The early melting of closed duplex DNA: analysis by banding in buoyant neutral rubidium trichloroacetate

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ABSTRACT

Aqueous RbTCA permits the buoyant banding of both native and denatured DNA at room temperature and neutral pH. A unique property of this solvent is the buoyant resolution of closed circular, underwound DNA (I) from the corresponding nicked (II) species. Conditions are reported here in which PM-2 DNA I is physically resolved from native PM-2 DNA II, the buoyant separation being 1.27 mg/ml in 3.3 M RbTCA at 25°C. The separation between nicked and closed DNAs increases with temperature up to 35.5°C, at which PM-2 DNA II cooperatively melts and subsequently pellets. The isothermal buoyant density of a closed DNA increases linearly as the linking number (Lk) of the closed DNA decreases. The early melting of closed DNA may be monitored with high precision by buoyant banding in RbTCA, it being possible to detect the disruption of as few as 40 base pairs in PM-2 DNA (10,000 base pairs). The constraint that the linking number be conserved in closed DNA requires that a change in duplex winding be accompanied by a compensating change in supercoiling. We estimate the linking number deficiency of PM-2 DNA I to be 0.094 turns per decibase pair. This result permits the estimation of the EtdBr unwinding angle, ϕ , by comparison with alternative determinations of the linking number deficiency which depend upon the value of ϕ . The result obtained here is that $\phi = 27.7^\circ \pm 0.5^\circ$ and is approximately independent of temperature over the range 15° - 35°.

INTRODUCTION

Aqueous solutions of RbTCA* possess the appropriate chemical and physical characteristics which permit the buoyant banding of DNA in either the native or the denatured form at room temperature and neutral pH (1). Native DNA is buoyant at approximately 3.3 M salt at 25°C. As the concentration of this chaotropic salt is increased the DNA eventually denatures and exhibits a pronounced elevation of the buoyant density; denatured linear or nicked circular DNAs are buoyant at 4.5 M salt, also at 25°C. The density shift for the complete denaturation of PM-2 DNA II under these conditions is 174 mg/ml (1). In the present communication we monitor the chaotropic salt-induced helix-coil transition for both closed and nicked DNA in RbTCA by buoyant equilibrium sedimentation. By comparing the RbTCA buoyant density

of a partially denatured DNA with that of the native species, it is possible to detect a single-stranded region comprising as little as 0.4% of the molecule.

We find that the buoyant density of cdDNAs depends linearly upon the topological underwinding and is normally greater than that of ncDNA. The topological winding or linking number, Lk (2-4), is invariant in cdDNA; and all naturally occurring cdDNAs are underwound (reduced Lk) with respect to ncDNA. In an underwound cdDNA the duplex winding resists alternation from the thermodynamically preferred B form. Since Lk must remain constant, compensating supercoils are generated. With the possible exception of highly underwound DNAs, the number of supercoils is approximately equal to the linking deficiency.

Closed, underwound DNAs begin to denature in aqueous RbTCA, with a reduction in the number of supercoils, at temperatures and salt concentrations well below those required to produce the helix-coil transition in ncDNA. A similar non-cooperative early melting of cdDNAs in aqueous $NaClO_4$ and $Mg(ClO_4)_2$ has been described (5,6). This loss of supercoiling is primarily due to local base pair disruption. The reduction in supercoiling is therefore proportional to the RbTCA-induced buoyant separation of cdDNA from ncDNA. By selecting the appropriate buoyant salt and temperature conditions, we are consequently able to calculate the linking number deficiency of any closed DNA. This value is then compared to that determined by an EtdBr titration (which depends upon the EtdBr unwinding angle). By combining these results we estimate that the intercalative unwinding angle of this dye is $27.2^\circ \pm 0.5^\circ$.

MATERIALS AND METHODS

The preparation and aqueous properties of RbTCA have been described (1).

A. Source of DNAs. Bacteriophage PM-2 DNA was isolated and purified as described previously (1). SV40 DNA I was the generous gift of Robert Watson.

