Purification of Closed Circular \( \lambda \) Deoxyribonucleic Acid and Its Sedimentation Properties as a Function of Sodium Chloride Concentration and Ethidium Binding

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SUMMARY

The sedimentation of circular \( \lambda \) DNA suggests that the molecule undergoes significant changes in shape and supercoiling as the NaCl concentration increases. Closed circular \( \lambda \) DNA, species I, isolated and purified from superinfected immune bacteria, sediments in sucrose gradients of low ionic strength at a rate 2.0 times faster than linear \( \lambda \) DNA, species III. The addition of ethidium causes the sedimentation rate of species I DNA to decrease until enough dye is bound to remove 121 supercoils per molecule. At this point, species I co-sediments with nicked and nonsupercoiled species II. Further additions of ethidium cause the sedimentation rate to increase until the relative rate of species I is again at least twice that of species III.

This classical behavior is altered when NaCl is present in the buffer. In 1.0 M NaCl the changes in S are complex. Initially, species I sediments 1.55 times faster than species III. Titration with ethidium causes a decrease in S to an early minimum value, then an increase to a first maximum, followed by a decrease to the S of species II. At this point enough dye has intercalated to remove 208 superhelical turns. Further additions of dye introduce supercoils and cause S to increase again. In 0.1 to 0.4 M NaCl the relative S of species I is 1.69 and 1.59, respectively. If titrated with ethidium, S first increases to a maximum value then decreases to the minimum rate when enough dye is bound to remove 158 and 183 supercoils, respectively.

The results indicate an increase in the superhelix density from 0.026 turns per 10 base pairs in buffer alone to 0.045 in the same buffer with 1.0 M NaCl. If this change in superhelix density results from a concomitant change in the average rotation angle between base pairs in the Watson-Crick helix, the addition of 1.0 M NaCl alters the rotation angle by 0.68° per base pair.

Increasing the ionic strength causes a decrease in the sedimentation rate of circular \( \lambda \) DNA (1, 2) but has relatively little effect on the sedimentation rate of linear or nicked circular \( \lambda \) DNA. Since the molecular weight is constant, shape or flexibility changes in the supercoiled molecule must account for the rather pronounced changes in the sedimentation velocity. Experiments reported in the preceding paper suggest that the number of potential supercoils, as estimated from ethidium dye binding affinity, increases as the sodium chloride concentration is raised from 0.1 to 1.0 M (3). Since we had not expected an increase in supercoiling to cause a lowering of the S value, the sedimentation velocity of circular \( \lambda \) DNA was examined in greater detail as a function of both ionic strength and the number of supercoils.

The sedimentation rate of purified circular \( \lambda \) \([\text{H}]\)DNA is measured relative to a linear \( \lambda \) \([\text{H}]\)DNA marker as a function of bound ethidium and (thereby the number of supercoils) at four NaCl concentrations, 0.0, 0.1, 0.4, and 1.0 M in 0.01 M Tris-HCl plus 10 mM sodium-EDTA buffer. In each solution and at each dye concentration, the number of potential supercoils present in a circular \( \lambda \) DNA molecule is calculated from the number of ethidium molecules bound relative to the number which are required to remove all supercoils, i.e. the number bound when

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1 We refer to circular DNA molecules whose component single strands form two intertwined but unbroken circles of phosphodiester bonds as simply circular DNA molecules (frequent synonyms: species or form I, covalently closed circular DNA). Molecules in which the chains of phosphodiester bonds have been broken by hydrolysis or other means are referred to as nicked circular DNA (frequent synonyms: species or form II, open circles). The latter is not a homogenous collection since the number and location of chain interruptions may vary within the population of molecules. Species or form III is linear duplex DNA. Single-stranded circular molecules do not enter this discussion.
circular λ DNA (species I) co-sediments with nicked circular λ DNA (species II).

MATERIALS AND METHODS

Media and Solutions

All media and buffer solutions were made with distilled de-ionized water and sterilized before use.

Tryptone broth, containing 1% (w/v) Bacto-tryptone and 0.86 M NaCl (0.5% w/v).

λ-dil contained 0.01 M potassium phosphate, pH = 7.1, 0.01 M MgSO₄, and 10 μg per ml of bovine serum albumin. The buffer was sterilized separately. After cooling, sterile MgSO₄ and albumin were added.

Tris buffers were prepared from the free base (Trizma base, Sigma) and HCl. Buffer A is 0.01 M Tris-0.001 M EDTA (pH 7.1). Buffer B is 0.02 M Tris-0.02M EDTA (pH 7.1). Buffer C is 0.01 M Tris-0.01 M MgSO₄ (pH 8.0).

Chemicals

Trona CsCl (American Potash and Chemical Corp.) was used for phage purification. A saturated stock solution (at 23°) was prepared in Buffer C and filtered both before and, while hot, after addition of zinc and lye NaHCO₃, and then stored at -17°.

Sarcosyl NL97 (sodium lauroyl sarcosinate, Geigy Chemical Co.) were added. Prepared in Buffer C and filtered both before and, while hot, after addition of zinc and lye NaHCO₃, and then stored at -17°.

Suitable volumes of 3H- or 32P-labeled DNA solutions were placed on glass fiber disks (Whatman GF/C, 2.4 cm), dried under the addition of zinc and lye NaHCO₃, and then stored at -17°.

Refractive Index Measurements

The refractive index, nD, of CsCl, NaCl, and sucrose solutions was measured using a Bausch and Lomb refractometer connected to a circulating cooling bath set at 20°.

