curvature or protein-induced bending.

The current ring-closure assay system is extremely well-suited for investigations of DNA structure for a number of reasons. (1) The molar cyclization factor j_M is sensitive to very small changes in fractional twist and persistence length. For example, a reduction in persistence length from 500 Å to 450 Å (a 10% decrease: 1 Å = 0·1 nm) results in a 60% increase in j_M (Shimada & Yamakawa, 1984; Hagerman & Ramadevi, 1990). (2) Because cyclization and linear dimer formation take place in the same reaction vessel, data can be gathered even under salt conditions that lead to significant loss of ligase activity (this work); consequently, the assay is valid for salt concentrations inaccessible to other sensitive techniques (e.g. electro-optic methods). (3) The use of a series of closely related DNA molecules, spanning at least one turn of helix, allows one to study DNA flexibility and/or curvature without the need to assume either particular values for the helical repeat or the absence of any influence of solution conditions on the helical repeat.

In this work, the ring-closure methodology is used to examine the influence of monovalent salt (NaCl) concentration on three properties of the DNA helix, namely, its bending rigidity, its torsional rigidity and its helical repeat. We have observed that all three properties of the helix are quite insensitive to NaCl concentrations below about 200 mM (+1 mM-$MgCl_2$). These observations are important for an understanding of the influence of salt on the solution structure of DNA, as well as its influence on protein–DNA interactions. The latter issue is of particular interest in light of numerous studies suggesting that such interactions might involve bending and/or twisting of the DNA helix by the associated binding protein.

2. Theory

The propensity for cyclization of DNA molecules is defined (Jacobson & Stockmayer, 1950; Wang & Davidson, 1966*a*,*b*; Shore *et al.*, 1981) as the ratio of two equilibrium constants, $j_M = K_C/K_D$, where K_C is the equilibrium constant for cyclization, K_D is the bimolecular equilibrium constant for the joining of two molecules to form a linear dimer, and j_M is the molar cyclization factor. So defined, j_M has units of molarity, and is related to a quantity we refer to as the cyclization (ring-closure) probability density factor, j (Hagerman & Ramadevi, 1990), through the relation:

$$j_M = j \times 10^{27}/(P^3 N_A).$$

In the above expression, the persistence length P has units of Å, and N_A is Avogadro's constant. It should be noted that the j employed by Shore *et al.* (1981), Shore & Baldwin (1983*a*,*b*) and Wang & Davidson (1966*a*,*b*) is equivalent to j_M. Others (Shimada & Yamakawa, 1985; Levene & Crothers, 1986*a*) define j in terms of a volume element $(2P)^3$, in which case the conversion to j_M involves an additional factor of 8; thus, $j = 8J$, where J is the cyclization factor of Shimada & Yamakawa (1984).

In their studies of T4 DNA ligase-catalyzed cyclization, Shore *et al.* (1981; Shore & Baldwin, 1983*a*,*b*) used separate reactions for the determination of the rate constants for cyclization and bimolecular joining (k_d) reactions; however, all of the information for the determination of both k_C and k_D can, under suitable conditions, be obtained from a single reaction, thus eliminating the uncertainties inherent in separate determinations of DNA and/or ligase concentrations, differences in ligase affinities for different DNA molecules and slight differences in buffer conditions. Our approach is based on the fact that, for a suitable range of DNA concentrations, linear monomer (M; molar concn M) is converted to linear dimer (D; molar concn D) and monomer circles (C, concn C) with initially comparable rates. The rates of circle and linear dimer formation are given by:

$$dC/dt = k_C(M_0 - C - 2D)$$

† Abbrevation used: bp, base-pair(s).

and:

$$dD/dt = 2k_D(M_0 - C - 2D)^2 - \text{terms}[O(D,M\ D,D^2 \ldots)],$$

where M_0 is the molar concentration of linear monomer at the start of the reaction, k_C is the first-order rate constant for cyclization, k_D is the elementary, bimolecular rate constant for the joining of two cohesive ends from different DNA molecules, and "terms[...]" represents avenues of loss of linear dimer to larger linear and cyclic species. The factor 2 in the expression for dimer formation represents 0·5 × (moles dimer/mole monomer) × 4 (number of ways of forming a dimer molecule from 2 monomers with identical ends). For a suitably small interval of time, dt, $dC = k_C M_0 dt$ and $dD = 2k_D M_0^2 dt$, such that:

$$\lim_{t \to 0} C(t) = k_C M_0 t$$

and:

$$\lim_{t \to 0} D(t) = 2k_D M_0^2 t.$$

It follows that:

$$j_M = 2M_0 \lim_{t \to 0} [C(t)/D(t)].$$

In order to obtain j_M experimentally, one therefore measures, for known M_0, the quantities $C(t)$ and $D(t)$ at several early time-points ($C, D \ll M$), followed by extrapolation of the ratio $C(t)/D(t)$ to $t = 0$. Since the ratio k_C/k_D is independent of ligase concentration over a wide range (Shore *et al.*, 1981; and see Results and Discussion, section (c)), ligase concentrations can be adjusted to allow convenient collection of data over a time-interval where the ratio $C(t)/D(t)$ is essentially constant.

3. Materials and Methods

(a) *Synthesis and purification of oligonucleotides*

Synthetic portions of the embedding sequence molecules were produced on controlled-pore glass supports using phosphotriester chemistry (Caruthers *et al.*, 1982; Atkinson & Smith, 1984) either manually (Hagerman., 1985) or using a Biosearch model 8600 automated DNA synthesizer. All oligomers were detritylated while still on the controlled-pore glass support. Cleavage of oligonucleotides from controlled-pore glass supports, base deprotection, and oligonucleotide purification were carried out as described by Hagerman (1985).

Concentrations of single-stranded oligomers were operationally defined by the relation 35 μg/ml = 1 A_{260} unit (50 μg/ml = 1 A_{260} unit for duplex DNA). Absorbance measurements were performed on a CARY 219 recording spectrophotometer (Varian Associates).

(b) *Purification of constructs and sequence verification*

Embedding sequence half-molecules were assembled by sequential rounds of directed ligation of several synthetic and natural DNA segments (Taylor, 1989). Embedding sequence sub-fragments, as well as the full-length embedding sequence fragments produced by ligation of half-molecules, were purified on preparative, 6% (w/v) polyacrylamide gels, E buffer (40 mM-Tris-acetate, 20 mM-sodium acetate, 1 mM-NaEDTA pH 7·9) at room temperature, and were subsequently eluted from gel slices using a preparative electroelution system designed is this laboratory (P.J.H., unpublished results) in which electrophoresing DNA is automatically diverted through, and collected on DEAE-cellulose (DE-52, Whatman) columns. Electroelutions were performed at room temperature in 0·5 × E buffer. DNA molecules were eluted from the DE-52 columns with 3 M-potassium acetate, and were stored as isopropanol slurries (50% (v/v) isopropanol, high-pressure liquid chromatography grade) at −20 °C.

Exact lengths and sequence compositions of the 8 final embedding sequence half-molecules were confirmed by the Sanger dideoxy sequencing approach (Sanger *et al.*, 1977), modified for sequencing of double-stranded DNA (Chen & Seeburg, 1985). Supercoiled plasmid DNA containing the embedding sequence half-molecule was denatured with alkali, and oligonucleotide-primed DNA synthesis allowed to occur in the presence of dideoxynucleotide triphosphates. Sequencing reagents were purchased as kits from New England Biolabs (NEB).