B. Preparation of HeLa Topoisomerase. A crude extract containing topoisomerase was isolated from HeLa cell nuclei (Fraction I of Vosberg *et al.* (7)). The enzyme preparation was frozen in a buffer containing 0.2 M potassium phosphate pH 7, 10% glycerol, 1 mM dithiothreitol, and 0.1 mM Na_3EDTA and stored in small aliquots at $-30^\circ C$. Activity was assayed by agarose gel electrophoresis (8).

C. Calculation of Linking Numbers. The value of Lk for cdDNA is the net number of interstrand revolutions. For a closed relaxed DNA, Lk_0 is approxi-

mately equal to the duplex winding number of ncDNA, N/ζ . Here N is the number of base pairs and ζ the duplex pitch, 10.0 bp/turn in drawn fibers (9) and 10.4 bp/turn in solution (10). The linking deficiency, ΔLk , is $Lk - Lk_0$ and is also invariant. In order to estimate ΔLk by a dye titration, v_c^0 is measured and $\Delta Lk = -(\phi/18) \zeta v_c^0$, where ϕ is the EtdBr unwinding angle (taken to be positive). The standard critical binding ratio, v_c^0 moles dye per mole nucleotide, is that needed to remove all supercoils at 37° and 0.2 M NaCl. This relationship is exact for cdDNAs prepared enzymatically under these conditions and is a good approximation otherwise. The binding ratio determined under CsCl/EtdBr buoyant conditions, v_c , is greater than v_c^0 by the constant value 0.016₇ (11). Finally, we define the specific linking deficiency as $\Delta Lk/N_{10}$, where the normalizing factor N_{10} is defined for convenience as $N/10$.

D. Preparation of PM-2 DNAs of altered Linking Number. PM-2 DNA I_L was prepared by incubating up to 50 μ g of PM-2 DNA I with a minimal volume of the topoisomerase preparation in incubation buffer (0.2 M NaCl, 0.02 M Tris pH 8.0, 2.5 mM Na_3EDTA , 1 μ g/ml BSA) for 30 min. at 37°C. The reaction was stopped by rapidly cooling to 4°, Na_3EDTA added to a final concentration of 10 mM, and NaCl added to a final concentration of 2.0 M. The DNA was pelleted in a Beckman SW65 rotor at 60 K $\times$ for 4 hr at 4°C then resuspended in NET buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.5, 2.5 mM Na_3EDTA). DNA I_L is completely relaxed under standard conditions defined as 0.2 M NaCl and 37° (11, 12) such that $\Delta Lk = 0$.

PM-2 DNAs of decreasing Lk were prepared by a similar incubation with topoisomerase in the presence of increasing concentrations of EtdBr (13). The DNA was isolated by buoyant banding in CsCl/EtdBr (14). Values of v_c for the resulting cdDNAs were determined from the EtdBr-induced buoyant separation from marker PM-2 ncDNA in CsCl (15, 49) and corrected to v_c^0 as described (12). The closed, underwound PM-2 DNAs thus prepared are listed in Table I.

E. Analytical Ultracentrifugation. Equilibrium buoyant density ultracentrifugation at 25° was performed as described previously (1). Equilibrium buoyant temperature shift experiments were conducted by establishing equilibrium at 15°C, then raising the temperature in 2.5° or 5° increments to 37.5°C. Generally 36-48 hr were required to reach equilibrium at each new temperature. No evaporation occurred over the course of the experiment, the radial position of the solvent meniscus having remained unchanged.

The practical buoyant densities (16) of all closed DNAs were determined

using PM-2 ncDNA as a marker (17) and employing the RbTCA buoyant density gradient (1). The apparent buoyant density, θ^a (the buoyant density at temperatures other than 25°C), was determined for ncDNA by calculating the solution density at band center employing the compositional density gradient (1), then applying the pressure correction (1, 17). The resulting value of θ^a is not corrected for the minor effects of solvent expansion (18). Values of θ^a for cdDNA species were calculated using the RbTCA buoyant density gradient (1) with PM-2 ncDNA as a marker. These calculations assume that all physical density gradients are independent of temperature.