Scintillation Spectroscopy

Suitable volumes of ^H- or ^P-labeled DNA solutions were placed on glass fiber disks (Whatman GF/C, 2.4 cm), dried under a heat lamp for 20 min and counted in 10 ml of scintillation fluid, prepared by adding g of a pre-mixed fluor, containing 2% p-bis-(o-Methylstyril)benzene and 98% 2,5-diphenyloxazole, to a liter of reagent grade toluene. Alternatively, DNA was precipitated from solutions with an equal volume of cold 10% trichloroacetic acid, collected on glass fiber disks, washed with cold water, dried, and counted as above.

Purification of Unlabeled λ Phage

A clear plaque mutant of lambda, λ40, was used throughout this study. Escherichia coli W3101 was grown in 1.0 or 1.5 liters of tryptone broth at 37° to a cell density of 5 × 10⁸ per ml and infected with λ40 added in 100 ml of Buffer C at a multiplicity of 0.1. Cells were incubated further in a 6-liter flask in a gyratory water bath (37°) until lysis was complete (120 to 140 min after phage infection). The lysate was chilled in ice, 0.5 ml of CHCl₃ added, and then centrifuged at 13,000 × g for 60 min to remove cell debris. Final concentrations of 0.10% (w/v) sodium dextran sulfate (mol wt = 5 × 10⁶, Pharmacia), 0.01% (w/v) polyethylene glycol glycerol (Carbowax 6000, Union Carbide), and 0.07 M NaCl were achieved by adding 30.0 g of NaCl, 10.0 ml of 20% (w/v) dextan sulfate, and 250 ml of 30% (w/v) polyethylene glycol to each measured liter of lysate. The mixture was stored at 4° overnight during which time the phage accumulated in the polymer interphase. The use of a separation funnel facilitated the next step. The bottom phase, containing dextran sulfate, and the interphase were collected and centrifuged at 10,000 × g for 15 min. Contaminating bottom and top phases were removed and discarded. The buff-colored interphase material was dissolved in 15 ml of Buffer C and 5 ml KC1 was added to a final concentration of 0.41 M. The white cloudy mixture was stored overnight at 4° to permit maximal precipitation of the dextran. The precipitate was removed by centrifugation for 15 min at 10,000 × g. The supernatant was collected and the phage purified further by sedimentation into a step-gradient of CsCl in Buffer C. The gradient tubes were prepared by pipetting in order: 10 ml of a solution with a density of 1.7 g per ml (ρ1 = 1.4000), 16 ml of 1.5 g per ml (ρ2 = 1.3850), 22 ml of 1.3 g per ml (ρ3 = 1.3645), and 0.9 ml of phage suspension. After centrifugation in the Spinco SW 25.2 rotor for 3 hours at 22,000 rpm at 5°, the phage band formed at approximately two-thirds down the length of the tube. It was collected by bottom puncture and cut in three chambers (2 ml each) by either the Dicine (5) procedure measuring purine deoxypentose, or by the microphosphate procedure of Chen et al. (6). The concentration of dilute solutions of labeled closed circular λ DNA was determined from the known specific activities (3H or [32P] counts per min per μg) of their purified linear counterparts.

Radiolabeled Phage

For the preparation of 3H-labeled λ, E. coli CR34 thy' was grown in tryptone broth supplemented with 0.5 μg per ml of thymidine. At the time of infection, 1 mCi of [methyl-3H]thymidine was added. All following steps were performed as described for unlabeled λ. For preparation of 32P-labeled λ, E. coli W3101 was grown in tryptone broth to a density of 2.1 × 10⁹ per ml. One generation time before infection, H₃2PO₄ (New England Nuclear) was added to yield a final activity of 38 μCi per ml. Cells were infected and [3P]λ40 was purified as described for the unlabeled preparations.

Purification of λ DNA

Linear λ DNA is obtained by phenol extraction of purified λ phage as described by Kaiser and Hogness (4). For analysis, the λ DNA used in fluorescein studies (3) and the [3H]DNA used as a standard of purity for circular λ DNA was purified further by CsCl equilibrium density gradient centrifugation in a Spinco 30 rotor, 20,000 rpm, 75 hours, 5°. The initial density of the DNA solution was 1.7 g per ml (ρCS = 1.4000). This procedure increased the purity, based on counts per absorbance at 230 nm (A230) of labeled DNA preparations by only a few per cent. After dialysis in three 2-liter changes of Buffer A, the DNA was stored at 4°.

Estimation of DNA Concentration

Routinely, DNA concentrations were estimated from the A260 using a value of 47 μg of DNA per ml of solution with A260 equal to 1.0. In addition, the concentration of stock solutions of linear or circular λ DNA was also determined by a procedure measuring purine deoxypentose, or by the microphosphate procedure of Chen et al. (6). The concentration of dilute solutions of labeled closed circular λ DNA was determined from the known specific activities (H or [3P] counts per min per μg) of their purified linear counterparts.