(c) *Enzymes*

Restriction endonucleases, *Eco*RI, *Hin*dIII, *Hae*III, *Kpn*I, *Pvu*II and *Cfo*I were obtained from Bethesda Research Laboratories (BRL), and were used according to the manufacturer's protocols at 37 °C unless otherwise indicated (bovine serum albumin was not added to any enzymatic reactions in this study; its presence was therefore limited to amounts present in the original enzyme stocks). Polynucleotide kinase and T4 DNA ligase were obtained from NEB, and, for cloning and labeling experiments, were used according to the manufacturer's protocols, with slight modifications; both kinase and ligase reactions (for constructions) were performed in 50 mM-Tris·HCl, 10 mM-$MgCl_2$, 1 mM-ATP and 5 mM-dithiothreitol (pH 7·8). Ligase-catalyzed cyclization reactions were generally carried out in 20 mM-Tris·HCl (pH 7·8), 1 mM-$MgCl_2$, 1 mM-ATP, and added NaCl as specified. Units of T4 DNA ligase activity are those specified by NEB: 1 NEB unit is defined as the amount of enzyme required to give 50% ligation of *Hin*dIII fragments of bacteriophage lambda DNA in 30 min at 16 °C in the reaction mix specified by NEB. This unit definition corresponds to 0·015 Weiss unit (Weiss *et al.*, 1968). It should be noted that Shore *et al.* (1981) defined their units in terms of protein mass, not activity (1 unit = 1·3 ng of protein, >97% pure by SDS/polyacrylamide gel profile). A rough correspondence between the mass units of Shore *et al.* (1981) and the NEB unit can be made by noting that 1 NEB unit generally corresponds to approx. 0·5 ng of protein (>95% pure by SDS/polyacrylamide gel). Therefore, 1 Shore *et al.* unit would correspond roughly to 2·6 NEB units. Such a correspondence assumes that both enzyme preparations have equivalent activities/unit mass (an unsupported assumption). The NEB unit of activity will therefore be used throughout this paper.

(d) *Cloning procedures*

The *Escherichia coli* strain HB101 (F⁻, *lacY*, *gal*, *leu*, *pro*, *endA*, *hsdR*, *hsdM*, *recA1*, *rpsL*) (Bolivar *et al.*, 1977) was used for transformation and production of plasmid

Table 1

Plasmids containing the Eco*RI–*Hind*III fragments used in the current ring-closure experiments*

Plasmid	*Eco*RI–*Hind*III fragment length (bp)	Number of tandem copies	Portion of final embedding sequence
pESR/MC	159†	20	R
pESL6	191	1	L
pESL5	189	1	L
pESL4	187	1	L
pESL3	185	1	L
pESL2	183	1	L
pESL1	181	1	L
pESL0	178‡	1	L

† The previously reported length of this fragment (Taylor & Hagerman, 1987) was 151 bp and was based on a reported value of 98 bp for the *Eco*RI–*Hae*III segment. This fragment is generated by *Hae*III digestion of a fragment, XA4, used by Shore *et al.* (1981), which has as its supposed ultimate source the 358 bp *Alu*I fragment 4 of ϕX174. Sequence analysis of this region revealed that the 98 bp fragment is in fact 106 bp in length and derived from the large *Alu*I fragment from pXH9 (Shore *et al.*, 1981).

‡ A missing G·C base-pair at the *Hae*III site of this insert has resulted in a 3 bp rather than a 2 bp difference in length from the next shortest member of the series.

DNA. An *Eco*RI/*Hind*III-ended derivative of pBR328 (Soberon *et al.*, 1980) was used as the plasmid vector. The 7 left-hand half-molecules, each of which contained 1 member of the phasing set, were cloned as single copies using general cloning procedures (Hagerman, 1984). The single right-hand half-molecule was cloned as 20 tandemly arranged copies in order to increase yields of this fragment, since 7 times as much of the latter molecule is required for the embedding sequence constructions. A multiple-copy cloning strategy devised for this purpose (Taylor & Hagerman, 1987) involves the use of synthetic, directional adapters to allow for control of fragment orientation during ligation, direct repeats being essential for clone stability (Sadler *et al.*, 1978). The designations of the resultant plasmids are listed in Table 1.

(e) *Plasmid isolation*

For large-scale preparation of embedding sequence half-molecules, cells were grown in a New Brunswick Scientific IF-250 fermentation system (250 l) according to a directly scaled-up version of the procedure described by Hagerman (1984), which itself constitutes a modification of the high-density fermentor protocol of Sadler & Tecklenberg, (1981).

A New Brunswick Scientific MF-1285, 28 l fermentor was used to grow cells in 17 l of Davis buffer (Sadler *et al.*, 1977), modified to contain 1·7% (w/v) glucose, 0·5% (w/v) Casamino acids, and thiamine·HCl (15 mg/l) to an A_{350} of 3·72. This 17 l growth was subsequently used to inoculate 170 l of the same medium in the IF-250 fermentor. IF-250 growth conditions were maintained at pH 7·0, with an air flow of 220 l/min, and agitation at 175 revs/min with reduction to 100 revs/min, 1 h following chloromphenicol amplification (170 mg/l) at $A_{350}=9{\cdot}0$), to reduce cell shearing. It should be noted that there was no detrimental effect on yield by eliminating the uridine amplification step; the addition of uridine had been reported to boost yields when added in log-phase growth (Norgard *et al.*, 1979; Sadler & Tecklenberg, 1981). This observation is of importance because the addition of uridine significantly inflates the cost of large-scale fermentations. Cells were collected using a Sharples AS-16 continuous-flow centrifuge (Pennwalt Co.) with approximately 80% recovery, yielding 1 to 1·5 kg/fermentor run. Cells were resuspended to a final concentration of 0·4 kg/l in 25% (w/v) sucrose/50 mM-Tris·HCl (pH 7·5), and lysed by the sequential addition (on ice) of (1) lysozyme (0·7 mg/ml Sigma) for 5 min; (2) EDTA to 35 mM final concn, for 5 min; and (3) 1/3 vol. 2% (w/v) Triton X-100, 62·5 mM-EDTA and 50 mM-Tris·HCl (pH 7·5). The clearing spins, polyethylene glycol fractionations and phenol/chloroform extractions were performed as described (Hagerman, 1984; Sadler & Tecklenberg, 1981). Typical yields were approx. 300 mg of plasmid/170 l fermentor run.

(f) *Preparative purification of primary inserts from plasmids*

Purified plasmid DNA was digested with *Eco*RI and *Hind*III at 1 unit/50 μg DNA under otherwise standard reaction conditions. Progress of the cleavage reaction was monitored by horizontal mini-agarose gels. Lyophilized enzyme (BRL) was added directly to the reaction mix, thus avoiding the glycerol resuspension step. The resultant *Eco*RI/*Hind*III-ended inserts were separated from plasmid DNA by passage over a Sepharose 2B-CL (Pharmacia) gel filtration column (7 cm × 55 cm). The column capacity is approx. 80 mg, and on 2 passes yields insert DNA that is >95% pure. Additional purification of these fragments occurs during the process of the construction and purification of the full-length embedding sequence molecules (see above).

The 366 bp *Eco*RI-ended fragment was isolated from plasmid pXA4 (Shore *et al.*, 1981). The pXA4/HB101 transformant was kindly provided by David Shore.

(g) *Preparation of radiolabeled DNA*

Full-length embedding sequence fragments were treated for 20 min with calf intestinal alkaline phosphatase (Boehringer-Mannheim) at 0·02 unit/pmol 5′ ends of DNA (standard reaction conditions). The reaction mixtures were subsequently heated to 65°C for 10 min

followed by extraction with phenol. Residual phenol was removed by repeated extractions with diethyl ether.

