Supercoiling in Closed Circular DNA: Dependence upon Ion Type and Concentration†

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ABSTRACT: The winding of duplex DNA in solution has been systematically compared in the presence of the univalent counterions Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+ over the concentration range 0.05–0.30 M. The method employed was to relax closed circular viral PM-2 DNA with an extract of nicking-closing enzyme prepared from HeLa cells, thereby obtaining closed, nonsupercoiled DNA. The number of superhelical turns generated upon transfer of the product DNA from incubation medium containing ion X^+ to a standard test medium, following inactivation of the enzyme, is a measure of the relative effect of each ion upon the duplex winding of the DNA. This method relies upon the invariance of the topological winding (linking number) in closed DNA in the absence of a strand scission. Two test media were employed to determine the number of superhelical turns generated by transfer: band counting in agarose gel electrophoresis and buoyant banding in CsCl/propidium diiodide. We find that the duplex rotation angle, ψ, varies with the counterion and increases in the order $Na^+ < K^+ < Li^+ < Rb^+ < Cs^+ < NH_4^+$, independent of the test medium. As the salt concentration increases, in each case, ψ correspondingly increases. At a concentration of 0.2 M under relaxation conditions, for example, the NH_4^+ salt of PM-2 DNA contains an excess of 12 duplex turns compared with the Na^+ salt. The Mg^{2+} salt of PM-2 DNA was similarly investigated over the ionic strength region 0.06 M to 0.15 M, with effects comparable to the Cs^+ salt. No effect was observed upon anion substitution at any concentration examined. The anions investigated include ClO_4^-, SCN^-, Cl_3CCOO^-, I^-, Cl^-, Br^-, HCO_3^-, $CH_3CO_2^-$, NO_3^-, SO_4^{2-}, citrate$^-$, and HPO_4^{2-}.

Closed duplex DNA is characterized by the conservation condition that the net interstrand winding (or linking) is unaltered by all structural changes except for backbone chain scissions. The simplest algorithm for counting α, the linking number, is to constrain the duplex axis to a plane; α, an integer, is then the number of complete revolutions made by either strand (Vinograd and Lebowitz, 1966; Vinograd et al., 1968; Pohl, 1968). Other, more complex algorithms may be used (Pohl, 1968; Fuller, 1971; Crick, 1976). A *relaxed* closed DNA is defined by $\alpha = \beta_0$, where β_0 is the number of duplex turns in the environment chosen and is unaffected by a chain scission. In the simplest case $\beta_0 = N/10$, where N is the number of base pairs. Most naturally occurring closed DNAs (DNA I[1]) are experimentally found to have $\alpha < \beta_0$. Exceptions are the replicative intermediates of plasmid (Crosa et al., 1976; Timmis et al., 1976), certain bacteriophage (Horiuchi and Zinder, 1976), and mitochondrial (Berk and Clayton, 1974, 1976) DNAs, for which $\alpha = \beta_0$.

The linking number may be altered only if one of the duplex strands is broken and rejoined following net interstrand rotation. Three enzymatic procedures are now available to accomplish this alteration: endonuclease scission followed by DNA ligase (Wang, 1969); action of an ω (Wang, 1971; Burrington and Morgan, 1976) or N/C (Champoux and Dulbecco, 1972; Keller, 1975; Vosberg et al., 1975; Bauer et al., 1977) enzyme; and incubation with DNA gyrase and ATP (Gellert et al., 1976a,b; Marians et al., 1977). The primary result of the first two reactions is an increase in α (thereby releasing energy), while the third decreases α (at the expense of the hydrolysis of ATP). In the present investigation we have employed the N/C enzyme isolated from HeLa cells to increase α to the value of β_0 which is determined by the environmental conditions of interest.

Various reports have appeared in the literature which indicate that the DNA duplex winding depends upon counterion type and concentration (Wang, 1969; Upholt et al., 1971; Hinton and Bode, 1975; Shure and Vinograd, 1976). The magnitude of this effect has not been systematically explored, particularly in the physiologically important salt concentration region. We present here the results of such an investigation, encompassing all the commonly occurring 1:1 electrolytes and Mg^{2+}, over the ionic strength range 0.05–0.30 M. We find that relatively large changes in β_0 can be produced by ionic substitution. In the case of PM-2 DNA, the substitution of NH_4^+ for Na^+ increases the superhelix density of DNA I by 10%. Alternatively, the proper choice of ionic conditions during the N/C closure may be used to generate closed DNAs containing a relatively large number of positive superhelical turns. For example, the relaxation of PM-2 DNA in 0.2 M NH_4Cl followed by transfer to 0.05 M NaCl results in the introduction of 14 positive turns at 20 °C. If the relaxation is at 4°, the same DNA will contain 20 positive turns at 25 °C. These molecules will be useful for the investigation of the properties of positively supercoiled DNA (Anderson and Bauer, in preparation).

We also propose that 0.2 M NaCl, 37 °C, be adopted as the standard state for reporting linking numbers and superhelix densities. Numerical values are tabulated which may be used to correct to this state from a variety of other salt and temperature conditions.

Materials and Methods

Incubation Solutions and Conditions. Incubation buffer containing 5 mM Tris–acetate, pH 8.3, was added to an appropriate amount of each salt to obtain 0.5 M stock solutions.

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[1] Abbreviations used are: DNA I, covalently closed circular duplex DNA; DNA II, nicked circular duplex DNA; N/C enzyme, nicking/closing enzyme; EDTA, disodium salt of ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminoethane; EtdBr, ethidium bromide; $PrdI_2$, propidium diiodide; $NaDodSO_4$, sodium dodecyl sulfate; NT, an equimolar mixture of Na^+ and $Tris^+$.

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Reagent grade potassium acetate was purchased from Mallinckrodt and optical grade CsCl from Harshaw. Cl_3CCOOK was prepared and purified as was described previously for $Cl_3CCOORb$ (Burke and Bauer, 1977). All other salts were purchased from Fisher as reagent grade. The pH of each solution was 8.3 with the exception of NH_4Cl, which was 7.6. Native, covalently closed circular PM-2 DNA was prepared by the procedure of Espejo and Canelo (1968a,b). Nicking-closing enzyme was isolated from HeLa cell nuclei by the procedure of Vosberg et al. (1975) and was purified to fraction I before use.

PM-2 DNA I was incubated at 20 °C in 40-μL reaction mixtures containing 1 μg of PM-2 DNA, approximately 50 units of N/C enzyme, and an aliquot of the appropriate stock salt solution. Following Vosberg et al. (1975) and Bauer et al. (1977), we define the unit of activity as that amount of enzyme required for the relaxation of 50% of 1 μg of PM-2 DNA in 0.5 h at 37 °C. After incubation for 12 h to assure complete relaxation, $NaDodSO_4$ was added to 0.1% and the solution was immediately frozen in dry ice/acetone. The solutions were then adjusted to 10% (w/v) sucrose, 0.008% (w/v) bromophenol blue, and analyzed by gel electrophoresis.