Purification of Superhelical λ DNA from Superinfected Lysogens

Step 1—E. coli 1100, an endonuclease I deficient mutant (7) was lysozymed for λ to serve as an immune host for superinfection. Cultures of E. coli 1100 (λ) were grown to a density of 5 × 10⁹ cells per ml at 37° in tryptone broth supplemented with 0.02 mg per ml of thiamin. The cells were sedimcnled 9,000 rpm, Sorvall GS3 rotor, 1 hour, 5°. The cell paste from 1- to 5-liters of culture was suspended in 500 ml of 0.01 M EDTA (pH 7.1) and homogenized with a motor-driven tissue homogenizer (maximum of 3 liters or 1.5 × 10⁹ total cells). Purified λ40 phage suspended in 400 ml of cold λ-dil were added at a multiplicity of 10 and allowed to adsorb for 10 min at 0°. The complex was shaken for 10 min in a 6-liter flask in a water bath at 37° to allow infection (almost 70% of the radioactivity in the input phage is injected). One hundred milliliters of 10% tryptone broth supplemented with 0.2 mg per ml of thiamin were added, and the incubation continued for 15 min to permit maximum conversion of superinfecting DNA to species λ. After chilling in an ice bath, the cells were sedimcnted, washed by suspension in 400 ml of 0.01 M Tris-HCl (pH 7.1), resedimented, and suspended in 160 ml of ice-cold 30% sucrose in Buffer B. After resuspension, 100 mg of lysozyme (muramidase, twice crystallized, Worthington) in 80 ml of cold Buffer B were added and spheroplast formation was allowed to proceed at 0° for 10 min with occasional shaking. The spheroplasts were sedimented at 12,000 rpm for 30 min at 5° in the Sorvall Centrifuge 40B.
was dialyzed exhaustively against Buffer A to remove phenol. Extractions with Buffer A-saturated phenol. The aqueous phase residual ethidium was quantitatively removed by three serial in rooms regularly lighted with fluorescent lamps.) After dialysis, 5 to 25% sucrose gradients containing Buffer A. When indicated, Fraction I. caution was taken even though very little, if any, breakage of the Fraction IV. Fraction IV was dialyzed in the dark. (This pre-

was obtained in 4 to 5 ml, and the total volume of the pooled DNA gradient. Fractions containing the more dense circular DNA-dye nm was measured to determine the distribution of ethidium in the identify those fractions which contained the labeled circular X [3H]DNA. These fractions along with the bottom fractions, which contained primarily circular DNA, were pooled. The pool

for 75 hours at 26,000 rpm, 5°. The RNA pelleted, protein and Sarcoeyl floated, and all of the DNA was concentrated as a band near the middle of the gradient. The tubes were punctured on the side opposite the RNA pellet about 2 cm from the bottom using an insert pin wired to the tip of a soldering gun. From each gradient, those fractions which contained the host DNA and phage DNA were pooled. Usually a majority of the DNA from a single tube was obtained in 4 to 5 ml, and the total volume of the pooled DNA was about 45 ml. This DNA pool, Fraction II, was dialyzed against two 2-liter changes of 10^{-2} M Tris-HCl, low 3 M EDTA, and

5% sucrose in order to prevent collapse of the tubes. After sedimentation, the tubes were punctured on the side 2 to 3 cm from the bottom and the solution above this level was discarded. The solution which remained in the tube below the puncture contained virtually all of the circular λ DNA and most of the contaminating E. coli DNA. The total volume of the concentrated Fraction III varied between 15 and 20 ml. Sucrose was removed by exhaustive dialysis against Buffer A.

Step 4—After dialysis, solid CsCl and ethidium bromide were added to give a density of 1.65 g per ml (ρ_{CsCl} = 1.3870) and a final ethidium bromide concentration of 100 μg per ml in a total volume of 36 ml. When more than 1 liter of cells was infected, the DNA pool was divided into two 29-ml fractions after the addition of ethidium bromide and CsCl.) The mixture was transferred to clear 30-ml polycarbonate centrifuge tubes, overlaid with 1- or 2-ml of light mineral oil and centrifuged to equilibrium in the Spinco 30 rotor (70 to 75 hours at 26,500 rpm, 5°). The procedure was that of Rudoff et al. (8) with minor modifications. The lower temperature increases the binding affinity of dye to DNA by about 2-fold. The gradient was fractionated into 1-ml fractions, as described previously for Fraction II, and samples were counted to identify those fractions which contained the labeled circular λ DNA. The refractive index was measured on selected fractions to check the CsCl concentration, and for selected gradients, A_{240} nm was measured to determine the distribution of ethidium in the gradient. Fractions containing the more dense circular DNA-dye complex were pooled (3 to 5 ml per gradient) and constituted Fraction IV. Fraction IV was dialyzed in the dark. (This pre-

Sedimentation Velocity Ethidium Bromide Titrations The sedimentation velocity of circular λ DNA was measured in 5 to 25% sucrose gradients containing Buffer A. When indicated, either 0.1, 0.4, or 1.0 M NaCl and various concentrations of ethidium bromide were included in the gradients. The sample (1 ml) layered on a gradient contained circular λ [3H]DNA and linear λ [32P]DNA in Buffer A containing the same NaCl concentration as the gradient. The sample also contained sufficient ethidium to yield, after some bound to DNA, a concentration of free dye identical with that in the gradient. As little as 0.2 μg of circular DNA and equally small concentrations of linear λ [32P]DNA were required, therefore the bound dye is usually a small fraction of the total except for conditions of low ionic strength, i.e. Buffer A alone, or values of r (moles of ethidium bound per mol of nucleotide), less than 0.01. Samples were layered on 29-ml (SW 25.1) or 36-ml (SW 27.1) gradients and sedimented for 4 to 7 hours at 20°. The time depended on the sedimentation rate of species I DNA in the particular gradient. Gradients were fractionated by bottom punctures, 1.1 to 1.2-ml fractions were collected, and a 1.0-ml portion of each was acid precipitated for scintillation counting. The fluorescence of layered samples, and the ethidium distribution in the fraction after sedimentation was measured using an Aminco-Bowman spectrophotofluorometer (480 or 530 nm excitation and 590 nm emission) as previously described (3). Since low concentrations of species I DNA were used in most of the gradients, the concentration of ethidium bound to DNA could not be measured directly above the background fluorescence of the free dye. It was calculated based on the free dye concentration, the DNA concentration and the appropriate binding constant (3). At certain free dye concentrations, e.g. when species I DNA co-