Dephosphorylated DNA was 5′-end labeled with ^{32}P by incubation with [γ-^{32}P]ATP (approx. 100 μCi/μg DNA; 7000 Ci/mmol ATP) and phage T4 polynucleotide kinase (New England Biolabs; approx. 20 units of enzyme/μg DNA) for 40 min at 37°C (100 μl/ng DNA, 50 mM-Tris·HCl, 10 mM-$MgCl_2$, 5 mM-dithiothreitol, 0·1 mM-spermine and 0·1 mM-Na_2EDTA, pH 7·6). Labeling was followed by a 10 min chase with cold ATP (0·1 mM final ATP concn), and a 2nd portion of T4 kinase to ensure that all molecules were fully phosphorylated. Unincorporated label was removed by 2 3-min spins (900 *g*) over a 6 cm column (Sephadex G-50; Pharmacia) packed in 1 ml tuberculin syringes (Becton, Dickinson & Company). We have demonstrated that the molecules thus prepared are both fully phosphorylated and without internal nicks, by overnight ligation at low DNA concentration (<1 μg/ml), followed by gel electrophoresis in 4% agarose with added ethidium bromide (10 mg/ml). In the above gels, nicked circles have lower gel mobility than linear, unligated DNA; intact, covalently closed circular molecules have higher mobility. Between 90% and 100% of the DNA molecules prepared as described above are without nicks, as determined by this simple ligation/gel test.

For cyclization experiments, (5′-^{32}P)-labeled DNA and unlabeled DNA were mixed (>95% unlabeled fraction) to yield a total DNA concentration of 3 μg/ml (total 20,000 cts/min) in 80 μl (50 mM-Tris·HCl, 10 mM-$MgCl_2$, 1 mM-ATP, pH 7·8) at 20(±0·5)°C. The reactions were initiated by addition of T4 DNA ligase, followed by manual mixing. At specific time-intervals, 10 μl portions were removed and quenched with 5 μl of stop solution (100 mM-EDTA, 0·04% (w/v) bromophenol blue 2% (v/v) glycerol) and heat (65°C, for 10 min).

(h) *Analytical agarose gel electrophoresis*

Analytical (vertical) gels used in the kinetics experiments were 4% (w/v) composite agarose: 2% electrophoresis grade agarose (BRL) plus 2% NuSieve agarose (FMC Bioproducts). Use of NuSieve agarose facilitates the pouring of high-percentage gels, which are required to give optimal resolution of linear and circular products. However, high-percentage NuSieve gels are brittle and fragment easily, thus being awkward to handle. The composite gel provides both the superior resolution of the NuSieve and the superior mechanical properties of regular agarose. The composite gels were all run at room temperature in E buffer. Following electrophoresis, the gels were dried under vacuum at 80°C, and were subjected to autoradiography using Kodak XAR-5 film.

(i) *Scanning densitometry*

Autoradiographs were scanned with an EC910 scanning densitometer (EC Apparatus Corp.), and peak areas measured using the Hoefer Scientific Instruments GS360 electrophoresis data system software and an IBM PC/AT computer. Control lanes containing bands of serially decreasing intensity were also scanned for each gel in order to ensure linearity of the densitometer response.

(j) *Analysis of the molar cyclization factors*

All j_M values were analyzed by application of the results of the Monte Carlo computations presented in the accompanying paper (Hagerman & Ramadevi, 1990). In particular, experimental log j_M values were subjected to linear least-squares fits to the corresponding, computed j_M values, with persistence length P, torsional elastic constant C, and helical repeat, h_R, being the variable parameters. For each set of log j_M *versus* L data, a global (3-parameter) least-squares fit was obtained by scanning C ($0{\cdot}1 \times 10^{-19}$ erg cm increments; 1 erg = 10^{-7} J), P (1 Å increments), and h_R (0·01 bp/turn increments). It was observed that the least-squares value for each parameter was quite insensitive to values of the other 2 parameters, and discussions in the text are couched in terms of the effects of single-parameter variations. Least-squares analyses were performed on a VAXstation II GPX computer.

4. Results and Discussion

(a) *Design and construction of DNA molecules for the measurement of ligase-catalyzed ring-closure*

Our choice of the length range for DNA molecules used in the present work is based on the following considerations. Under conditions compatible with T4 DNA ligase-catalyzed ring-closure, the maximum value of j_M (corresponding to maximal rates of cyclization) occurs for DNA molecules having lengths in the vicinity of 500 bp (Shore *et al.*, 1981; Hagerman, 1985; Shimada & Yamakawa, 1984; Hagerman & Ramadevi, 1990). However, because this length is more than three times the persistence length of DNA under ligation conditions, the effects of introducing regions of curvature or alterations in torsional alignment would be reduced significantly by background flexibility. Furthermore, j_M becomes less sensitive to changes in persistence length as the length of the DNA molecule is increased. On the other hand, in the absence of intrinsic curvature, the rates of ligase-catalyzed cyclization are dramatically reduced for molecules shorter than approximately 250 bp (Shore *et al.*, 1981; Shore & Baldwin, 1983*a*). In consideration of the above, the current studies utilize DNA molecules having lengths in the range of 330 bp to 360 bp.

In order to facilitate the incorporation of additional sequences of interest (e.g. sequences possessing intrinsic curvature), the DNA molecules developed for the current studies contain a centrally located, nested set of restriction sites (Fig. 1). Additional sequences may therefore be embedded within the original molecules at these sites; hence the designation "embedding sequences" for the parent molecules.

As demonstrated (Shore & Baldwin, 1983*a*; Shimada & Yamakawa, 1984; Hagerman & Ramadevi, 1990) for DNA molecules in the 250 bp to 400 bp size range, there is also a large effect on j_M of the extent of torsional alignment of the ends of the molecule being cyclized. Thus, in order to allow for the effect of fractional twist (itself a sequence-dependent property), a set of seven DNA molecules was constructed wherein each member of the set differed from the others only in the variable length of a "phasing sequence" insert (Fig. 1). In this

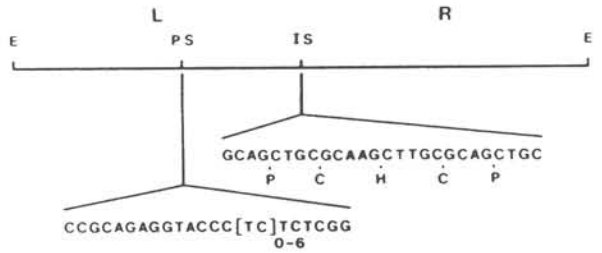

Figure 1. Organization of the embedding sequence molecules used in the current study. The set of 7 *Eco*RI (E)-ended molecules differ in length from one another in increments of 2 bp, due to the presence of phasing inserts of differing lengths in the left-hand half of each molecule (phasing set; PS). A nested set of restriction sites was installed at the center of each molecule to facilitate incorporation of other segments of DNA in future studies (insertion site; IS). Restriction sites include *Pvu*II (P), *Cfo*I (C) and *Hin*dIII (H). The embedding sequence molecules were cloned as *Eco*RI–*Hin*dIII half-molecules, there being 7 versions of the left-hand half (L) and a single version of the right-hand half (R).