Gel Electrophoresis. The electrophoretic analysis was performed in 1% agarose slab gels (10 × 14 × 0.35 cm) in an electrophoresis buffer containing 40 mM Tris-acetate, 20 mM sodium acetate, 4 mM EDTA, pH 8.3. Current was applied at 20 V for 18 h, with recirculation of buffer between reservoirs. Following electrophoresis, gels were stained for 1 h in E buffer containing EtdBr (Calbiochem) at a concentration of 0.5 μg/mL. Gels were then illuminated from below with a short-wave UV lamp obtained from Ultraviolet Products, San Gabriel, Calif., and photographs taken with Kodak Royal Pan film. The negatives were scanned with a Joyce-Lobel recording microdensitometer.

Preparative Ultracentrifugation. Solutions of 3 mL total volume were prepared containing 0.1 μg of PM-2 DNA I, 0.1 μg of PM-2 DNA II, 0.1 μg of PM-2 DNA relaxed in various salts as described above, 1.565 g/mL CsCl, and 330 μg/mL $PrdI_2$ (Calbiochem). These solutions were then centrifuged at 44 krpm, 20 °C, for 42 h in a Beckman SW 50.1 rotor. The resulting solutions were illuminated with a long wavelength UV light source and photographed. Distances between bands were measured from the resulting negatives with a Nikon microcomparator. The analysis of the bands to obtain the superhelix densities of the relaxed species was performed as described by Burke and Bauer (1977).

Results

The linking number may be written as the sum $\alpha = \beta_0 + \tau_0$, where the parameter τ_0 is interpreted to be the number of superhelical turns. On an intensive basis the superhelix density, σ_0, is defined at $10\tau_0/N$. We recognize that the above formulation represents a special case since both β and τ depend upon the DNA tertiary structure in a complex way (Bauer and Vinograd, 1968; White, 1969; Pohl, 1968; Fuller, 1971; Crick, 1976). In the absence of knowledge of the disposition of the DNA duplex in solution, it is not possible to calculate the corresponding more general structural parameters, the writhing number and the twist (Crick, 1976). We employ the symbol τ_0 to specify the number of titratable superhelical turns (Bauer and Vinograd, 1968); i.e., the number obtained by any method which measures the degree of unwinding required to reduce β_0 to α without a chain scission. Such methods include the alkaline buoyant titration (Vinograd et al., 1968; Wang, 1974), various dye-based techniques (for a review, see Bauer and Vinograd, 1971), and band counting in electrophoresis (Keller and Wendel, 1974; Shure and Vinograd, 1976).

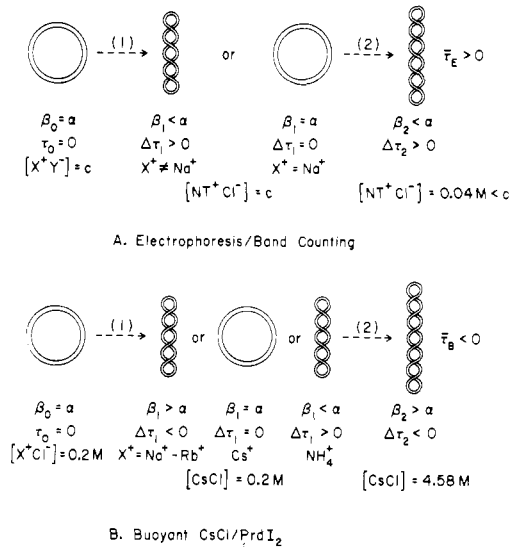

FIGURE 1: Diagrammatic representation of the methods used to determine the effects of ion type and concentration upon the duplex winding number. (A) Band counting following transfer to E buffer. The transfer is conceived as occurring in two steps, indicated by the dashed arrows: (1) change of ion at constant concentration, c; and (2) reduction in concentration to that of E buffer. (B) Equilibrium sedimentation in buoyant $CsCl/PrdI_2$. The two conceptual processes 1 and 2 are as in A, except that the transfer is to Cs^+Cl^- instead of to NT^+Cl^-.

The two general procedures used to probe the variation of τ_0 with counterion type and concentration are illustrated in Figure 1. In both cases the closed DNA is relaxed and the N/C enzyme subsequently inactivated. In method A the number of bands generated upon transfer from incubation buffer to electrophoresis buffer, $\bar{\tau}_E$, is measured. This process may be conceived as occurring in two steps, as indicated by the dashed arrows: (1) replacement of the incubation salt by the electrophoresis buffer at constant concentration; and (2) adjustment of the salt concentration to 0.04 M. In method B the superhelix density is measured in buoyant $CsCl/PrdI_2$. In these experiments, which were conducted at an incubation salt concentration of 0.2 M only, the corresponding conceptual steps are (1) transfer from XCl to CsCl at 0.2 M; and (2) increase in the CsCl concentration to the buoyant 4.58 M.

Variation of Duplex Winding with Ionic Environment. The relaxation of closed circular PM-2 DNA was carried out in a number of different 1:1 salt solutions, each ranging in concentration from 0.05 M to 0.30 M. At any given temperature, DNA relaxed by treatment with N/C enzyme forms a Gaussian distribution of species which differ in the number of superhelical turns, centered about the mean value $\bar{\tau}_0 = 0$ (Pulleyblank et al., 1975). The distribution so obtained is identical with that formed by treatment of lightly nicked circular duplex DNA with DNA ligase (Depew and Wang, 1975; Pulleyblank et al., 1975). The distribution formed following N/C treatment in each solvent was then transferred to standard E buffer, Figure 1A, the composition of which was described above. This change in counterion, combined with the concomitant reduction in ionic strength (to approximately 0.04 M), introduces positive superhelical turns into each species of DNA. The entire Gaussian distribution then increases in mobility to an extent proportional to the number of superhelical turns so introduced.