RESULTS

A. Buoyant Density of Nicked and Closed Virion PM-2 DNAs in RbTCA. The practical buoyant density of native PM-2 ncDNA in 3.3 M RbTCA, 25°, is $1.480_8 \pm 0.001$ g/ml (1). Virion PM-2 DNA I, buoyant at $1.493_5 \pm 0.001$ g/ml under the same conditions, is completely resolved from the nicked species. Equilibrium buoyant profiles of PM-2 DNA I and of the corresponding ncDNA in RbTCA are presented in Figure 1, panel A. This separation of untitrated, chemically unmodified DNA I from native ncDNA is a unique property of aqueous neutral RbTCA density gradients. An analogous buoyant separation, but of much smaller magnitude, is obtained between partially titrated closed DNA and untitrated nicked DNA in alkaline CsCl over the very narrow pH range 11.4-11.8 (19, 20). In neutral CsCl at 20°, a very small increase in buoyant density (2 mg/ml) was reported with a closed PM-2 DNA sample of very low Lk (20). The buoyant shift was ascribed to the possible existence of denatured, untitrated regions in this highly stressed molecule. The chaotropic potency of RbTCA is sufficient to bring about the loss of most supercoils in PM-2 DNA I at 25° by a combination of two effects: a salt-associated reduction in duplex stability, and the concomitant release of the free energy associated with supercoiling.

B. Dependence of the Buoyant Density upon the Extent of Topological Underwinding. To determine the buoyant density of a closed DNA as a function of the extent of underwinding, a family of closed PM-2 DNAs was prepared as described above. The buoyant banding of highly underwound DNA I_E ($\nu_C^0 = 0.098$), with PM-2 ncDNA as a marker, is shown in Figure 1B. DNA I_E, banding at 1.501 g/ml, is 20.6 mg/ml denser than nicked DNA and 7.8 mg/ml denser than PM-2 DNA I. When a cdDNA with a relatively small winding deficiency is banded in RbTCA it has a characteristically lower buoyant density, as shown in Figure 1C. Here DNA I_B ($\nu_C^0 = 0.045$), buoyant at 1.488₁ g/ml, is not

Figure 1. Buoyant banding in RbTCA of a family of closed circular PM-2 DNAs with nicked circular PM-2 DNA as marker. The location of the PM-2 ncDNA band ($\theta = 1.480$, g/ml), detected by absorbance at 280 nm, is radially aligned in all three panels. The radial position of this band in each case was (A) 6.535 cm; (B) 6.530 cm; and (C) 6.589 cm. The lower abscissa indicates the radial distance relative to PM-2 ncDNA (Δr) and the upper abscissa the corresponding buoyant density shift ($\Delta\theta$). Approximately $0.5 \mu\text{g}$ DNA per band was added to 0.38 ml of solution at an initial solution density of 1.49 g/ml. The samples were centrifuged for 36 hr at 44 Krpm, 25°C . The cdDNAs employed and the relative buoyant density increments are: (A) virion PM-2 DNA I, 12.7 mg/ml; (B) PM-2 DNA I_E, 20.6 mg/ml; and (C) PM-2 DNA I_B, 7.3 mg/ml.

completely separated from the ncDNA marker band. For a family of cdDNAs, the RbTCA buoyant density increases as the linking number, L_k , decreases (ν_c^0 increases). The RbTCA buoyant densities and the specific linking defi-

ciencies of the cdDNAs employed here are listed in Table I. Values of ΔLk were calculated from v_c^0 as described in Materials and Methods.

The RbTCA-induced separation between closed and nicked DNAs most likely arises from a difference in the extent of intact base pairing. Underwound cdDNAs undergo an early helix-coil transition (23), driven by the positive free energy of supercoiling, at temperatures below T_m of the cognate nicked DNAs (19, 24). At the buoyant density of PM-2 ncDNA at 25°, RbTCA is sufficiently concentrated to induce the early melting transition in most underwound cdDNAs. The extent of duplex disruption and the associated buoyant density increase are determined by the magnitude of the free energy which drives the early melting. The free energy increases approximately with the square of the number of supercoils (24), thus a highly underwound closed DNA will be more extensively denatured upon transfer to RbTCA and will exhibit a greater buoyant density than a closed DNA with only a few initial supercoils. As a result the buoyant density of closed DNA in RbTCA varies inversely with Lk . The loss of duplex winding due to denaturation must be accompanied by a concomitant diminution in the number of supercoils. Partially melted closed DNA, buoyant in RbTCA, therefore has at most a small number of residual supercoils and contains one or more denatured regions.