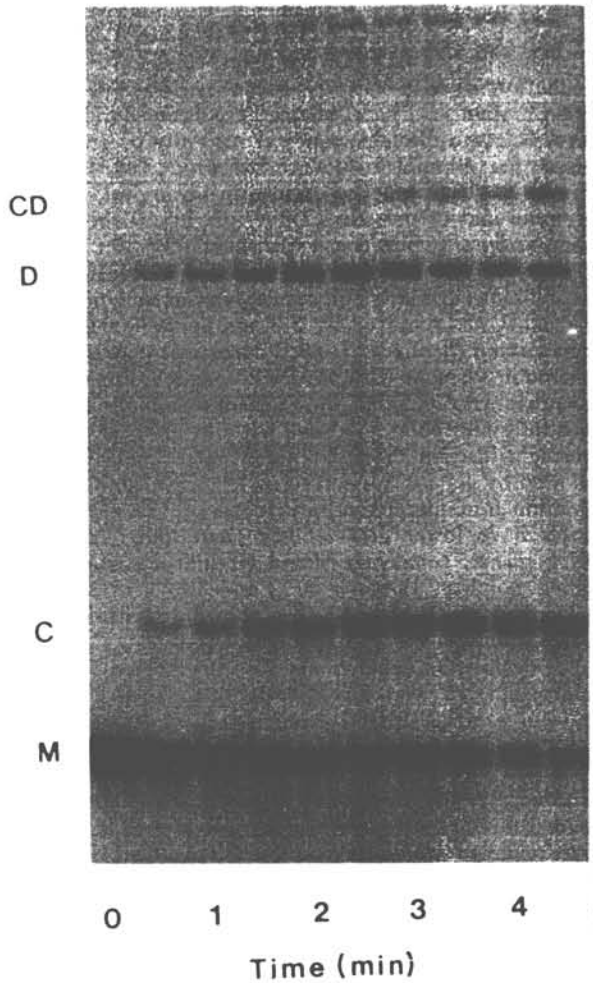

Figure 2. Autoradiograph showing progressive ligation of the linear 366 bp DNA (M) to form covalently closed circular DNA monomer (C) and linear dimer (D). Circular dimer (CD), as well as higher multimers are also visible at later time-points. DNA concentration, 2·1 μg/ml; T4 DNA ligase concentration, 310 units/ml; in 50 mM-Tris·HCl, 10 mM-$MgCl_2$, 1 mM-ATP and 5 mM-dithiothreitol, pH 7·8; temperature 20°C.

work, the phasing sequence insert lengths varied in 2 bp increments (and one increment of 3 bp) over a range of 13 bp (slightly more than 1 turn of the helix). Thus, measurement of j_M for all members of the series yields the torsional component of j_M directly (Shore & Baldwin, 1983*a*; Shimada & Yamakawa, 1985; Hagerman & Ramadevi, 1990).

The joining of the *Hin*dIII end of the standard right-hand half-molecule (159 bp; derived from pESR/MC) to that of each of the seven left-hand half-molecules (178 to 191 bp; derived from the corresponding pESL plasmids) resulted in a set of seven molecules (337 to 350 bp) that were used for most of the subsequent ring-closure measurements. One major advantage of using such a closely related set, as opposed to a series of molecules of similar length but unrelated sequence, is that determinations of helical repeat as well as torsional and flexural elastic constants can be related to particular sequences.

(b) *Experimental determination of the molar cyclization factor* j_M

The molar cyclization factor, j_M, can be expressed directly in terms of the ratio ($R_{c/d}$) of the integrated intensities of the autoradiograph bands corresponding to circular monomer, C, and linear dimer, D (Fig. 2). Since $C/D = 2R_{c/d}$, independent of the fraction of DNA molecules that are labeled (see Appendix):

$$j_M = 4M_0 \operatorname*{Lim}_{t \to 0} R(t)_{c/d}.$$

Thus, j_M values are determined directly from the initial molar concentration of linear monomer M_0 and the extrapolated ratio $R(0)_{c/d}$ of the band intensities for cyclic monomer and linear dimer species (Fig. 3). For the ring-closure experiment displayed in Figures 2 and 3 (366 bp fragment), the value of j_M is $5{\cdot}8 \times 10^{-8}$ M, in good agreement with the determined value of $5{\cdot}6 \times 10^{-8}$ M (Shore *et al.*, 1981; Shore & Baldwin, 1983*a*).

In order to improve the precision of the ring-closure experiment, M_0 can be adjusted to yield $R(0)_{c/d}$ values that fall within the range of approximately 0·2 to 5·0, thus reducing the errors associated with the determination of the relative band intensities. We have confirmed (data not shown) that $R(0)_{c/d}$ is directly proportional to $1/M_0$ over a 20-fold range. In addition, final DNA concentrations can be established with greater precision by adding a small amount of labeled monomer (1 to 5% by mass) to unlabeled monomer, the concentra-

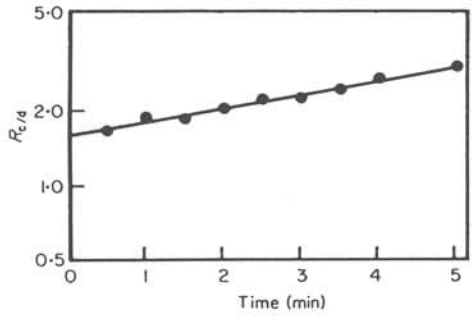

Figure 3. Time-dependence of the ratio $R_{c/d}$ of circular monomer to linear dimer band intensities for the autoradiograph in Fig. 2. Circle and dimer bands are quantified by scanning densitometry of the autoradiograph (see Materials and Methods, section (i)). Extrapolation of $R_{c/d}$ to $t=0$ yields the quantity $R(0)_{c/d}$ used in the determination of j_M values.

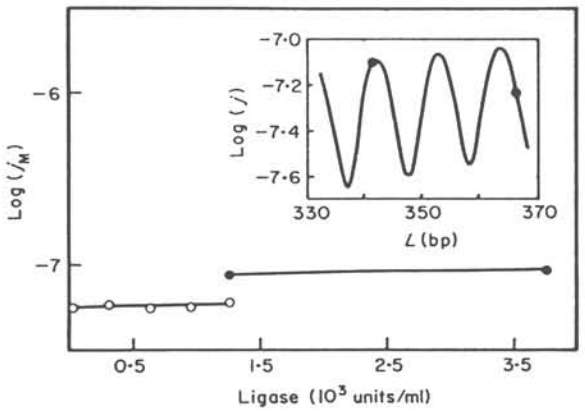

Figure 4. Dependence of the molar cyclization factor, j_M, on T4 DNA ligase concentration. These plots demonstrate that j_M does not vary with ligase concentration over a 100-fold range (30 to 3000 units/ml). Initial measurements were performed on the 366 bp *Eco*RI-ended fragment (○) (≤1000 units ligase/ml), and subsequently, using the 344 bp embedding sequence molecule (●) (≥1000 units/ml). It is of interest to note that, although shorter, the 344 bp molecule exhibits a larger value for j_M. This is due to the differences in the fractional twist of these 2 fragments required for cyclization (see the text). Inset: theoretical j_M curve (Shimada & Yamakawa, 1984) using the following values; $P=449$ Å, $C=1{\cdot}8\times10^{-19}$ erg cm and helical repeat $h_R=10{\cdot}38$ bp over the length interval 330 to 370 bp. The j_M values predicted for the 344 bp and 366 bp molecules are indicated.

tion of the latter being determined by ultraviolet light absorption in the absence of label (for convenience of handling). This last operation is validated by the fact that the correspondence between C/D and $R_{c/d}$ is independent of the labeled fraction (see Appendix).

(c) *The molar cyclization factor* j_M *is independent of ligase concentration*

In order for cyclization measurements to reflect the intrinsic properties of the DNA molecules being studied, it is essential that j_M be independent of ligase concentration. Control measurements were therefore made over a 100-fold range of ligase concentration, with resultant j_M values proving to be invariant over this range (Fig. 4). Our results are consistent with those of Shore *et al.* (1981), who demonstrated that the apparent first-order rate constant for ligase-catalyzed cyclization was directly proportional to ligase concentration over a tenfold range, and that the rate of bimolecular joining (dimer formation) was also directly proportional to ligase concentration.