Figure 2 illustrates the effects of such a transfer for increasing incubation salt concentrations of CsCl (A) and

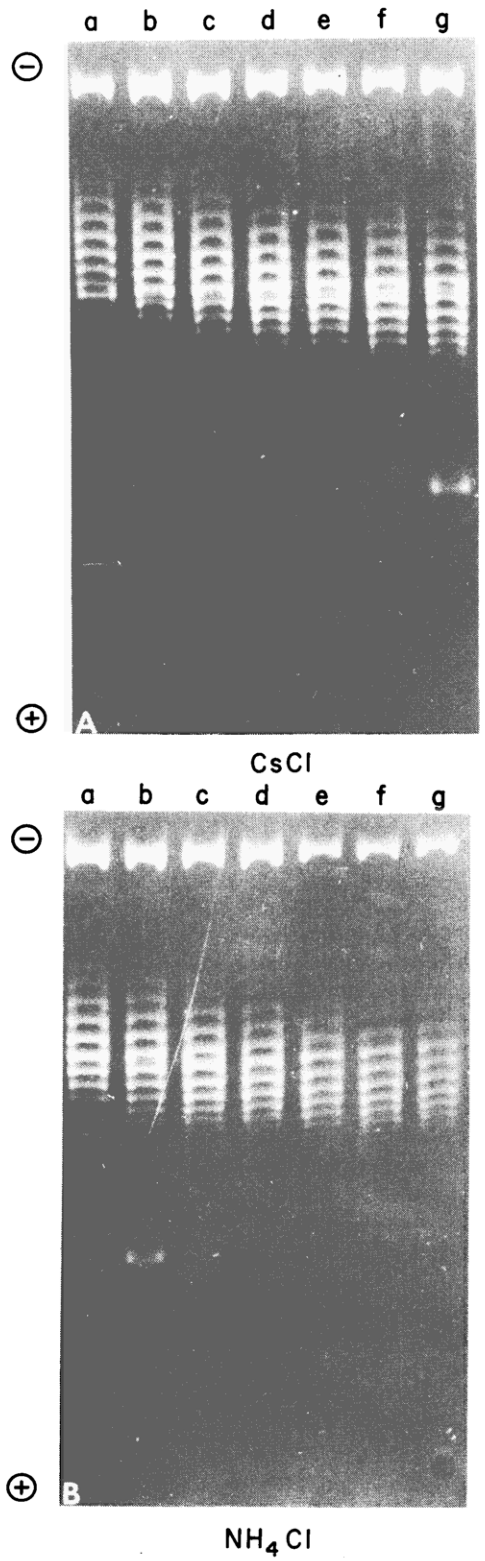

FIGURE 2: Electrophoresis in 1% agarose gels following 12-h incubations with N/C enzyme at 20 °C. Electrophoresis was carried out at 20 °C. (A) CsCl and (B) NH_4Cl at concentrations of: (a) 0.04 M; (b) 0.08 M; (c) 0.12 M; (d) 0.16 M; (e) 0.20 M; (f) 0.25 M; (g) 0.30 M.

NH_4Cl (B). In each case positive superhelical turns are introduced as a consequence of the transfer from incubation to electrophoresis conditions, the magnitude of the effect being somewhat greater for NH_4Cl than for CsCl at comparable salt concentrations. Since the activity coefficients of all 1:1 electrolytes used in this study differ but little over the concentration range of interest (Robinson and Stokes, 1959), no attempt was

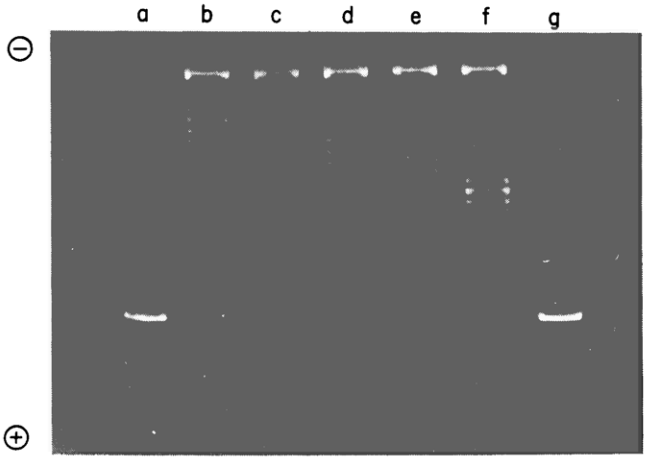

FIGURE 3: Electrophoresis in a 1% agarose gel following 12-h incubations with N/C enzyme at 20 °C. Electrophoresis was carried out at 16 °C. Channels a and g contain untreated PM-2 DNA I. The remaining channels contain PM-2 DNA relaxed in 0.16 M concentrations of: (b) KCl; (c) LiCl; (d) RbCl; (e) CsCl; (f) NH_4Cl.

made to correct for nonideality. The mobility of each Gaussian band set increases with incubation salt concentration in both cases, the effect being more pronounced at low ionic strengths.

In these experiments the DNA is initially neutralized by the cation present in the incubation medium, X^+. In the very early stages of the electrophoresis the net mobility of the DNA will therefore be determined by the equivalent conductivities of both X^+ and of the polyanionic DNA. The limiting equivalent conductivity of Cs^+ is 67 $cm^2\ \Omega^{-1}\ equiv^{-1}$ and that of NH_4^+ is 64 $cm^2\ \Omega^{-1}\ equiv^{-1}$, both at 18 °C (Robinson and Stokes, 1959). These two ions should therefore similarly affect the net mobility of the DNA in the initial stages of the experiment. The DNA very soon moves into the $Na^+/Tris^+$ electrophoresis buffer and henceforth migrates with a mobility corresponding to a species partially neutralized by $Tris^+$ and partly by Na^+. The equivalent conductivity of $Tris^+$ is unavailable, but that of Na^+ is 43 $cm^2\ \Omega^{-1}\ equiv^{-1}$ at 18 °C. The DNA should therefore increase in mobility following the transfer from either Cs^+ or NH_4^+ to Na^+. An increase in the incubation salt concentration for either Cs^+ or Na^+ might therefore decrease the apparent mobility of the closed DNA bands, but Figure 2 demonstrates that the mobility actually increases with increasing incubation ionic strength; hence the counterion exchange process itself can only act to diminish the magnitude of the differential effects reported here. The DNA mobility showed no dependence upon the ionic strength of the applied solution over the range 0.05–0.30 M.

Figure 3 presents the results of a series of incubations with N/C enzyme, all at 0.2 M salt and 20 °C, for the salts KCl, LiCl, RbCl, CsCl, and NH_4Cl. The corresponding large differences in electrophoretic mobility following transfer to E buffer at 16 °C demonstrate clearly that the extent of winding of the DNA duplex depends upon the choice of counterion at constant ionic strength. In this particular series the reduction in duplex winding number relative to Na^+, upon transfer to E buffer, is greatest for NH_4^+ and least for K^+.