C. Determination of the Extent of Early Melting for Closed DNAs. The buoyant density increment accompanying the DNA helix-coil transition is much

Table I: Buoyant Densities of DNAs of Decreasing Linking Number

DNA	v_c^0	$\Delta Lk/N_{10}^+$	θ_{25} (g/ml)
I_L	0	0	1.480 ₈
I_A	0.014 ₆	0.017 ₃	1.479 ₈
I_B	0.044 ₇	0.061 ₅	1.488 ₁
SV40 I	0.044 ₈	0.061 ₇	1.488 ₆
I_C	0.049 ₀	0.067 ₈	1.488 ₃
I_D	0.058 ₀	0.081 ₀	1.490 ₆
I	0.067 ₃	0.094 ₄	1.493 ₅
I_E	0.098 ₄	0.139 ₆	1.501 ₄
I_F	0.127 ₈	0.182 ₄	1.509 ₅
I_G	0.141 ₈	0.202 ₈	1.513 ₀

[†]The value $\phi = 26^\circ$ was used to calculate the specific winding deficiency from v_c^0 . N_{10} for PM-2 DNA is 1000 (21) and for SV40 DNA is 523 (22).

greater in RbTCA than in other buoyant salt systems (1). Buoyant banding in RbTCA is therefore a sensitive monitor of the helix integrity in a molecule containing both single-stranded and duplex regions. For the present case of cdDNA, the RbTCA buoyant density shift from marker native nicked DNA may be quantitatively related to the extent of early melting.

The fraction of bases unpaired, f , for a cdDNA buoyant in RbTCA may be calculated from the relative buoyant density shift, R , assuming that sequence heterogeneity may be neglected (19).

$$f = \frac{R}{k + (k - 1) R} \quad (1)$$

The parameter R is the ratio of the buoyant increment accompanying partial denaturation to that accompanying complete denaturation, $R = (\theta_I - \theta_{IIn}) / (\theta_{IId} - \theta_{IIn})$. Here θ_I is the buoyant density of the closed DNA, θ_{IIn} the buoyant density of native ncDNA, and θ_{IId} the buoyant density of denatured ncDNA. In equation (1), the factor k is the ratio $(M_{IIn}^{\theta_{IIn}} / M_{IId}^{\theta_{IId}})$, where M_{IIn} and M_{IId} are the solvated molecular weights of the neutral nucleotide subunits (including the counterion) in native and denatured DNA. The value of M for the native case, including the preferential hydration, is $M_{IIn} = M_3 (1 + \Gamma_{IIn})$. In general a subscript arabic numeral refers to a parameter associated with component 1, water; component 2, salt; or component 3, neutral anhydrous macromolecule (25). For RbDNA, M_3 is 393.35 daltons. The preferential hydration for native DNA, Γ_{IIn} (moles of water preferentially associated per mole of anhydrous neutral nucleotide), may be calculated from the appropriate buoyant density, θ_{IIn} , as (26)

$$\Gamma = \frac{M_3 (1 - \theta \bar{v}_3)}{M_1 (\theta \bar{v}_1 - 1)} \quad (2)$$

The partial specific volume of anhydrous RbDNA, $\bar{v}_3$, is 0.516 ml/g (27), and the partial specific volume of water, $\bar{v}_1$, is taken to be unity.

The fractional extent of denaturation, $f_{25,u}$, for a series of closed circular PM-2 DNAs is presented as a function of v_c and of the titratable superhelix density, N_T/N_{10} (12), in Figure 2. Here N_T is the apparent number of superhelical turns as determined by titration at the indicated temperature and salt concentration. The subscript u denotes that the result is uncorrected for the additional denaturation induced by the greater concentration of salt at the closed DNA band center relative to that at the marker. The magnitude of this effect is calculated below. Above a threshold value of N_T/N_{10} , the fraction of duplex denatured in the early melting transition is a linear function of N_T .