It is of interest to note that, of the two molecules used in these control measurements, the shorter (344 bp) yields a larger value for j_M than the longer molecule (366 bp). This is opposite to predictions based on the known general length dependence of j_M (Shore *et al.*, 1981) and is, in fact, due to differences in the torsional alignment of the ends of these molecules (Shore & Baldwin, 1983*a*; Hagerman & Ramadevi, 1990). In order to underscore this point, the inset to Figure 4 displays a theoretical curve for j_M over the short length interval 330 to 370 bp, generated according to the analytical treatment of Shimada & Yamakawa (1984) using a value of 10·38 bp for the helical repeat of the two related DNA fragments. This observation further underscores the importance of including a consideration of torsional alignment effects on the interpretation of measured ring-closure rates.

The overall rates of both cyclization and dimerization reactions are affected by increasing the ligase concentration, thus influencing the nature of the $R_{c/d}$ plots. For a ligase concentration of 1250 units/ml (1 mM-ATP, 20 mM-Tris·HCl, 1 mM-$MgCl_2$, pH 7·8), there is essentially no change in the concentration of linear monomer over a two-minute period. Consequently, the rate of dimer formation, which is second-order with respect to monomer concentration, remains constant, as does the ratio $R_{c/d}$. However, for higher concentrations of ligase, both reactions proceed much further within the same period, and the relative net rate of dimer formation decreases due to reduced concentration of the monomer and to loss of dimer to higher multimers. Nevertheless, the extrapolated ratio $R(0)_{c/d}$, and therefore j_M, remain constant. This situation is represented in Figure 5. It should be noted that at the highest ligase concentration tested (12,000 units/ml), a lower/$R(0)_{c/d}$ value was obtained, perhaps due to the onset of weak, nonspecific binding of protein (either ligase or a minor non-ligase impurity in the commercial ligase preparation).

The general observation that, over a wide range of ligase concentrations, j_M values are independent of ligase concentration is important for another reason; experiments can be carried out under conditions where ligase activity is significantly diminished (e.g. high salt concentration), and hence,

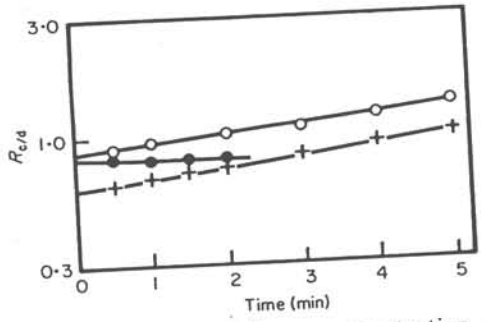

Figure 5. Influence of ligase concentration on the nature of the $R_{c/d}$ *versus* time plot for the 344 bp embedding sequence molecule. (●) 1250 units ligase/ml; (○) 3000 units ligase/ml; (+) 12,000 units ligase/ml. Reaction conditions are otherwise as specified in Materials and Methods, with NaCl added to 54 mM. DNA concentration = 2·98 μg/ml.

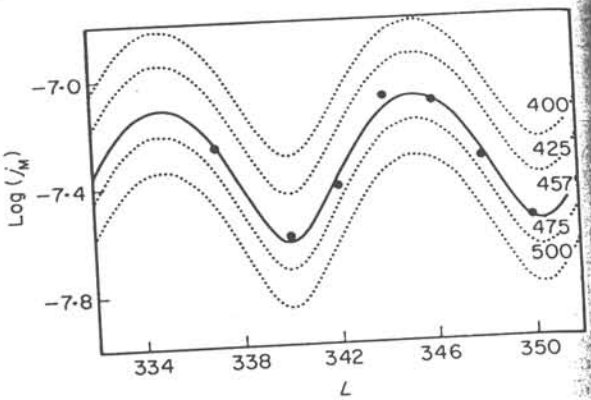

Figure 6. Plot of log j_M *versus* L for various values of P, holding C and h_R constant. The continuous line (P = 457 Å) represents the best fit to the experimental ring-closure data at 54 mM-NaCl (global, 3-parameter fit). Helical repeat, h_R = 10·46 bp/turn; $C = 2{\cdot}0 \times 10^{-19}$ erg cm.

where higher concentrations of ligase are required. Moreover, our observations demonstrate that for ligase concentrations up to approximately 2 μg enzyme per millilitre, any non-specific interactions between ligase and DNA that may occur do not alter the properties of the helix. In addition, for the series of seven experiments performed with the 336 bp fragment, we observed a standard deviation in log j_M of 0·018, which corresponds to a ±4% variation in j_M from experiment to experiment, thus attesting to the precision of the current approach.

Finally, since the joining of cohesive ends has been shown to be a fast pre-equilibrium reaction and not slow relative to closure by ligase (Shore *et al.*, 1981), rates of covalent joining, intramolecular or otherwise, will depend on the equilibrium fraction of associated ends and not on ligase or associated components of the ligation reaction (e.g. ATP). It should be noted that the ATP present in the ligation reaction buffer is present in vast excess ($\sim 10^5$-fold molar excess over linear monomer); the values obtained for j_M should therefore not depend on ATP concentration. In accord with this last assertion, our value for j_M (366 bp fragment, 1·0 mM-ATP) is essentially identical with that of Shore *et al.* (1981) for the same fragment in 0·25 mM-ATP.

(d) *Analysis of the torsion angle-dependent j_M values for the DNA molecules comprising the embedding sequence set*

One of the attractive features of ring-closure measurements is the ability to extract information pertaining to both torsional and bending stiffness as well as the helical repeat of the sequences being studied (Shore & Baldwin, 1983*a*; Shimada & Yamakawa, 1984; Hagerman & Ramadevi, 1990). In order to underscore the utility of the approach more fully, j_M values were determined (as described in section (b), above) for each of the seven *Eco*RI-ended embedding sequence molecules (Fig. 6). It is clear that the j_M values vary dramatically over a period of approximately 10 bp, as expected from both experimental (Shore & Baldwin, 1983*a*) and theoretical (Shimada & Yamakawa, 1984; Hagerman & Ramadevi, 1990) studies.

A detailed analysis of the curve presented in Figure 6, using the analytical approach of Shimada & Yamakawa (1984) (see Hagerman & Ramadevi, 1990), yields values for the persistence length P, torsional elastic constant C, and helical repeat h_R of the set of embedding-sequence molecules. Two features of these results should be noted. First, the value determined for P (=457 Å) in 1 mM-Mg^{2+} is within 10% of the corresponding values obtained previously under similar conditions through the use of hydrodynamic methods (Hagerman, 1981; Elias & Eden, 1981), thus providing a consistency check for the two approaches. Second, the value determined for C ($= 2{\cdot}0 \times 10^{-19}$ erg cm) is reasonably close to the previous estimates presented by Shore & Baldwin (1983*a*), Horowitz & Wang (1984) and Frank-Kamenetskii *et al.* (1985).

As indicated in Figure 6, the resistance of DNA molecules to bending deformations, represented by P, can be determined in a sensitive fashion from the overall vertical position of the log j_M *versus* L curve. In this example (54 mM-added NaCl), the log j_M *versus* L curve yields a best-fit value for P of 457 Å, with an associated uncertainty of much less than 5%. Moreover, as discussed previously, experimental values of log j_M, and hence P, are not subject to errors in the delivery of ligase to the reaction vessels, since cyclization and dimer formation are determined in the same reaction. It should also be noted that P is rather insensitive to errors in the determination of DNA concentration: a 20% error in the measured DNA concentration for a 300 bp fragment with a nominal value for P of 500 Å would result in only an approximately 5% error in P.

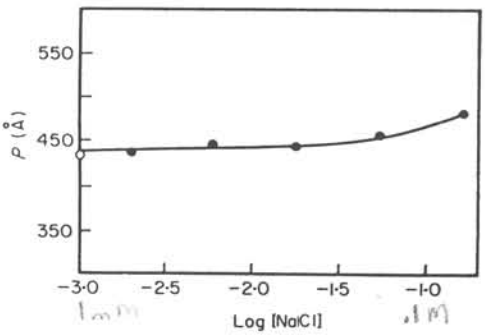

Figure 7. Experimentally determined values for P, plotted as a function of NaCl concentration. Each point represents the results of 7 independent ring-closure measurements at NaCl concentrations ranging from 0 to 162 mM. (○) No added NaCl; (●) NaCl added as indicated. The standard deviations in P are all less than ±14 Å (circle diameter = 10 Å), and were determined from the variation in log j_M for each L.