Microdensitometer scans of DNA relaxed in 0.16 M CsCl and in 0.16 M NH_4Cl (cf. Figure 3) are shown in Figures 4A and 4B, respectively. Here the difference in mobility can be readily visualized. The most intense peak in each family is denoted τ_m, and all other peaks are counted as $\tau_m + i$, where i takes on the values +1, +2, +3 . . . for peaks to the right of the central maximum and −1, −2, −3 . . . for peaks to the left

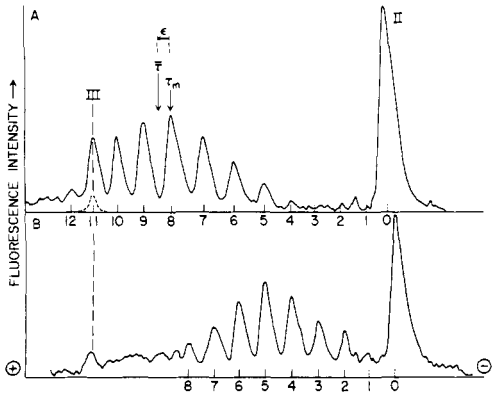

FIGURE 4: Microdensitometer tracings of the electrophoresis gel shown in Figure 3. (A) CsCl, channel e of Figure 3; (B) KCl, channel b of Figure 3. II indicates the position of nicked circular PM-2 DNA, and III indicates the position of contaminating linear PM-2 DNA. The dashed peak at the III position in A represents an estimate of the amount of this material, by comparison with B.

of the central maximum. Since the distribution is Gaussian (Depew and Wang, 1975; Pulleyblank et al., 1975) the relative concentrations of species containing τ_m and $\tau_m + i$ turns may be calculated from (Depew and Wang, 1975)

$$\frac{-RT}{i} \ln \frac{[\tau_m + i]}{[\tau_m]} = A(i + 2\epsilon) \qquad (1)$$

where A is a constant which depends upon environmental conditions and ϵ is the deviation of τ_m from the actual center of the Gaussian; that is, $\bar{\tau} = \tau_m - \epsilon$. Least-squares linear fits of the left side of eq 1 vs. i were used in each case to estimate values of ϵ, given by the ratio of the intercept to the slope. These values were used to obtain the fractional part of $\bar{\tau}$ plotted in Figure 5 and listed in Tables I (as superhelix densities) and II, below.

Each homogeneous band within a Gaussian distribution is assumed to arise from DNA containing a characteristic number of superhelical turns, and adjoining bands are assumed to differ in winding by a single turn (Depew and Wang, 1975). In order to determine the absolute value of τ_m in each case, the distance migrated, measured from DNA II, was plotted as a function of assumed winding number with the requirement that the line so obtained pass linearly through the origin. It was observed that the apparent separation between adjoining bands depends somewhat upon the extent of overlap with these neighboring species, thus introducing some curvature into the plots. This effect was relatively small, however, and in no case led to ambiguity in the selection of τ_m. This procedure is illustrated in Figure 4, in which the locations of the band centers are indicated on the abscissa for each family of curves. The dotted bars represent the assumed locations of species present in amounts below the limit of detectability and were obtained by linear interpolation. The nicked DNA comigrates with relaxed closed DNA under the electrophoresis conditions. A similar approach employing band counting following the closure of nicked circular DNA with polynucleotide ligase has been recently used to estimate the extent of unwinding of DNA by RNA polymerase (Wang et al., 1977).

Variation of Duplex Winding with Ionic Strength. The product of the N/C enzyme reaction is, in each incubation solvent, a Gaussian distribution of closed species centered around $\tau_0 = 0$. The number of superhelical turns generated upon shift to E buffer, $\bar{\tau}_E$, is plotted as a function of ionic strength in Figure 5A for the above series of Cl^- salts. All values of $\bar{\tau}_E$ become more positive with increasing incubation

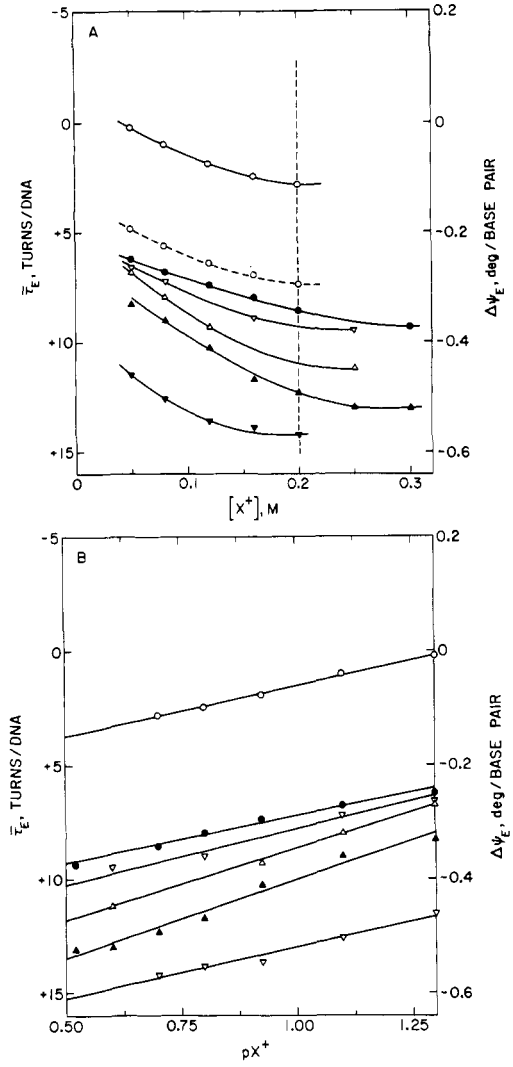

FIGURE 5: The relative change in the number of superhelical turns induced in transfer to electrophoresis buffer (20 °C) vs. the concentration in the relaxation mixture (20 °C) of: (○) NaCl; (●) KCl; (▽) LiCl; (△) RbCl; (▲) CsCl; (▼) NH_4Cl. The right ordinate gives the associated alteration in the duplex winding angle, $\Delta\psi_E$, in degrees per base pair (see eq 2). The vertical dashed line indicates the 0.20 M concentration level at which comparisons with the buoyant density estimates were made (Figure 6). The transfer of DNA relaxed in NaCl introduced very few superhelical turns under these conditions and the resulting Gaussian distributions were very poorly resolved from DNA II. The concentration series for NaCl was therefore obtained by incubation at 4 °C followed by electrophoresis at 20 °C to improve resolution of the bands (- - ○ - -). The location of the NaCl line at 20 °C was obtained by subtracting 4.8 turns from each datum obtained at 4 °C (see text).

cation concentration, especially in the region below 0.2 M. In the case of Na^+, the transfer from incubation buffer at 20 °C to E buffer at 20 °C produces a shift which is too small to measure directly in the presence of nicked DNA. The ionic strength series for NaCl was therefore obtained by relaxing the DNA at 4 °C and performing the electrophoresis at 20 °C, with the results shown in the horizontal dashed line of Figure 5A. At an incubation NaCl concentration of 0.05 M, nearly identical with E buffer, the transfer from 4 to 20 °C results in the generation of +4.8 superhelical turns. This value is in agreement with the previously determined temperature dependence of the duplex winding angle, approximately 1.1×10^{-2} deg per °C per bp (Wang, 1969; Depew and Wang, 1975), based upon titration with EtdBr and assuming a dye unwinding angle of 26°. Parallel experiments with $Tris^+$, also performed at 4 °C, indicated that the substitution of this ionic