Figure 2. The fractional extent of denaturation at 25°C, $f_{25,u}$, of cdDNAs plotted as a function of the critical dye binding ratio, v_c , (lower abscissa), or the associated change in the titratable superhelix density, N_T/N_{T0} , where N_T is the titratable number of superhelical turns under the given experimental conditions (upper abscissa). The value of $f_{25,u}$ is calculated from the RbTCA buoyant densities presented in Table I using Eq. 5, and is uncorrected for the denaturing effects of the salt gradient (see text). All DNAs banded were PM-2 cdDNAs except for the darkened circle, which represents SV40 DNA I.

These experimental conditions, 3.3 M RbTCA and 25°C, are insufficiently denaturing to remove all supercoils, as demonstrated by the absence of a buoyant density shift for PM-2 DNA I_A. In order to follow the early melting transition of PM-2 DNA I to completion (defined by removal of all supercoils), more potent denaturing conditions are required. The salt concentration cannot be varied due to the requirement of buoyancy. We therefore vary the experimental temperature.

D. Temperature Dependence of Buoyant Density Increment. If the buoyant density difference between nc and cdDNAs arises from partial denaturation, then the magnitude of the buoyant separation should increase with temperature as additional base pairs are disrupted. The buoyant densities of PM-2 DNA I and of the ncDNA were determined at a series of temperatures between 15° and 37°C. The resulting buoyant band profiles, obtained at the temperatures 15°, 20°, 30°, 35° and 35.5°, are presented in Figure 3. Similar

Figure 3. Buoyant banding of PM-2 DNAs I and II at 15°, 20°, 30°, 35°, and 35.5° in RbTCA. Equilibrium was first attained at 15°C, and the temperature then raised sequentially to the indicated values. The radial distance from the center of rotation is shown on the abscissa. Scans were obtained with a Hewlett Packard 7004B X-Y recorder at 282 nm except for the 35°C profile, which was measured at 285 nm. The very sharp spike is the air-solution meniscus.

experiments were performed at 17.5° and 25°C (data not shown), with comparable results. Both DNA bands move to a region of greater solution density following a temperature increase. Below 35°C, the increment in buoyant density accompanying a temperature increase is less for PM-2 ncDNA, the lighter of the two species, than for PM-2 DNA I. Since $d\theta_{II}/dT < d\theta_I/dT$, the separation between native nicked and closed DNAs becomes greater with increasing temperature. The maximum resolution between bands, 1.24 mm, occurs at 30°C. At 35°C, the nicked species undergoes pronounced band broadening and a relatively greater density gain than at lower temperatures. Both these changes are characteristic of the initiation of denaturation. When the temperature is raised an additional 0.5°, PM-2 ncDNA completely denatures and pellets, while PM-2 DNA I remains buoyant. The midpoint of the melting transition for PM-2 ncDNA is 35.0°C in 3.29 M RbTCA at atmospheric pressure, as determined by monitoring absorbance changes (28). In contrast to the hypersharp melting of nicked DNA, the early region of the helix-coil transition for the closed molecule is noncooperative.

A similar multi-temperature ultracentrifuge melting experiment was performed employing SV40 DNA I and three additional PM-2 DNAs of different Lk. The apparent buoyant density, θ^a , for these DNAs is presented as a function of temperature in Figure 4. The buoyant density of all DNA species increases as a linear function of temperature. The value of the slope ($d\theta^a/dT$) is not constant but increases with decreasing Lk, as documented in Table II. The buoyant shift accompanying a temperature change is smallest for DNA I_L and is indistinguishable from that obtained for PM-2 ncDNA. PM-2 DNA I exhibits an intermediate dependence of the buoyant density upon temperature and the highly underwound PM-2 DNA I_E shows the largest such increase. The melting transition for PM-2 ncDNA, and the resultant large increase in θ_{II}^a near 35°, is indicated by the dashed line in Figure 4. At 35°C the solvent is sufficiently denaturing so that even the slightly supercoiled DNA PM-2 DNA I_L begins to melt. The density increment for this DNA is consequently greater at 35°C than at lower temperatures.