evaluation of purification schemes did not give reproducible yields and sufficiently large amounts of species I λ DNA free of E. coli DNA. Table I summarizes the results from two preparations utilizing the procedure described in detail above. Approximately 50 μg

| Purification step | Total A units | Total A units of λ DNA | Per cent recovery | Fold purification |
|------------------|--------------|------------------------|------------------|------------------|
| 1. Fraction I (crude extract) | A 3500 | 15 | 20% | 0 |
| 2. Fraction II (CsCl step) | B 260 | 19 | 79 | 13 |
| 3. Fraction III (sucrose step) | A 17 | 2.3 | 15 | 30 |
| 4. Fraction IV (ethidium bromide-CsCl step) | A 7.9 | 7.5 | 6 | 220 |

* Absorbance due to λ DNA is calculated from the known specific activity, (14 H counts per min per A_{260} unit), of λ[H] DNA purified from a sample of the superinfecting phage.

A and B refer to two different preparations purified at different times. A contained 10^{10} bacteria in 2 liters and B contained 1.5 × 10^{10} bacteria in 3 liters; both had been infected with λ[H] DNA at a multiplicity of 10.

* Recovery of λ DNA relative to the input of λ DNA

* Recovery of λ DNA in Steps 2 to 4 is relative to λ DNA in Fraction I.
of species I λ DNA was obtained routinely from 10⁹ bacteria superinfected at a multiplicity of 10.

The distribution of circular λ [PH]DNA, linear λ [32P]DNA, and ethidium in the final CsCl gradient of the purification is shown in Fig. 1. The λ [PH]DNA molecules must be in the species I form to band in the more dense peak. Based on the counts per min per ml to A⁶⁰⁰ ratio, or the presence of labeled linear marker DNA, the peak fraction is contaminated by no more than 5% with molecules from the less dense band of linear and nicked circular molecules. Nevertheless, after removal of CsCl and ethidium bromide, its composition was 70% species I and 30% species II.

Sedimentation Velocity as Function of Bound Ethidium—
Several studies, using various circular DNA molecules and ethidium intercalation, have employed sedimentation velocity to monitor the unwinding and winding of superhelices (9-15).

Most measurements were made in high salt concentrations, preferably 2.83 M CsCl, using the analytical ultracentrifuge. Sucrose gradients and the preparative centrifuge were used here. This permitted ready variation of the salt concentration, direct fluorescence measurement of free, and in some cases bound, ethidium, and a simultaneous determination of the sedimentation behavior of linear λ [32P]DNA and circular λ [PH]DNA as a function of bound ethidium. Although measured directly in a few critical instances, the latter was estimated routinely using the Scatchard equation² (16).

The ethidium binding isotherms at the four ionic strengths used in this study were measured in several concentrations of sucrose. The inclusion of sucrose did not alter the value of K or n. Table II lists the values for these constants determined in 25% sucrose. The corresponding values determined in the absence of sucrose can be found in Table II of the preceding paper (3).

Contrary to the results reported by Le Pecq and Pualetti (17), sucrose did affect the fluorescence of free ethidium. Fluorescent intensity increased about 15% when the buffer contained 25% rather than 5% sucrose. This was accompanied by a slight red shift in the emission spectrum. Sucrose did not change the fluorescent yield from ethidium when it was bound to DNA.

Table III summarizes the conditions used in 12 gradients with varying amounts of ethidium and no added NaCl. Routinely, the amount of λ DNA sedimented was less than 2 µg, and the amount of bound dye was calculated from the free dye concentration in the gradients using the Scatchard equation. To check the validity of the methods, larger amounts of marker DNA or circular DNA were added to samples 6 through 12. This permitted the direct measurement of bound ethidium by fluorescence. Since the DNA concentrations are known from the free dye concentration only. The two gradients displayed in detail in Fig. 2 present results from a detailed study of the gradients.

The results indicated that even under these conditions, where greater than normal amounts of DNA moved through the upper portion of the linear sucrose gradient, the free dye concentration remained constant at the initial value throughout the tube during centrifugation. As verified by the direct measurement of bound dye, Fig. 2 and Table III, the amount of ethidium which binds and sediments with λ DNA is accurately calculated using the free dye concentration.

TABLE II

| Ionic strength | Solutions | K (liters/mol) | n |
|----------------|-----------|---------------|---|
| 0.016 Buffer A + 25% sucrose | 29 | 0.18 |
| 0.12 Buffer A + 0.10 M NaCl + 25% sucrose | 6.8 | 0.18 |
| 0.42 Buffer A + 0.40 M NaCl + 25% sucrose | 2.2 | 0.18 |
| 1.02 Buffer A + 1.0 M NaCl + 25% sucrose | 0.98 | 0.18 |

² r/K = K(n - r), where r is the moles of ethidium bound per mol of DNA nucleotide, ρ is the molar concentration of ethidium free in solution, K is the association constant in liters per mol, n is the maximum number of binding sites per nucleotide.