TABLE I: Effects of Ion Type and Concentration upon Superhelix Density and Average Duplex Rotation Angle at 20 °C.[a]

Counterion, X^+	$\bar{\sigma}_E$, 0.2 M[b] (× 100)	$d\sigma_E/dpX^+$ (× 100)	$\Delta\psi$[c] (deg/bp)	$d\psi/dpX^+$ (deg/bp)	ΔT_{equiv}[d] (°C)
Na^+	0.284	−0.451		−0.162	
K^+	0.882	−0.420	0.22	−0.151	20
Li^+	0.944	−0.450	0.24	−0.162	22
Rb^+	1.117	−0.644	0.30	−0.232	27
Cs^+	1.249	−0.696	0.35	−0.251	32
NH_4^+	1.442	−0.467	0.42	−0.168	38

[a] Slopes and intercepts obtained from Figure 5B, as described in Figure 1A. [b] Values of $\bar{\sigma}$ are those generated upon transfer of the DNA from incubation buffer to E buffer at 20 °C. These values were calculated from the relation $\bar{\sigma}_E = 10\bar{\tau}_E/N$, with N = 9850 bp for PM-2 DNA. [c] This quantity represents the change in duplex rotation angle from NaDNA at 20 °C, 0.2 M salt, and was obtained by applying eq 2 to each salt in turn. Alternatively, this value may be read from Figure 5A by subtracting the ordinate $\Delta\psi_E$ for ion X^+ from that for Na^+ along the vertical dashed line. [d] The temperature decrement required to induce the same duplex rotation angle changes at constant salt as those produced isothermally and listed under the column headed $\Delta\psi$ (see text).

species for Na^+ overwinds the duplex by approximately 0.5 superhelical turn. We therefore regard the effects of $Tris^+$ and Na^+ as indistinguishable. The plot for $\bar{\tau}_E$ in NaCl at 20 °C shown in Figure 5A was derived by subtracting 4.8 turns from each determination at 4 °C. The right-hand abscissa in Figure 5A shows the reduction in the average duplex rotation angle upon transfer from incubation medium to E buffer

$$\Delta\psi_E = \psi(\text{E buffer}) - \psi(X^+DNA^-, \text{incubation}) \quad (2a)$$

The two ordinate scales are related by

$$\Delta\psi_E = -(\bar{\tau}_E/N)360 \quad (2b)$$

where N = 9850 base pairs in PM-2 DNA (Wang, 1974).

Figure 5B presents a plot of the same shifts in $\bar{\tau}_E$ and in $\Delta\psi_E$ upon transfer to E buffer as a function of incubation pX^+ for each species. Each curve is linear over this salt concentration range and the slopes and the intercepts (in terms of $\bar{\sigma}_E$) at 0.2 M ($pX^+ = 0.70$) are tabulated in Table I. These results demonstrate that a relatively large alteration in supercoiling can be induced by counterion substitution. Here the difference in $\bar{\tau}_E$ between NH_4^+ and Na^+ is +11.4 superhelical turns in this DNA containing 985 duplex turns (the NH_4 DNA being more highly supercoiled in E buffer). The corresponding difference in superhelix density following transfer to E buffer is +0.012, and in the duplex rotation angle, $\Delta\psi_E$, is −0.42 deg/bp. The slopes of these curves fall into two families, the ions Na^+, K^+, Li^+, and NH_4^+ showing significantly less concentration dependence than Cs^+ and Rb^+. This phenomenon is discussed in greater detail below. Table I also lists $\Delta\psi$, the change in the absolute duplex rotation angle for each salt relative to NaDNA at 0.2 M, 20 °C, in the incubation medium. It should be emphasized that $\bar{\tau}_0 = 0$ in this medium. The quantity ψ is related to the duplex winding number by $\beta_0 = N\psi/360$. The ionic strength dependence of ψ for each salt, expressed as $d\psi/dpX^+$, is also tabulated. The last column lists the temperature decrement which would be required to bring about a duplex overwinding of $\Delta\psi$ at constant ionic strength.

Evidence That Ionic Effects Are DNA Mediated. It is possible that a salt-mediated partial inhibition of the N/C enzyme leads to incomplete relaxation in the presence of some counterions. This would, in turn, produce the observed apparent shifts in duplex winding. To examine this possibility, PM-2 DNA was relaxed in 50 mM LiCl, KCl, and NH_4Cl and subsequently examined in an electrophoresis experiment in which the usual E buffer was replaced by 50 mM KCl, 5 mM Tris-Cl (pH 8.3). The results unambiguously demonstrate that the DNA relaxed in KCl is devoid of superhelical turns following transfer, the DNA relaxed in LiCl contains perhaps a few turns, and that relaxed in NH_4Cl has $\bar{\tau}_E$ of approximately +5. These winding alterations, all relative to K^+, agree with corresponding differences which may be read from Figure 5. This result, involving a different electrophoresis buffer, shows that the method (Figure 1A) is independent of the counterion employed in the electrophoresis buffer and that the DNA is completely relaxed in the presence of K^+. While all possible salt combinations were not investigated systematically in this manner, in no case did the counterion appear to influence either the rate or the extent of the N/C reaction.

Comparison of the Band Counting Results with Superhelix Density Determinations in CsCl/PrdI$_2$. The superhelix densities of DNAs relaxed in 0.2 M salt were determined for a number of monovalent cations by transfer to buoyant CsCl/$PrdI_2$, as described in Figure 1B. Values of $\bar{\tau}_B$, interpreted as $\Delta\tau_1 + \Delta\tau_2$ of Figure 1B, were calculated by the method of Burke and Bauer (1977) with the $PrdI_2$ duplex unwinding angle taken to be 26 ± 2.6° (Wang, 1974). The values of $\bar{\tau}_B$, all subject to an uncertainty of ±1, are −18.7 for Na^+; −12.8 for K^+; −11.8 for Li^+; −9.8 for Rb^+; −8.6 for Cs^+; and −7.3 for NH_4^+. The result for Cs^+ is in close agreement with the estimate of −8.2 turns upon transfer from 0.1 M to 3 M CsCl (Wang, 1969, 1974). Figure 6 presents a plot of $(\bar{\tau}_{X^+} - \bar{\tau}_{Na^+})_B$ as a function of the corresponding estimates of $(\bar{\tau}_{X^+} - \bar{\tau}_{Na^+})_E$ determined by band counting, the solid line being drawn at 45°. The agreement between the two methods, involving entirely different test media, supports the use of $\bar{\tau}_{X^+} - \bar{\tau}_{Na^+}$ as a measure of the magnitude of the duplex winding alterations in the presence of ion X^+ relative to Na^+ in the incubation solvent. It should be emphasized that all DNA species are fully relaxed in this solvent, thereby eliminating potential structural complexities due to supercoiling.