For both closed and nicked DNAs, the temperature dependence of the buoyant density arises from two separate effects: i) solvent expansion ($d\rho/dT$), an apparent buoyant density increase; and ii) a decrease in preferential hydration at constant water activity $(\partial\Gamma_n/\partial T)_{a_1}$, an actual buoyant density increase (18, 29). At temperatures within the melting region there is an additional contribution to the buoyant density increase due to the dehydration associated with denaturation, $(\partial\Gamma/\partial N_C)_T$, where N_C is the number

Figure 4. The apparent buoyant density (θ^a) for a series of DNAs in RbTCA plotted as a function of temperature. In order of increasing buoyant density the DNAs are PM-2 DNA I (▲), PM-2 DNA II (●), PM-2 DNA I_A (□), SV40 DNA I (■), PM-2 I (○), and PM-2 DNA I_E (Δ). The apparent buoyant densities were calculated as described in the text. At 25°C the apparent buoyant density is the same as the practical buoyant density, θ . The dashed line indicates the cooperative denaturation of ncDNA.

of nucleotide bases transferred from helix to coil. The magnitude of this dehydration is extraordinarily large in RbTCA (1). At any temperature below $T_{m,II}$, the buoyant density increment between a marker native ncDNA and a ccdNA therefore arises only from the early melting of the latter. The assumption is made that the loss of preferential hydration due to a change

Table II: The Temperature Dependence of RbTCA Buoyant Density for Circular PM-2 DNAs

DNA	Lk(Turns/DNA)*	$d\theta^a/dT(\text{g/ml}^\circ\text{C})^\dagger$ ($\times 10^4$)
II	(1000)	3.23 ± 0.11
I _L	1000	3.07 ± 0.14
I _A	982	3.50 ± 0.19
I	906	7.46 ± 0.25
I _E	860	8.55 ± 0.15

* Calculated from the data in Table 1.

† The apparent buoyant density uncorrected for the minor effect of solvent expansion. The slopes were determined for temperatures below 35°C.

in temperature is the same for native and for denatured DNA. Tunis and Hearst (29) have shown $(\partial\Gamma_d/\partial T)_{a_1}$ is slightly less than $(\partial\Gamma_n/\partial T)_{a_1}$ in KTFA. However in the present case the dominant contribution to the density increase arises from denaturation, and $(\partial\Gamma/\partial N_c)_T \gg (\partial\Gamma/\partial T)_{N_c}$. In addition, since the total fraction of base pairs denatured in the early melting of closed DNA does not exceed 23%, the contribution of a small difference between $(\partial\Gamma_n/\partial T)_{a_1}$ and $(\partial\Gamma_d/\partial T)_{a_1}$ to the total hydration change may be neglected.

E. Temperature Dependence of the Early Melting of Closed DNA in Buoyant RbTCA. The extent of denaturation of a cdDNA buoyant in RbTCA depends upon the temperature, salt concentration, and initial winding deficiency of the cdDNA. It was shown above (see Fig. 2) that in Buoyant RbTCA $f_{25,u}$ is a linear function of N_T . The quantity $f_{25,u}$ denotes the fraction of base pairs disrupted at 25° and at the salt concentration at the center of the buoyant band of the cdDNA. This salt concentration is, in general, greater than that at the location of the marker PM-2 ncdDNA. The salt concentration gradient is of sufficient magnitude so that closed DNAs of different initial superhelix density undergo an extent of early melting which depends upon their distance from the marker nicked DNA. This effect arises because, in addition to a density gradient, a substantial denaturation gradient is associated with the increase in chaotropic salt concentration. A closed DNA, buoyant at a greater solution density than the nicked species, is melted to a greater extent than would have occurred in the presence of the denaturation gradient.

At equilibrium the buoyant band will be located at the unique radial position at which the hydrated polymer density is the same as the solution density (30). The quantity θ may be expanded to express its dependence on the extent of denaturation induced by the radially increasing salt concentration.

$$\frac{d\theta}{dr} = \frac{\partial\theta}{\partial N_c} \times \frac{\partial N_c}{\partial [TCA]} \times \frac{\partial [TCA]}{\partial \rho} \times \frac{d\rho}{dr} \quad (3)$$

The requirement for band stability is that the increase in polymer density due to denaturation be less than the associated increase in solution density; that is, $d\theta/dr < d\rho/dr$. By combining this with equation (3) and rearranging, the condition for band stability may be expressed as

$$\frac{\partial N_c}{\partial [TCA]} < \frac{\partial N_c}{\partial \theta} \times \frac{\partial \rho}{\partial [TCA]} \quad (4)$$