(e) *Dependence of DNA flexibility on monovalent salt (NaCl) concentration*

Values for P, derived from seven independent sets of ring-closure measurements at NaCl concentrations ranging from 0 to 162 mM, are plotted in Figure 7. These results demonstrate that DNA flexibility is not significantly increased (reduced P) by increases in NaCl concentration over the above range, with P remaining in the vicinity of 450 to 500 Å. This range of P values is entirely consistent with the earlier ring-closure results obtained by Shore *et al.* (1981), performed in 10 mM-$MgCl_2$ in the absence of added NaCl, where $P = 470$ to 490 Å (Shimada & Yamakawa, 1985; Hagerman & Ramadevi, 1990). Moreover, for moderate salt concentrations (20 to 200 mM-NaCl; 1 mM-$MgCl_2$), the absence of any significant decrease in P with increasing salt concentration is entirely consistent with the earlier studies by Elias & Eden (1981), Hagerman (1981). Rizzo & Schellman (1981) and Maret & Weill (1983), which all demonstrated a plateauing of P in the 450 to 500 Å range for the higher salt concentrations. This behavior is well-described by the simple plateau model for P (Odijk, 1977; Skolnick & Fixman, 1977), which represents P as a sum of electrostatic and non-electrostatic (i.e. salt-independent) components. The model predicts that the electrostatic component of P is significant only at low counterion concentrations ($[Mg^{2+}] <$ 0·5 mM; $[Na^+] < 10$ to 20 mM), in accord with observations of the current study. It should be noted that, while the notion of a non-electrostatic component of P is intuitively reasonable, the intrinsic term could well reflect electrostatic interactions within the helix that simply are not sensitive to external counterion concentration.

The current results contrast sharply with the observations of Borochov *et al.* (1981), Borochov & Eisenberg (1984), Kam *et al.* (1981), and Cairney & Harrington (1982), which are characterized by a continued strong decrease in P with increasing NaCl concentration to values as low as 260 Å at 2 M-NaCl. The most plausible explanation for the observations of those authors is the extreme sensitivity of their results to excluded volume effects, coupled with the absence of an accurate means to correct for such effects (Manning, 1981; Schurr & Allison, 1981). It should be noted that excluded volume corrections are not required for the ring-closure approach, due to the small sizes of the molecules studied.

At this point, several issues pertaining to the presence of 1 mM-$MgCl_2$ in the ligation buffers (required for ligase activity) need to be addressed. First, we (Hagerman, 1981) and others (Elias & Eden, 1981) have demonstrated that small amounts of $MgCl_2$ (50 to 100 μM) are sufficient to reduce P to a value of 450 to 500 Å, and that further increases in $MgCl_2$ concentration have no significant effect on P. Second, our intent has been to demonstrate that P is no longer sensitive to counterion concentration at moderate salt concentrations (10 mM to 200 mM-NaCl), a result that is evident upon inspection of Figure 7. One might conceivably argue that the presence of magnesium counterions in the vicinity of the DNA helix somehow blocks the effects of sodium counterions on P, thus preventing further reductions of P with increasing NaCl concentration, a suggestion we regard as being highly unlikely (see below, this section).

Finally, the results presented in Figure 7 actually demonstrate a slight but significant increase in P upon going from 0 mM to 162 mM-added NaCl. A possible explanation of this observation is suggested by the work of Lohman *et al.* (1980), who investigated the salt-dependence of pentalysine–DNA interactions. Those authors observed that pentalysine–DNA binding constants became much less sensitive to Mg^{2+} concentration with increasing Na^+ concentrations, and were able to obtain an apparent equilibrium binding constant for Mg^{2+}: $\log K_{obs}^{Mg} = -1{\cdot}7 \log[NaCl] + 0{\cdot}3$ (eqn (17) of Lohman *et al.*, 1980). Using their expression (eqn (6) of Lohman *et al.*, 1980),

$$D/D_0 = (1/2)\{1 + (1 + 4K_{obs}^{Mg}[Mg^{2+}])^{1/2}\},$$

for the probability of finding a DNA molecule that has only sodium ions in the thermodynamically bound counterion layer, we find that at 162 mM-NaCl (1·0 mM-$MgCl_2$), $D/D_0 = 0{\cdot}959$. In other words, at the highest NaCl concentration used in our study, essentially all of the DNA molecules would be surrounded exclusively by Na^+ in the bound counterion layer. This latter result suggests that, while Mg^{2+} is important for ligase activity, at the higher NaCl concentrations, the DNA is behaving as if it were in a pure Na^+ environment. The slight increase in P between 10 mM and 162 mM-NaCl might therefore reflect the transition from a local counterion layer of mixed valence to one comprising solely Na^+. This latter effect, while of interest, does not directly pertain to the major

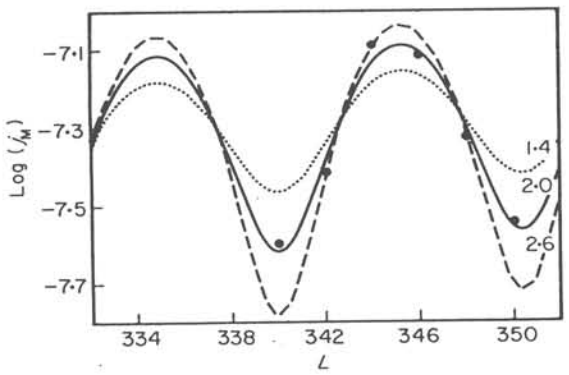

Figure 8. Plot of log j_M *versus* L for various values of C for same dataset as displayed in Fig. 6 (54 mM-NaCl). (1·4) $C = 1{\cdot}4 \times 10^{-19}$ erg cm; (2·0) continuous line representing the best fit to the experimental ring-closure data ($C = 2{\cdot}0 \times 10^{-19}$ erg cm); (2·6) $C = 2{\cdot}6 \times 10^{-19}$ erg cm. h_R = 10·46 bp/turn; P = 457 Å.

conclusions of this work, which are clear in providing no evidence for any significant reduction in P between 10 mM and 200 mM-NaCl.

(f) *The torsional stiffness of DNA is not significantly dependent on NaCl concentration for moderate salt concentrations*

Using the set of seven embedding sequence molecules, we have determined values for the torsional elastic constant C as a function of NaCl concentration over the range, 0 to 162 mM. The dependence of the log j_M *versus* L curve on C is illustrated in Figure 8 for a single NaCl concentration; the results of the entire analysis are presented in Table 2. The C values display no significant salt dependence over the range of NaCl concentrations employed in the current study, with an average value for $C = 2{\cdot}0\ (\pm 0{\cdot}2) \times 10^{-19}$ erg cm. This result is entirely consistent with the absence of a strong change in either the persistence length or the helical repeat (see below) over this same range of NaCl concentration. Furthermore, the absence of any dependence of C on NaCl concentration establishes the validity of a direct comparison between the results of Shore & Baldwin (1983*a*) and of Horowitz & Wang (1984) (see below), those two studies being performed in 50 mM-NaCl and 0 mM-NaCl, respectively.