The data obtained from band counting are independent of those from the buoyant method; but only the latter depend upon the $PrdI_2$ duplex unwinding angle, ϕ. We set $y_{26} = y_B \cdot (26/\phi) \equiv (\bar{\tau}_{X^+} - \bar{\tau}_{Na^+})_B$ and $y_E \equiv (\bar{\tau}_{X^+} - \bar{\tau}_{Na^+})_E$; the variance about the 45° line of Figure 6 is then given by $v = \Sigma(y_B - y_E)^2$ and the best least-squares estimate, ϕ_{mp}, is determined by the condition $dv/d\phi = 0$. The result is $\phi_{mp} = 26(\Sigma y_{26}y_E)/(\Sigma y_{26}^2)$ and, numerically, $\phi_{mp} = 25.0°$. A plot of the standard deviation, $SD = (v/5)^{1/2}$, vs. ϕ is included in the insert to Figure 6, demonstrating that the minimum is well defined. The dashed line in Figure 6 lies at an angle of 46.1° and represents the best least squares fit at $\phi = 25°$. The difference between the two lines is not regarded as significant.

Effects of Various Anions. Corresponding band counting experiments were performed employing a variety of anions. The chaotropic anions ClO_4^-, SCN^-, Cl_3CCOO^-, and I^- were examined over the concentration range 0.04 M to 0.10

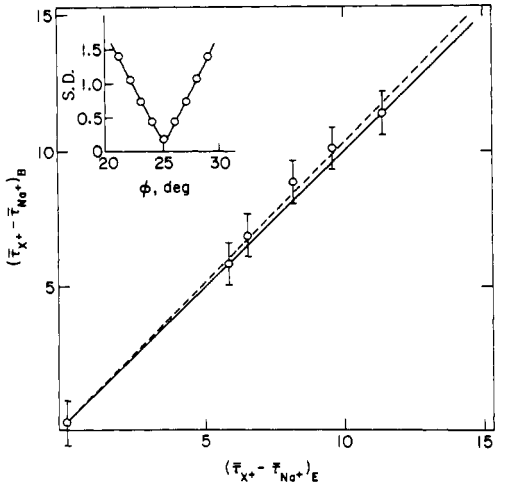

FIGURE 6: Comparison of relative differences in superhelical turns between cation X^+ and Na^+ as obtained by two different methods (Figure 1). Cation X^+ is, from left to right: Na^+, K^+, Li^+, Rb^+, Cs^+, and NH_4^+. The solid line is at 45° and signifies complete accord between the two methods. The error bars represent the 10% uncertainty in the unwinding angle of 26° as estimated by Wang (1974). The inset shows the variation in the standard deviation from the solid line as a function of the value taken for the $PrdI_2$ unwinding angle, ϕ. The dashed line in the figure lies at 46.1° and represents the best least squares fit for the value $\phi = 25°$.

M. The N/C enzyme was inhibited by concentrations of SCN^- above 0.14 M, ClO_4^- above 0.18 M, I^- above 0.20 M, and Cl_3CCOO^- above 0.25 M. The results, documented in Table II, show no significant difference between any of these ions and the Cl^- ion. The K^+ salts of the nonchaotropic anions Br^-, HCO_3^-, $CH_3CO_2^-$, NO_3^-, SO_4^{2-}, citrate$^-$, and HPO_4^{2-} were also compared with KCl at a concentration of 0.2 M. The N/C enzyme was active in all cases and no significant differential shifts compared to Cl^- were observed upon transfer to E buffer. We conclude that the nature of the anion does not influence the duplex rotation angle, at least over this salt concentration region.

Effect of the Mg^{2+} Ion. It has been reported (Wang, 1974; Depew and Wang, 1975) that the addition of mM concentrations of Mg^{2+} to DNA in 1:1 electrolytes brings about an increase in ψ and a concomitant generation of negative superhelical turns. We have confirmed and extended this result by conducting a series of N/C incubations in the presence of increasing concentrations of $MgCl_2$, followed by transfer to E buffer and electrophoretic analysis as described above (Figure 1A). At all $MgCl_2$ concentrations examined above 0.02 M, $\bar{\tau}_E$ was constant and equal to +10.5 turns/DNA. These experiments included concentrations of 0.03, 0.04, and 0.05 M, corresponding to ionic strengths of 0.09, 0.12 and 0.15 M. At a $MgCl_2$ concentration of 0.02 M (ionic strength 0.06 M), $\bar{\tau}_E$ appeared to be somewhat less positive, approximately +8.8 turns/DNA. On this basis, the Mg^{2+} ion appears to be most nearly comparable to the Cs^+ ion (at the same ionic strength) in its effect upon the duplex rotation angle. Shure and Vinograd (1976) estimated that $\Delta\sigma_0$ upon transfer of SV40 DNA from 1 mM Mg^{2+} to 0.2 M NaCl is +0.0067, using $\phi = 26°$. This would correspond to the generation of +6.6 superhelical turns in PM-2 DNA and is consistent with our results.

Discussion

The average values of the structural parameters of the B form of DNA in solution appear to depend upon a number of factors including base composition and sequence (Bram, 1971, 1973); temperature in the premelting region (Wang, 1969; Depew and Wang, 1975); the substitution of Na^+ for Cs^+ (Wang, 1969; Upholt et al., 1971); the salt concentration of NaCl or of CsCl above 1.0 M (Upholt et al., 1971); the addition of chaotropic solvents at relatively high molarities (Bauer, 1972a,b; Burke and Bauer, in preparation); and the substitution of Mg^{2+} for Na^+ (Bauer, 1972b; Pulleyblank et al., 1975) or for Cs^+ (Wang, 1974). The principal experimental techniques employed in the above investigations include wide angle x-ray scattering, sedimentation velocity measurements, and effects upon the tertiary structure of superhelical DNA. These latter experiments have been primarily based upon EtdBr-mediated winding alterations compared to those of the factor in question. The cumulative effect of this research is the impression that the free energy minimum of the B structure of DNA in solution is fairly broad with respect to variation in at least the duplex rotation parameters and that considerable distortion can occur without induction of a major structural transition (such as denaturation). The A structural form of DNA appears, on the other hand, to be substantially more rigid (Bram, 1973; Hamilton et al., 1959; Cooper and Hamilton, 1966). Fully comparable solution studies with the A form are, however, not available.