The product $\partial N_c / \partial \theta \times \partial \rho / \partial [TCA]$ is nearly constant at constant temperature, except for a possible slight dependence upon pressure. Equation (4) predicts that a partially denatured species remains buoyant so long as the cooperativeness of the melting transition does not exceed a critical extent. Since the early melting of closed DNAs is noncooperative, helix-coil transition intermediates are observed as stable bands (Fig. 3). Similarly, at the onset of the helix-coil transition for nicked DNA the value $\partial N_c / \partial [TCA]$ is small enough so that partially melted DNAs remain buoyant. Such a transition intermediate for nicked DNA at 35° is also shown in Fig. 3. As denaturation proceeds in a highly cooperative manner for nicked DNA, the value $\partial N_c / \partial [TCA]$ increases rapidly. The stability condition (Eq. 4) is eventually no longer satisfied and the DNA pellets. The thermally induced melting transition is expected to be even more cooperative in the presence of a destabilizing gradient than in a solution of uniform concentration, a prediction also borne out in alkaline CsCl gradients (31).

We next estimate the magnitude of the denaturation gradient,

$$\frac{dN_c}{dr} = \frac{\partial N_c}{\partial [TCA]} \times \frac{d[TCA]}{dr} \quad (5)$$

and calculate the extent of melting which would have occurred had all closed DNAs been buoyant at the salt concentration at the band center of PM-2 ncDNA. The dependence of the extent of denaturation for closed DNA upon the chaotropic salt concentration is given by

$$N_{c,r_{II}} = N_{c,r_I} - \frac{\partial N_c}{\partial [TCA]} \times \frac{d[TCA]}{dr} \times (r_I - r_{II}) \quad (6)$$

Here $N_{c,r_{II}}$ and N_{c,r_I} are the number of disrupted bases in a closed DNA hypo-

thetically buoyant at the salt concentration found at the actual band locations of the nicked (r_{II}) and closed (r_I) species. The value of $N_{c,r_{II}}$ may be calculated, using equation (6), from measured apparent buoyant densities.

$$\theta_{r_{II}}^a = \theta_{r_I}^a - \frac{\partial \theta}{\partial N_c} \times (N_{c,r_I} - N_{c,r_{II}}) \quad (7)$$

Equations (6) and (7) are combined and rearranged to

$$\theta_{r_{II}}^a = \theta_{r_I}^a - \frac{\partial \theta}{\partial N_c} \times \frac{\partial N_c}{\partial [TCA]} \times \frac{d[TCA]}{dr} \times (r_I - r_{II}) \quad (8)$$

In the absence of information to evaluate directly $\partial N_c / \partial [TCA]$, we make use of the linear relationship between temperature and $[TCA]$ presented previously (28) to equate a difference in salt concentration to a difference in effective temperature, T_{eff} . Equation (8) then becomes

$$\theta_{r_{II}}^a = \theta_{r_I}^a - \frac{\partial \theta}{\partial T_{eff}} \times \frac{\partial T_{eff}}{\partial [TCA]} \times \frac{d[TCA]}{dr} \times (r_I - r_{II}) \quad (9)$$

The equilibrium salt redistribution, $d[TCA]/dr$, calculated for a 1.1 cm solution column at 44 Krpm and at the isoconcentration coordinate from the compositional density gradient (1, 32) is 0.605 M/cm at atmospheric pressure. The molar effectiveness of aqueous RbTCA as a denaturant, $dT_m/d[TCA]$, is 31.0°/M in the concentration range 3.1-3.9 M (28). This information permits a change in salt concentration to be related to the change in temperature which would cause the same extent of denaturation. We calculate for the equilibrium salt gradient that the effective temperature gradient is

$$\frac{dT_{eff}}{dr} = \frac{\partial T_m}{\partial [TCA]} \times \frac{d[TCA]}{dr} = 18.8^\circ/\text{cm} \quad (10)$$