Most measurements were made in high salt concentrations, preferably 2.83 M CsCl, using the analytical ultracentrifuge. Sucrose gradients and the preparative centrifuge were used here. This permitted ready variation of the salt concentration, direct fluorescence measurement of free, and in some cases bound, ethidium, and a simultaneous determination of the sedimentation behavior of linear λ [32P]DNA and circular λ [PH]DNA as a function of bound ethidium. Although measured directly in a few critical instances, the latter was estimated routinely using the Scatchard equation² (16).

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² r/K = K(n - r), where r is the moles of ethidium bound per mol of DNA nucleotide; ρ is the molar concentration of ethidium free in solution; K is the association constant in liters per mol; n is the maximum number of binding sites per nucleotide.
FIG. 2. Top, measuring $r$ directly using excess marker or circular $\lambda$ DNA. The $^3$H counts were associated with purified circular $\lambda$ DNA (5660 cpm per $\mu$g) which contained 15% nicked circular molecules. The $^32$P counts, $O-O$, were associated with a linear $\lambda$ DNA marker. Unlabeled linear $\lambda$ DNA was added to the marker before sedimenting, yielding a specific activity of 600 cpm per $\mu$g. The fluorescence, $\Delta-\Delta$, at 590 nm (530 nm activation) is primarily due to the dye bound to linear DNA. In the peak fraction of the $^32$P profile there was 0.60 $\mu$g per ml of linear $\lambda$ DNA and 0.36 $\mu$g per ml of bound ethidium bromide. Therefore, $r$ was 0.046. $S_{rel}$ of circular $\lambda$ DNA with respect to linear is 1.30 under these conditions. Table III, sample 9, gives further details on this gradient. Sedimentation: 4 hours, 20°, 25,000 rpm, SW 25.1 rotor. Bottom, the detailed conditions are given in Table III, sample 11. The circular $\lambda$ $[32P]$ DNA (5600 cpm per $\mu$g), $O-O$, contained 30% species II + III, $\times-\times$, linear $\lambda$ $[32P]$ DNA marker; $O-O$, fluorescence at 590 nm due to $32$P nm activation; $O-O$, per cent sucrose, established from the refractive index; $\Delta-\Delta$, fluorescence of free ethidium in selected; $\Delta-\Delta$, fluorescence after the addition of 50 $\mu$g of salmon sperm DNA in 0.50 ml of Buffer A to 0.50 ml of the selected fraction (intensity is multiplied by 2.0 to correct for the dilution); $\Delta-\Delta$, fluorescence of the sucrose solutions used to make the gradient (0.70 mg per ml of ethidium) after similar additions of salmon sperm DNA. The peak fraction of circular $\lambda$ DNA contained 0.86 $\mu$g of DNA and 0.062 $\mu$g of bound dye yielding an $r$ of 0.000. Sedimentation was for 5 hours, 20°, 25,000 rpm, SW 25.1 rotor.

Examples of more typical gradients performed to determine the relative sedimentation rate, $S_{rel}$, as a function of salt concentration and ethidium bound are shown in Fig. 3. $S_{rel}$ is the distance sedimented by the $\lambda$ $[3H]$-labeled species I divided by the distance sedimented by the linear $\lambda$ $[32P]$ DNA marker present in the same tube.

In the one gradient (top) enough dye was bound to remove 17 of the 208 negative supercoils believed to exist when no dye is present. In the other gradient, the DNA examined had been used for a complete binding study (3) and had sufficient dye bound to remove all negative supercoils and to introduce 132 positive supercoils. The $S_{rel}$ is 1.4 in the first case and 1.8 in the second.

Under all of the conditions examined in this study, one observes only two peaks of $\lambda$ $[3H]$-labeled circular molecules, the rapidly sedimenting supercoiled form and the nicked circles sedimenting 157, faster than the linear marker. The species I molecules sediment as a single sharp band suggesting that, at least after time-averaging, they are homogeneous with respect to dye intercalation and supercoiling.

The species I used for gradients where $S_{rel}$ was near minimal values, i.e. all supercoils removed, was further purified in a Buffer A sucrose gradient just prior to sedimentation in the ethidium containing gradient. This yielded a preparation that was 90% species I and avoided complications in interpreting gradients containing significant amounts of species II whose band pattern would overlap with that of unwound species I.

Relative Sedimentation Rate in Buffer A as Function of Bound Ethidium—The variation of $S_{rel}$ as dye is bound and supercoils are removed is seen in Fig. 4. The value of $S_{rel}$ decreases from a value of 2.0 (2, 18) to a value of 1.12 as $C_f$ increases from 0 to 0.38. Binding more dye introduces positive supercoils and causes $S_{rel}$ to increase.

A slight decrease in the $s_{20,W}$ value of species II or linear DNA as ethidium is bound was reported (9, 11, 19). Here, the linear
TABLE III
Sample and gradient condition for estimating $S_{rel}$ of circular $\lambda$ DNA at low ionic strength with various amounts of intercalated ethidium bromide (EB) in circular $\lambda$ DNA. (Calculated) (measured after sedimentation)