The average value for C determined in the current work falls within the range of $1{\cdot}1 \times 10^{-19}$ to $4{\cdot}1 \times 10^{-19}$ erg cm estimated by Barkley & Zimm (1979) and is slightly higher than the value of approximately $1{\cdot}5 \times 10^{-19}$ erg cm determined from studies employing luminescence depolarization (Millar *et al.*, 1982; Thomas *et al.*, 1980) or electron paramagnetic resonance (Hurley *et al.*, 1982). However, there are difficulties in the interpretation of results from the spectroscopic approaches as a consequence of the necessary assumption that there exists no DNA-independent movement of the probe. Most of the early depolarization studies also suffered from the use of poorly characterized DNA.

Table 2

Relationship between NaCl concentration and the torsional elastic constant C

[NaCl] (mM)	C (10^{-19} erg cm)
0	2·3
2	2·2
6	1·8
18	1·8
54	2·0
162	2·1
	Average quantity = 2·0 ± 0·2

C values were determined directly from the analysis of Shimada & Yamakawa (1984) (see Hagerman & Ramadevi, 1990).

Shore & Baldwin (1983*b*) and Horowitz & Wang (1984) have obtained data for the distribution of topoisomers for small DNA circles. Analysis of these data by Shimada & Yamakawa (1985) yielded values for C of $2{\cdot}9 \times 10^{-19}$ erg cm, and $3{\cdot}1 \times 10^{-19}$ to $3{\cdot}2 \times 10^{-19}$ erg cm, respectively. Frank-Kamenetskii *et al.* (1985) and Levene & Crothers (1986*b*) have employed the topoisomer approach to determine C from topoisomer data for longer DNA molecules, with resultant values of $3{\cdot}0 \times 10^{-19}$ erg cm and $3{\cdot}4 \times 10^{-19}$ erg cm, respectively. However, the result of Levene & Crothers appears to be an overestimate, due to an incorrect extrapolation of their computed values for the variance of writhe to the long chain limit.

By fitting a harmonic twisting potential to a set of j_M values for molecules varying in length from 237 to 254 bp, Shore & Baldwin derived a value of $2{\cdot}4 \times 10^{-19}$ erg cm for C. Re-examination of those data by Shimada & Yamakawa (1984) yielded a range of values for C of $2{\cdot}4 \times 10^{-19}$ to $3{\cdot}0 \times 10^{-19}$ erg cm, depending on how the data points were fitted. Levene & Crothers (1986*b*) also analysed the data of Shore & Baldwin (1983*a*), again using a simple harmonic twisting potential, and arrived at a yet higher value for C ($3{\cdot}8 \times 10^{-19}$ erg cm). This latter value would appear to be erroneously high, however, due to the misplotting, by Levene & Crothers, of five of the 12 data points of Shore & Baldwin (Fig. 10 of Levene & Crothers, 1986*b*).

In aggregate, investigations of the torsional rigidity of DNA indicate that, at equilibrium, fluctuations in helical twist are well-represented by an elastic model with a torsional elastic constant $C \geq 2{\cdot}0 \times 10^{-19}$ to $3{\cdot}0 \times 10^{-19}$ erg cm, even for relatively small DNA molecules (200 to 250 bp). Our own studies (this work; Hagerman & Ramadevi, 1990) yield values for C consistent with the above range, and demonstrate that C is not significantly dependent on salt concentration over the range examined in this work. Furthermore, our studies suggest that, as a corollary to the elastic model, fluctuations in

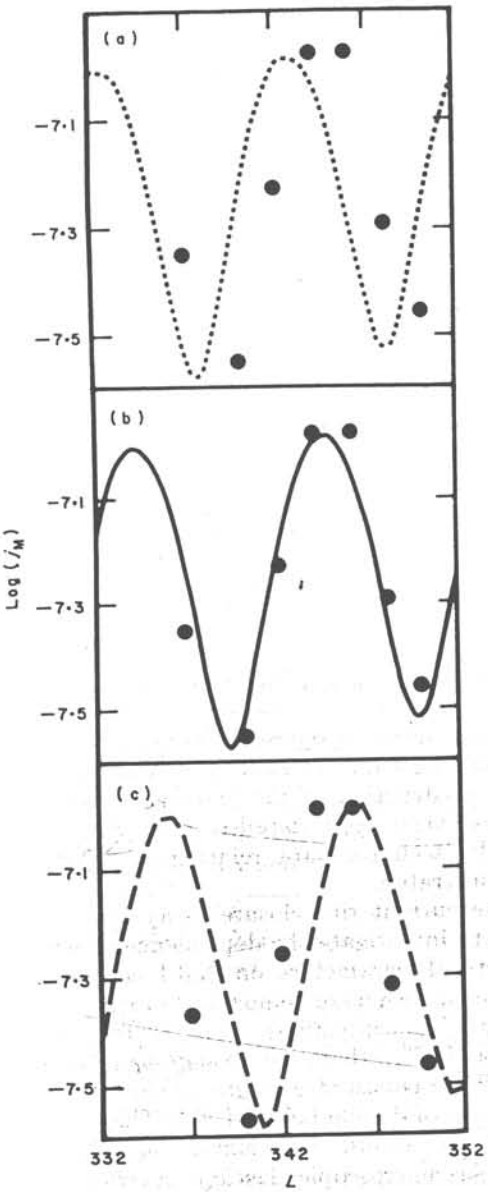

Figure 9. Comparison of computed log j_M *versus* L curves for various values of h_R with the experimental data (2 mM-NaCl). (a) 10·49 bp/turn, (b) 10·44 bp/turn, (c) 10·39 bp/turn. $P = 439$ Å; $C = 2{\cdot}2 \times 10^{-19}$ erg cm.

twist and writhe are, to a good approximation, separable, thus lending further support to the conclusions of Shore & Baldwin (1983*a*,*b*) and Horowitz & Wang (1984).

(g) *The helical repeat of DNA is essentially independent of NaCl concentration over the range, 0 to 162 mM*

From shifts of the positions of the local maxima of a log j_M *versus* L curve, one can determine changes in the helical repeat, h_R, of particular DNA sequences to a high degree of precision, as demonstrated in Figure 9. The results of the least-squares analysis of the experimental log j_M curves, determined for various NaCl concentrations, are given in Table 3. Our results indicate that, over the range, 0 to 162 mM-NaCl (1·0 mM-MaCl), there is no significant NaCl concentration-dependence of h_R, with a molecule-average value for h_R of 10·44 (±0·01) bp/turn for the embedding sequence molecules. Not surprisingly, we have found that the values for h_R determined from the log j_M curves are quite insensitive to the values determined for either P or C.

Table 3
Relationship between NaCl concentration and the helical repeat h_R

[NaCl] (mM)	h_R (bp/turn)
0	10·43
2	10·44
6	10·44
18	10·44
54	10·46
162	10·45
	Average quantity = 10·44 ± 0·01

It should be noted that the position (L) of a single local maximum of log j_M is formally consistent with a set of helical repeats, such that $h_R(m) = L/m$, where m is the number of helical turns. For example, for our value of $h_R = 10{\cdot}44$ bp/turn, the maximum in the log j_M curve (Fig. 9(b)) of 344·5 bp corresponds to 33 turns of helix. However, were the maximum to reflect 32 or 34 turns, the corresponding helical repeats would be 10·77 or 10·13 bp/turn, respectively. In fact, additional information can be obtained by fitting the entire log j_M *versus* L curve, and the global least-squares value of 10·44 bp/turn has been determined in this fashion. One potential caveat with this latter approach for the determination of absolute values for h_R, is that the local peak to peak separation reflects the helical repeat of the sequences used for phasing. However, our results are in excellent agreement with those of Shore & Baldwin (1983*a*,*b*: $h_R = 10{\cdot}45$ bp/turn), and of Wang (Wang, 1979; Peck & Wang, 1981; Horowitz & Wang, 1984: $h_R = 10{\cdot}54$ bp/turn); namely, that 10·77 bp/turn and 10·13 bp/turn can be rejected as alternatives to 10·44 bp/turn for the helical repeat of our molecules. Furthermore, having established $m = 33$, we estimate that alterations in h_R of fewer than 0·05 bp/turn could be detectable easily over the range of NaCl concentrations examined in the current study (Figs 9 and 10). The close agreement between the current results (10·44 bp/turn; 1 mM-$MaCl_2$) and those of Shore & Baldwin (1983*a*,*b*: 10·45 bp/turn; 10 mM-$MgCl_2$) further argues against a strong influence on helix structure of magnesium concentrations above 1 mM.