TABLE II: Effects of Various Anions upon Superhelix and Duplex Winding of PM-2 DNA.[a]

Anion, Y^-	$[K^+Y^-]$, M			
	0.04	0.06	0.08	0.10
ClO_4^-	2.33	3.02	3.64	4.21
SCN^-	2.70	3.48	3.75	4.70
Cl_3CCOO^-		3.44	3.65	4.66
I^-	2.88	3.33	3.66	4.50
Cl^-	2.55	3.55	3.60	4.56
Mean	2.59	3.33	3.66	4.53
SD	±0.27	±0.23	±0.05	±0.19

[a] The entries represent the number of superhelical turns generated upon transfer to standard E buffer at 15 °C.

The present investigation is concerned with the dependence of the DNA duplex rotation angle upon ionic strength in the presence of a variety of electrolytes. The principle of the method employs the basic topological constraint $\alpha = \bar{\beta}_0 + \bar{\tau}_0$, characteristic of closed circular DNA, as well as the ability of the mammalian N/C enzyme to reduce $\bar{\tau}_0$ to zero. This enzyme is readily obtained from HeLa cells and does not require Mg^{2+} or other divalent ions, thereby rendering it more suitable for these experiments than the otherwise equivalent combination of endonuclease followed by polynucleotide ligase (Wang, 1969; Depew and Wang, 1975; Shure and Vinograd, 1976). In the presence of the N/C enzyme in any solvent, $\bar{\tau}_0 = 0$ and therefore $\alpha = \beta_{XP}(c)$, where XP indicates the corresponding neutral DNA species at counterion concentration c in moles/liter. Upon transfer to test conditions, following inactivation of the enzyme, α is invariant and $\alpha = \bar{\beta}_{test} + \bar{\tau}_{test}$. The simple result remains $\beta_{XP}(c) = \bar{\beta}_{test} + \bar{\tau}_{test}$ and, since this latter quantity is directly measurable, $\beta_{XP}(c)$ may be determined with respect to $\bar{\beta}_{test}$ for any experimental conditions under which the enzyme remains active. We have in this manner been able to examine all the common alkali metal ions, as well as NH_4^+, over the concentration range 0.05 to 0.3 M.

Figure 1 includes a qualitative summary of changes in winding numbers which accompany each conceptual step of the two methods employed. The quantitative results are best expressed as the variation in duplex rotation with the logarithm of the salt concentration, plots of which are linear for each

counterion (Figure 5B). The slopes of the curves for Na^+, K^+, Li^+, and NH_4^+ do not vary significantly from the average value, $d\bar{\tau}_E/dpX^+ = -4.40$ turns. The slopes for Cs^+ and Rb^+, -6.86 and -6.34 turns, respectively, appear to be significantly more negative. The basis for this apparent division into two categories is not at present understood. The most straightforward interpretation of the linear decrease in $\bar{\tau}_E$ with pX^+ is, however, that the extent of charge shielding by associated counterions increases with the ionic strength and is therefore proportional to the chemical potential. This latter quantity contains, in turn, the principal component $-RT \ln [X^+]$. The data permit no basis for speculation regarding the question of whether or not such shielding is site specific. The results do clearly indicate, however, that the various cations exert significantly different effects upon the duplex winding. To our knowledge none of the existing theories for the behavior of DNA as a polyelectrolyte take such ion specificity into account.

Previous investigations of the dependence of the duplex rotation angle upon the ionic environment have been restricted primarily to higher salt concentrations. The results of Wang (1969) were based upon 3 M CsCl as a standard solution and employed 12° as the EtdBr unwinding angle. No effect was observed upon reducing the CsCl concentration to 2.0 M (a result later confirmed by Upholt et al., 1971). After correcting for the reestimated value of 26° for the EtdBr unwinding angle (Wang, 1974), the substitution of X^+ for Na^+ at 2.0 M changes ψ by 0° for K^+, by +0.09° for Li^+, and by +0.15° for Cs^+. Wang (1969) also showed that a reduction in the ionic strength from 3 M CsCl to 0.1 M CsCl decreases ψ by 0.30°. It is difficult to compare these estimates with the present results since the salt concentration ranges are so different. Figure 5A suggests that all ions apparently approach a plateau region at about 0.25–0.3 M; hence extrapolations of our data above 0.3 M salt are unwarranted. Qualitatively, both the high and the low salt data demonstrate that ψ is greater in Li^+ and in Cs^+ than in Na^+. This is also true in K^+ at low salt but perhaps not at high salt.

Since the superhelix density of a closed circular DNA varies with the environmental conditions, it is useful to employ a standard state value for purposes of comparing this quantity among different DNAs. We propose that the condition 0.2 M NaCl, 37 °C, pH 8.3, be used for this purpose and that the corresponding standard values of all winding variables be denoted with a superscript zero. (The symbol β° was previously employed as a normalizing factor and is equal to $N/10$. We suggest that this use be eliminated.) This would include the number of superhelical turns, τ°; the superhelix density, σ°; the duplex rotation, ψ°; and the duplex winding number, β°. The topological winding number, α, is independent of environmental condition changes. This choice of standard state is particularly advantageous because both the N/C enzyme and the polynucleotide ligase are active and DNAs may be obtained which contain exactly zero tertiary turns. Thus, DNAs containing $\alpha = \beta^\circ$ turns ($\bar{\tau}^\circ = 0$) are available as known reference standards. This choice of standard state would replace that previously proposed by Upholt et al. (1971), which was 2.85 M CsCl at 20 °C. This latter proposal was reasonable when the EtdBr sedimentation velocity titration was the method of choice for the determination of superhelix densities, but such is no longer the case. The presently proposed standard state has the additional advantage of being close to physiological conditions. The value of any winding variable at other temperatures, ionic environments and salt concentrations may then be calculated from the data presented here, Table I, and by Wang (1969). A comprehensive tabulation of the standard superhelix densities of the known closed DNAs will appear elsewhere (Bauer, 1978).