| Gradient | Input Sample | $r^*$ (Calculated) | $r$ (measured after sedimentation) |
|----------|--------------|---------------------|-----------------------------------|
|          | Circular $\lambda$$^{32}$P DNA | Unlabelled $\lambda$ DNA | Linear $\lambda$ DNA | Circular $\lambda$ DNA |
| (a) | (b) | (c) | (d) | (e) | (f) | (g) | (h) | (i) |
| 1 . . . | 0 | 1.14 | 0.50 | 0 | 0 | 0 | 0 | 0 | 2.0 |
| 2 . . . | 0.006 | 1.14 | 0.50 | 0 | 0 | 0.008 | 2.0 |
| 3 . . . | 0.010 | 1.14 | 0.50 | 0 | 0 | 0.013 | 2.0 |
| 4 . . . | 0.025 | 1.14 | 0.50 | 0 | 0 | 0.028 | 1.6 |
| 5 . . . | 0.030 | 1.14 | 0.50 | 0 | 0 | 0.034 | 1.4 |
| 6 . . . | 0.035 | 1.57 | 1.00 | 2.50 | 0 | 0.038 | 0.035 | 1.2 |
| 7 . . . | 0.038 | 0.64 | 2.50 | 20.0 | 0 | 0.040 | 0.037 | 1.1 |
| 8 . . . | 0.042 | 0.50 | 1.00 | 25.0 | 0 | 0.044 | 0.043 | 1.2 |
| 9 . . . | 0.045 | 0.64 | 2.50 | 12.5 | 0 | 0.046 | 0.046 | 1.3 |
| 10 . . . | 0.060 | 5.60 | 0.50 | 0 | 0.001 | — | 0.001 | 1.6 |
| 11 . . . | 0.070 | 5.60 | 0.50 | 0 | 0.063 | — | 0.060 | 1.9 |
| 12 . . . | 0.100 | 6.60 | 0.50 | 0 | 0.078 | — | 0.085 | 2.1 |

*The values listed are $r$ calculated using the Scatchard equation with the $C_f$ value of the gradient, $K = 3.0 \times 10^6$ liters per mol, and $n = 0.18$. The sample layered on the gradient had ethidium bromide (EB) added to yield this calculated $r$ and was checked by fluorescence. In every case the value agreed with the calculated $r \pm 10\%$ so that very little ethidium bromide was scavenged or released to the gradient by the sample. The $r$ value measured on these fractions after sedimentation also agreed well with the calculated values. At low ionic strength, binding is very high and the same $K$ value is appropriate for both circular and linear molecules (3).

marker $[^{32}P]DNA$ showed only slight changes in the number of centimeters it sedimented from the meniscus as the ethidium concentration was varied. The changes in $S_{rel}$ are attributed predominantly to changes in the rate at which species I sediments.

Sedimentation Velocity as Function of Bound Ethidium in 1.0 M NaCl—A complex and nonmonotonic variation in $S_{rel}$ was observed when 1 M NaCl was included in the Buffer A-ethidium sucrose gradients (20, 21). The results of 48 gradients performed with several different species I preparations are shown in Fig. 5. The intercalation of enough ethidium to remove 22 supercoils caused a decrease in $S_{rel}$ to a first minimum. This was followed by an increase in $S_{rel}$ as more ethidium bound. About 112 supercoils were removed at the midpoint of a first maximum. With additional ethidium, $S_{rel}$ decreased again to a second minimum, lower than the first, whose value is characteristic of molecules lacking supercoils. At this point enough ethidium was bound to remove 208 supercoils. In higher concentrations of ethidium, $S_{rel}$ increased to a second maximum, presumably due to the introduction of supercoils of the opposite (positive) handedness. Although it was not as well characterized as the first minimum, a third minimum in $S_{rel}$ appeared when enough dye was bound to both remove 208 negative supercoils and introduce 124 positive ones. The value of $S_{rel}$ then increased again and (not shown in the figure) was 1.90 at $C_f$ equal to 20 $\mu g$ per ml.

The $S_{rel}$ versus $C_f$ graph (Fig. 5) is the raw data and includes no assumptions or corrections related to dye binding affinity or binding capacity. Plots of $C_f$ versus $r$ for circular $\lambda$ DNA prepared from data in the previous paper (3) were used to obtain the moles of ethidium bound per mol of nucleotide at each free dye concentration. Values for the superhelix parameters at each of the maximums and minimums mentioned above were then calculated and are presented in Table IV.

Sedimentation Velocity of Species I as Function of Ethidium Concentration at Intermediate Salt Concentrations—The shape of the $S_{rel}$ versus $C_f$ curve at intermediate NaCl concentrations (Fig. 6) is intermediate between that seen for Buffer A alone and Buffer A plus 1 M salt. $S_{rel}$ in the absence of ethidium is lowered by the addition of salt. Binding ethidium causes an initial increase in the sedimentation rate of circular molecules, but as more dye is bound the loss of potential supercoils eventually leads to a minimal relative rate equal to 1.1. The relative sedimentation rates as a function of ethidium bound at the four ionic strengths examined are compared in Fig. 7. The values of $C_f$ and $r$ for the sedimentation minimum, where nicked and closed circular molecules co-sediment and the latter has no supercoils, are shown in Table V. Assuming a constant unwinding angle due to ethidium intercalation with a value equal to $-12^\circ$ (22), the superhelix parameters indicated in the table were calculated for circular molecules in the four different ionic environments.

**DISCUSSION**

As reflected by the sedimentation rate, the number of ethidium molecules which must intercalate to remove all supercoils from a circular $\lambda$ DNA is a function of the NaCl concentration. It was not possible to obtain good fluorometric data for Buffer A alone, but for the other salt concentrations, the free ethidium concentration at the major minimum in relative sedimentation rate is identical with the value of $C_f$ at the point of equivalent dye affinity determined fluorometrically (3). Although plots of $r$...
FIG. 4. Top, the relative sedimentation rate of circular \( \lambda \) DNA as a function of the free ethidium concentration in Buffer A (TE). Bottom, the relative sedimentation rate of circular \( \lambda \) DNA in Buffer A as a function of the moles of dye bound per mol of nucleotide (\( r \)). The values are listed in Table III: 0-0, Column f; O-O, Column h; \( \square - \square \), Column h.