The influence of NaCl concentration on helical twist has been studied by Hinton & Bode (1975) and by Anderson & Bauer (1978). Both investigations arrived at the same qualitative conclusion; namely, that increases in NaCl concentration are

accompanied by increases in helical twist (i.e. reduced h_R). Hinton & Bode (1975) studied the influence of counterion type and concentration on the superhelix density of circular bacteriophage lambda DNA. Those authors observed that the positions of $s_{20,w}$ minima (using ethidium bromide titration) of supercoiled bacteriophage lambda varied as a function of NaCl concentration; their data at 100 mM, 400 mM and 1 M-NaCl yield a value for the salt-dependence of h_R, $dh_R/d\log$ [NaCl] $= -0{\cdot}23$ bp/turn, following correction for an ethidium unwinding angle of 26° (Pulleyblank *et al.*, 1975; Wang, 1974; Liu & Wang, 1975). As can be seen by inspection of Figure 10, the apparent salt-dependence of h_R determined by Hinton & Bode (1975) represents a substantial overestimation, perhaps due to the influence of the salt-dependence of the binding of ethidium on the sedimentation results. Anderson & Bauer (1978) studied the influence of counterion type and concentration, using the band-shift method of Wang (1979). Their data suggest that, over the range 50 mM to 300 mM-NaCl, there is a constant increase in the helix twist of approximately $+0{\cdot}16°$/bp per log [NaCl]. For $h_R \approx 10{\cdot}44$ bp/turn, their observed change in twist would correspond to $dh_R/d\log$ [NaCl] $= -0{\cdot}05$ bp/turn. Our own results (Fig. 10) suggest that any change in the helical repeat occurring in the region below 200 mM-NaCl is significantly less than that observed by Anderson & Bauer (1978). In comparing our own results with those of Hinton & Bode (1975) and with Anderson & Bauer (1978), it should be noted that the former two studies were performed at somewhat higher NaCl concentrations than those used in the current investigation, although the concentration ranges do overlap.

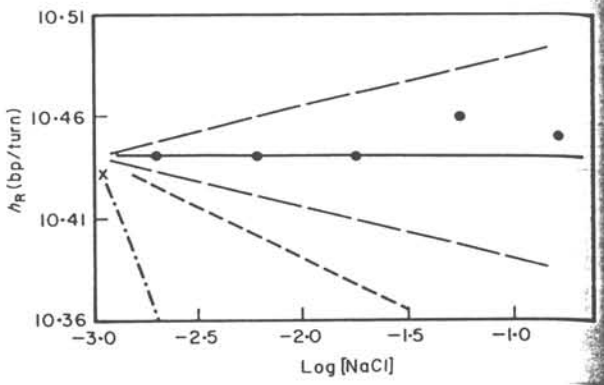

Figure 10. Plot of the helical repeat, h_R, as a function of log [NaCl]. (———) Horizontal line at the average value for our experimental data (●); (×) 0 mM-NaCl); (— — —) $\pm 0{\cdot}025$ bp/turn per log [NaCl]; (– – – – –) $-0{\cdot}05$ bp/turn per log [NaCl] extrapolated from data of Anderson & Bauer (1979) to 1 mM-NaCl; (— — —) $-0{\cdot}23$ bp/turn per log [NaCl] from data of Hinton & Bode (1975).

5. Conclusion

The purpose of this work has been to develop a convenient approach for the application of ring-closure measurements to the study of DNA structure. Towards this goal, we have developed a method that permits the simultaneous assessment of cyclization (k_C) and bimolecular joining (k_D) rates in a single reaction vessel. In contrast, previous methodologies required the independent determination of k_C and k_D using different molecules and separate experiments, thus requiring identical buffer conditions as well as precise knowledge of relative ligase activities. These difficulties are entirely avoided in the current approach, since both circle and dimer formation take place concurrently in the same reaction vessel. The current ring-closure method has been directly compared (using a 366 bp fragment) to the method employed by Shore *et al.* (1981), and has been shown to give essentially identical results. Our experimental results are independent of ligase concentration over a 100-fold range, and are independent of reactant DNA concentration.

We have also presented a complete assay system for the practical application of ring-closure measurements to structural studies, one that involves the use of a series of reference molecules known as the embedding sequence molecules. The concept, design and construction of the embedding sequence molecules have been detailed, and their ability to supply accurate data relating to DNA structure demonstrated.

The current ring-closure assay system has been used to investigate the dependence of several DNA structural parameters on NaCl concentration. In particular, we have demonstrated that over a range of salt concentrations from 0 to 162 mM-NaCl (1 mM-$MgCl_2$), there are no significant reductions in either the persistence length or the torsional elastic constant of double-helical DNA. These observations imply, in accord with simple theoretical models, that the macroscopic elastic properties of DNA are no longer sensitive to bulk, monovalent counterion concentration above 10 to 20 mM. Moreover, there is no significant change, over the same range of NaCl concentration, in the average helical repeat, h_R, of the DNA molecules used in this study, thus suggesting that previous studies have overestimated the effect of salt on helical twist.

Finally, we are continuing to develop the ligase-catalyzed cyclization method with a view toward its use in measurements of both sequence-directed and protein-induced curvature of DNA. The embedding sequence molecules have been designed with central restriction sites for the insertion of additional specific sequences. For example, in studies of sequence-directed DNA curvature (Wu & Crothers, 1984; Hagerman, 1985; Koo *et al.*, 1986), sequences of interest (10 to 50 bp) would be inserted into the center of the embedding-sequence molecules, and the effects of these insertions on j_M values determined. A similar approach could be applied to the examination of bending induced by sequence-specific, DNA-binding proteins.

Appendix

Conversion of Relative Band Intensities for Monomer Circles and Linear Dimers to their Corresponding Molar Concentrations

For g equal to the fraction of DNA molecules that have been subjected to labeling, and f equal to the efficiency of labeling within that fraction, the probabilities, P_i, of having i labeled ends are given by:

$$P_{M,0}=(1-g)+g(1-f)^2$$
$$P_{M,1}=2gf(1-f)$$
$$P_{M,2}=gf^2$$

for the monomer (M), and:

$$P_{D,0}=P_{M,0}^2$$
$$P_{D,1}=2P_{M,0}P_{M,1}$$
$$P_{D,2}=2P_{M,0}P_{M,2}+P_{M,1}^2$$
$$P_{D,3}=2P_{M,1}P_{M,2}$$
$$P_{D,4}=P_{M,2}^2$$

for the dimer (D).

The ratio, $R_{c/d}$, of circular monomer (c) to linear dimer (d) band intensities is related to their respective molar concentrations (C, D) by the following relation:

$$R_{c/d}=[\langle n_M\rangle/\langle n_D\rangle]\,(C/D),$$

where $\langle n_M\rangle$ is the average number of labeled ends per monomer, $\langle n_D\rangle$ is the corresponding quantity for dimer molecules, and where:

$$\langle n_M\rangle/\langle n_D\rangle=\sum_{i=0}^{2} iP_{m,i}/\sum_{j=0}^{4} jP_{D,j}.$$

By simple algebra, one can show that the right-hand side of the above expression is equal to 1/2, independent of f and g. Thus:

$$C/D=2R_{c/d}.$$

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