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References

Bauer, W. (1972a), *J. Mol. Biol. 67,* 183.
Bauer, W. (1972b), *Biochemistry 11,* 2915.
Bauer, W. (1978), *Annu. Rev. Biophys. Bioeng. 7* (in press).
Bauer, W, and Vinograd, J. (1968), *J. Mol. Biol. 33,* 141.
Bauer, W. R., Ressner, E. C., Kates, J., and Patzke, J. V. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 1841.
Berk, A. J., and Clayton, D. A. (1974), *J. Mol. Biol. 86,* 801.
Berk, A. J., and Clayton, D. A. (1976), *J. Mol. Biol. 100,* 85.
Bram, S. (1971), *Nature (London), New Biol. 232,* 174.
Bram, S. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2127.
Burke, R. L., and Bauer, W. (1977), *J. Biol. Chem. 252,* 291.
Burlington, M. G., and Morgan, A. R. (1976), *Can. J. Biochem. 54,* 301.
Champoux, J. J., and Dulbecco, R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 143.
Cooper, P. J., and Hamilton, L. D. (1966), *J. Mol. Biol. 16,* 562.
Crick, F. H. C. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2639.
Crosa, J. H., Luttropp, L. K., and Falkow, S. (1976), *Nature (London) 261,* 516.
Depew, R. E., and Wang, J. C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4275.
Espejo, R. T., and Canelo, E. S. (1968a), *Virology 34,* 738.
Espejo, R. T., and Canelo, E. S. (1968b), *J. Bacteriol. 95,* 1887.
Fuller, F. B. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 815.
Gellert, M., Mizuchi, K., O'Dea, M. H., and Nash, H. A. (1976a), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3872.
Gellert, M., O'Dea, M. H., Itoh, T., and Tomizawa, J.-I. (1976b), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4474.
Hamilton, L. D., Barklay, R. K., Wilkins, M. H. F., Brown, G. I., Wilson, H. K., Marvin, D. A., Ephrussi-Taylor, H., and Simmons, N. S. (1959), *Biophys. Biochem. Cytol. 5,* 397.
Hinton, D. M., and Bode, V. C. (1975), *J. Biol. Chem. 250,* 1071.
Horiuchi, K., and Zinder, N. D. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2341.
Keller, W., and Wendel, I. (1974), *Cold Spring Harbor Symp. Quant. Biol. 39,* 199.
Marians, K. J., Ikeda, J.-E., Schlagman, S., and Hurwitz, J. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 1965.
Pohl, W. F. (1968), *J. Math. Mech. 17,* 975.
Pulleyblank, D. E., Shure, M., Tang, D., Vinograd, J., and Vosberg, H.-P. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,*

4280.
Robinson, R. A., and Stokes, R. H. (1959), Electrolyte Solutions, 2nd ed, London, Butterworth.
Shure, M., and Vinograd, J. (1976), *Cell 8,* 215.
Timmis, K., Cabello, F., and Cohen, S. N. (1976), *Nature (London) 261,* 512.
Uphold, W. B., Gray, H. B. Jr., and Vinograd, J. (1971), *J. Mol. Biol. 61,* 21.
Vinograd, J., and Lebowitz, J. (1966), *J. Gen. Physiol. 49,* 103.
Vinograd, J., Lebowitz, J., and Watson, R. (1968), *J. Mol. Biol. 33,* 173.
Vosberg, H.-P., Grossman, L. I., and Vinograd, J. (1975), *Eur. J. Biochem. 55,* 79.
Wang, J. C. (1969), *J. Mol. Biol. 43,* 25.
Wang, J. C. (1971), *J. Mol. Biol. 55,* 523.
Wang, J. C. (1974), *J. Mol. Biol. 89,* 783.
Wang, J. C., Jacobson, J. H., and Saucier, J.-M. (1977), *Nucleic Acids Res. 4,* 1225.
White, J. H. (1969), *Am. J. Math. 41,* 693.

Ethidium Bromide Binding to Transfer RNA: Transfer RNA as a Model System for Studying Drug–RNA Interactions†

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ABSTRACT: The interaction of ethidium bromide (EB) with tRNA has been examined by optical (absorption spectra, fluorescence intensity and lifetime) and ^{1}H nuclear magnetic resonance (^{1}H NMR) methods. The optical measurements show that the strongest EB binding site is intercalative. At higher EB levels about three additional nonintercalative sites are occupied. The ^{1}H NMR experiments gave the following information about the strong intercalative site. Results for mixed and pure species of transfer RNA (tRNA) showed that none of the residues involved in the tertiary structure are adjacent to, or disrupted by the strongly bound EB. The strong binding site in yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Val}_1$ is adjacent to the sixth base pair of the amino acid acceptor stem. Results for four other class I tRNAs are consistent with the strong binding site being located in the amino acid acceptor stem, but some other binding sites for these four tRNAs cannot be ruled out on the basis of the ^{1}H NMR results alone. Yeast $tRNA^{Leu}_3$, a class III tRNA, exhibits spectral changes on binding EB which are clearly different from those of the class I tRNA examined here. The results for yeast $tRNA^{Leu}_3$ are interpreted in terms of a unique EB binding site in the extra arm which stabilizes the base pairs of this stem. *E. coli* $tRNA^{fMet}$ appears to be different from the other tRNAs investigated here in that there seem to be several binding sites of similar binding strength. These results are consistent with the notion that the tertiary structure of tRNA restricts the binding of EB to a single site in the amino acid acceptor stem. Additional support for this notion is given by ^{1}H NMR results which show that EB and chloroquine, an intercalative drug, bind to the same unique site of *E. coli* $tRNA^{Glu}_2$.

The interaction of EB[1] with tRNA is of general interest in connection with questions regarding drug–nucleic acid interactions since EB is known to have a number of effects on the biological and biochemical properties of polynucleotides (Waring, 1965, 1975). For example, EB inhibits RNA (Kramer et al., 1974) and DNA (Loeb, 1974) polymerases and the digestion of DNA by DNase I (Eron and McAuslan, 1966). EB also has antibiotic properties and is mutagenic (Waring, 1975). Kramer et al. (1974) report that binding of EB affects the rate of in vitro replication of a small replicating RNA molecule and that mutational changes of only three bases eliminated the EB inhibition. Their results suggested that at least one important EB binding site was eliminated by the three base changes since the number of binding sites in the mutant RNA is smaller than in the wild type (Kramer et al., 1974). Several recent papers have illustrated the utility of EB in probing nucleosome structure (Angerer et al., 1974; Ide and Baserga, 1976; Ballestra et al., 1976). Lurquin and Buchet-Mathieu (1971) have demonstrated that binding of EB affects the rate of aminoacylation of some tRNA, and this raises interesting questions about the relationship between EB binding properties and the effect on the rate of aminoacylation.

The studies mentioned above serve to illustrate the important role of EB in polynucleotide studies and to emphasize the need for more information about the factors which control the binding of EB to polynucleotides.

Because much is known about the secondary and tertiary structure of tRNA molecules in the crystal and in solution, these molecules provide useful "model" systems for exploring some of the factors which may influence the binding of EB to RNA. Since tRNAs exhibits a variety of tertiary structural features not found in double helical systems previously investigated they are of special interest.

Previous studies of the optical melting curves of tRNA in the presence and absence of EB show there is competition between EB binding and formation of tertiary structure such that at high EB levels the tertiary structure is disrupted (Urbanke et al., 1973). This is to be contrasted with the stabilization of

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[1] Abbreviations used: EB, ethidium bromide; DSS, sodium 2,2-dimethylsilapentane-5-sulfonate.

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