**TABLE IV**

Summary of sedimentation behavior of circular \( \lambda \) DNA in 1.0 M NaCl and corresponding superhelix parameters

| Sedimentation designation | \( S_{rel} \) | \( C_f \) | \( r \) | \( \rho_{app} (r_0 - r) \) | \( \rho_{app} (\rho_1 - \rho) \) |
|---------------------------|-------------|---------|------|----------------|----------------|
| Control (no ethidium bromide present) | 1.56 | 0 | 0 | -208 | -0.045 |
| First minimum | 1.4 | 0.07 | 0.006 | -186 | -0.040 |
| First maximum (midpoint between the first minimum and the second minimum) | 1.7 | 0.80 | 0.085 | 96 | 0.021 |
| Second minimum | 1.1 | 2.3 | 0.067 | 0 | 0 |
| Second maximum | 1.8 | 6.1 | 0.092 | +76 | +0.017 |
| Third minimum | 1.6 | 11 | 0.107 | +124 | +0.027 |

\( S_{rel} \) is the apparent number of superhelical turns originally present in the molecule, and \( \rho_0 \) is the corresponding superhelix density calculated as described in the preceding paper (3).

versus \( C_f \) determined fluorometrically are used to convert these values of \( C_f \) to the corresponding binding ratios for estimating the number of supercoils, the end point determinations themselves are independent of each other. Their coincidence demonstrates that equivalent dye affinity and the co-sedimentation of nicked and closed circles are achieved by intercalating the same number of dye molecules, \( i.e. \) by unwinding the primary helix the same number of turns. Both methods support the conclusion that increasing salt concentration increases \( \tau^0 \) and therefore must change the average pitch of the Watson-Crick helix. Using the value of \( r \) at the minimum in \( S_{rel} \) measured in Buffer A as the reference state, the magnitude of the change is 0.29° in 0.1 M NaCl, 0.48° in 0.4 M, and 0.68° in 1.0 M NaCl.

We use the term unwinding for the effect of ethidium on the primary helix and winding for the effect of increasing the NaCl concentration. This is in keeping with the sense and handedness of supercoils as originally discussed (9) and with the view that ethidium intercalation unwinds the helix by 12° (22) rather than winding it by about the same amount (23). Our data, however, only demand that the effects of salt and ethidium be of opposite sign.

There have been a number of reports on the linear expansion of duplex DNA at low ionic strength as visualized by electron microscopy (24, 25). Expansion at low salt is believed to occur.
by a uniform partial unwinding of the normal B configuration helix. A salt-dependent winding at high ionic strength and unwinding at low is consistent with the results obtained in these studies. If circular \( \lambda \) DNA is supercoiled because the primary helix already is underwound (as circular molecules from natural sources all appear to be), further winding of the Watson-Crick helix in high salt will lead to an increase in the number of supercoils.

Increased temperature also causes an unwinding of the helix which is measurable with circular molecules (15). Therefore in this and the previous paper a constant temperature at 20° was maintained during sedimentation and dye affinity measurements.

We use the term potential supercoils in relating the number of ethidium molecules intercalated to the number of supercoils removed because we cannot be certain that this number physically exists. The physical and energetic requirements of supercoiling at high superhelix densities might induce changes in the primary helix which reduce the average rotation angle between base pairs (15, 26). The ethidium titration would yield the same end point as if the supercoils existed since it would require an equivalent unwinding to both return the helix to its normal parameters and remove the residual negative supercoils.

One reason for initiating this study was the conflict between the number of supercoils in \( \lambda \) DNA in high salt as estimated by electron microscopy (1) and by ethidium intercalation (15). When circular \( \lambda \) DNA was spread from a cytochrome \( c \) solution in 2 M NaCl, a majority of the molecules had fewer than 40 supercoils with an average of 12. Not only is this quantitatively very different from the 208 potential supercoils obtained here by ethidium intercalation and sedimentation studies, but it suggests an ionic effect of opposite sign.

Initially we were misled by the early minimum in \( S_{sel} \) (Fig. 5) and submitted an abstract indicating that the dye binding sedimentation data also suggested a lower number of supercoils at high salt concentrations (20). Fortunately, before the work was actually presented, the complete picture, as seen in Fig. 5, had emerged but the abstract remains in error. Since that report, the nonmonotonic sedimentation behavior in ethidium titrations of circular DNA with potentially high superhelix density has been observed by a number of other workers with circular DNA from several sources (13, 27–29). The shape and stiffness transitions reflected in the sedimentation rate pattern can also be detected by viscosity measurements (30).

From their electron micrographs of circular SV40 DNA,
(3 x 10^4 daltons) with various superhelix densities and from theoretical consideration of the hydrodynamic properties of circular DNA, Upholt et al. (27) proposed a model for changes in DNA tertiary structure as a function of superhelix density. It explains the nonmonotonic sedimentation patterns (similar to those presented here), on the basis of changes in looping, branching, and stiffness of the supercoiled DNA as a function of \( \sigma \).

Since it is clear that to remove all of the negative supercoils more dye must be bound at high salt concentrations than at low, the electron micrographs prepared from 2 M NaCl-cytochrome c solutions reported by Bode and MacHattie (1) remain as a discordant result. Those prepared from low ionic strength gave a reasonably consistent value for \( \tau^* \). Although additional experimental support would be necessary to establish the electron microscopy-result as other than an artifact of the method, other possibilities cannot be completely eliminated by the existing data.

A number of reports have suggested DNA may exist in solutions in one other than the B form (e.g. Refs. 31-33) and a forced transition in the secondary structure to a helix form, and sedimentation results in 1 M NaCl. When circular \( \lambda \) DNA supercoils more tightly, as it does in high salt, it might reach a state where it is energetically more favorable to alter the B form of the primary helix than to supercoil. As more bases per turn are accommodated in the distorted form of the primary helix, fewer supercoils are demanded and physically present. Nevertheless, removal of all supercoils by ethidium intercalation would require a complete reversal of the process as indicated in the discussion of actual and potential supercoils.

The properties of circular DNA can be acquired by any DNA under special conditions. When linear molecules are restricted at two points so that single strands of the duplex cannot rotate about each other, the region of DNA between the points behaves like a circle. In bacteria, topological division into a number of loops with independent supercoiling has been reported for the large folded chromosome of \( E. coli \) (34).

The shape transitions suggested by the change in \( S_{10} \) about the first local minimum (Fig. 5) occur over a relatively narrow range of superhelix density. Therefore, a topologically restricted region of linear DNA could be forced to undergo this change by a rather small variation in the local environment. It will be of interest to learn whether cells ever utilize this possibility in a biologically significant way to regulate the shape or function of their chromosomes.

REFERENCES

1. BODE, V. C. & MACIHATTIE, L. A. (1968) J. Mol. Biol. 32, 573-579
2. KIGER, J. A., YOUNG, E. T. & SINSHEIMER, R. L. (1968) J. Mol. Biol. 33, 599-613
3. HINTON, D. M. & BODE, V. C. (1975) J. Biol. Chem. 250, 1061-1070
4. KAISER, A. D. & HOGNESS, D. S. (1960) J. Mol. Biol. 27, 87-106
5. DISCHER, D. (1955) in The Nucleic Acids (CHARGAFF, E. & DAVIDSON, J. N., eds) Vol. 1, pp. 285-305, Academic Press, New York
6. CHEN, P. S., TOHBARA, T. Y. & WARNER, H. (1956) Anal. Chem. 28, 1756-1758
7. DURWALD, H. & HOFFMAN-BERLING, H. (1968) J. Mol. Biol. 34, 381-390
8. RADLOFF, W. R. & BAUER, W. R. & VINograd, J. (1967) Proc. Natl. Acad. Sci. U. S. A. 57, 1514-1521
9. BAUER, W. R. & VINOGRAD, J. (1968) J. Mol. Biol. 33, 141-171
10. BUJARD, H. (1968) J. Mol. Biol. 33, 503-505
11. CRAWFORD, L. V. & WARING, M. J. (1967) J. Mol. Biol. 25, 23-30
12. CRAWFORD, L. V. & WARING, M. J. (1967) J. Gen. Virol. 1, 297-300
13. GRAY, H. B., UPHOLT, W. B. & VINograd, J. (1971) J. Mol. Biol. 62, 1-19
14. WANG, J. C. (1969) J. Mol. Biol. 43, 263-272
15. WANG, J. C. (1969) J. Mol. Biol. 43, 25-39
16. SCATCHARD, G. (1949) Ann. N. Y. Acad. Sci. 51, 666-672
17. LE PECQ, J.-B. & PAOLETTI, C. (1967) J. Mol. Biol. 27, 87-106
18. BODE, V. C. & KAINER, A. D. (1965) J. Mol. Biol. 14, 399-417
19. LERMAN, L. S. (1961) J. Biol. Chem. 236, 18-30
20. HINTON, D. M. & BODE, V. C. (1969) Fed. Proc. 28, 532
21. HINTON, D. M. & BODE, V. C. (1967) Fed. Proc. 30, 1065
22. PIGRAM, W. J., FULLER, W. & DAVIES, M. E. (1973) J. Mol. Biol. 80, 361-365
23. PAOLETTI, C. & LE PECQ, J.-B. (1971) J. Mol. Biol. 69, 43-62
24. LANG, D., BUJARD, H., WOLFF, B. & RUSSELL, D. (1967) J. Mol. Biol. 25, 163-181
25. INMAN, R. B. (1967) J. Mol. Biol. 25, 200-210
26. MAESTRE, M. F. & WANG, J. C. (1971) Biopolymers, 10, 1021-1030
27. UPHOLT, W. B., GRAY, H. B., JR. & VINograd, J. (1971) J. Mol. Biol. 62, 21-38
28. BAUER, W. R. (1972) J. Mol. Biol. 67, 183-198
29. DENHARDT, D. T. & KATO, A. C. (1973) J. Mol. Biol. 77, 479-494
30. RÉVENT, B. M. J., SCHMIR, M. & VINOGRAD, J. (1971) Nature New Biol. 239, 10-13
31. BAUM, V., PILET, J., LAN, T. P., & HILL, L. R. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 3352-3355
32. BEAM, S. (1971) J. Mol. Biol. 59, 777-788
33. GORDON, C. N. (1972) J. Mol. Biol. 78, 601-615
34. WORCEL, A. & BURGI, E. (1972) J. Mol. Biol. 71, 127-147
Purification of closed circular lambda deoxyribonucleic acid and its sedimentation properties as a function of Sodium chloride concentration and ethidium binding.
D M Hinton and V C Bode

J. Biol. Chem. 1975, 250:1071-1079.

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