For example, at 25°C the effective temperature difference between marker PM-2 ncDNA and PM-2 DNA I is 2.0°C, corresponding to a salt concentration difference of 0.06 M. Under the same conditions, the effective temperature increase for DNA I_E is 3.1°C (0.1 M). The value of T_{eff} at the center of the closed DNA band is equal to $[T_{r_{II}} + \frac{dT_{eff}}{dr} (r_I - r_{II})]$. For any closed DNA with a denatured region sufficiently large so that end effects may be neglected, the slope $\partial \theta / \partial T_{eff}$ is nearly constant. The degree of denaturation at constant temperature and salt concentration, f , calculated for closed DNAs using Eqs. (1) and (9), is plotted as a function of v_c for several temperatures in Figure 5. For a closed DNA with a large denatured region such that the nucleation free energy required to initiate melting may be neglected, df/dv_c is nearly constant and independent of temperature. The best least squares values of this slope at 25°, 30°, and 35°, respectively, are 1.51 ± 0.02 , 1.57 ± 0.04 , and 1.47 ± 0.03 . The slopes at 15° and at 20°, while not

Figure 5. The degree of denaturation, f , for closed DNAs buoyant in RbTCA at a series of temperatures plotted as a function of the critical dye binding ratio, ν_c , or the change in N/N_{10} . The equilibrium buoyant banding temperatures employed are 35° (○); 30° (△); 25° (□); 20° (×); and 15° (●). The calculation of f from the RbTCA buoyant density is discussed in the text.

evaluated, are not greatly different based upon the available data. The limit of precision in these experiments for the determination of $\Delta\theta$ is ± 0.5 mg/ml, corresponding to a fractional degree of denaturation of 0.004. If the number of base pairs in PM-2 DNA is taken to be 10,000 (21), then the limit size for detection of denaturation from the buoyant shift in RbTCA is approximately 40 base pairs. This calculation demonstrates the extreme sensitivity of the RbTCA buoyant method to changes in secondary structure.

F. The Intercalative Unwinding Angle of EtdBr. The EtdBr unwinding angle may be calculated by comparing estimates of ΔLk determined from the early melting in RbTCA with those based upon measuring the amount of dye required to unwind the superhelix. The method of calculation is similar to that employed in the alkaline buoyant titration in CsCl (19, 20). The buoyant and dye binding methods are related by

$$f = \frac{\zeta}{180} (\phi) (\nu_c) \quad (11)$$

where ζ is the number of base pairs per duplex turn under the RbTCA banding

conditions. This assumes that the residual superhelix density for closed DNAs in buoyant RbTCA is the same for all DNAs and is given by the abscissa intercept where $f = 0$. Employing a straight-forward thermodynamic analysis, Wang (20) has shown that this assumption is valid near the endpoint of the early melting transition. It should be emphasized that the unwinding angle refers to the conditions of the ν_c - based assay, buoyant CsCl/EtdBr at 20°. Using the value of the slope ($df/d\nu_c$) calculated from the data of Figure 5 and Eq. (11), and taking $\zeta = 10.4$ (10), we calculate that $\phi = 27.2^\circ \pm 0.5^\circ$.

DISCUSSION

Melting of Closed DNA. A linking number deficiency in cdDNA dramatically alters many of the fundamental physical and chemical properties of this molecule compared to both relaxed cdDNA and to ncDNA (12, 20, 23, 24, 33-35). All naturally occurring superhelical DNAs behave as if the duplex winding were incomplete at the time of covalent ring closure. The restoration of the optimal duplex winding generates supercoiling. The associated positive free energy may be released via a variety of pathways. For example, the relative binding affinity towards intercalating dyes is enhanced or inhibited for a closed DNA depending on whether supercoils are removed or formed during the reaction. In addition, closed underwound DNA is more susceptible to cleavage by single-strand specific nucleases, has a greater initial affinity for single-strand specific reagents and binding proteins, and initiates thermal or alkaline denaturation at a lower temperature or pH.

The difference in stability between closed and nicked duplex DNAs is clearly demonstrated by comparing their thermal or chaotropic salt-induced melting profiles. As demonstrated previously (28) the helix-coil transition for nicked DNAs in 4 M RbTCA is cooperative and occurs over a narrow temperature range of 5.6°C. The same transition for closed DNA is substantially broadened, to greater than 45°C, and begins at a lower temperature. The spectroscopic technique employed in the earlier work (28), while covering the entire melting transition for both closed and open DNAs, reveals little detail of the early melting region. The method of band velocity sedimentation permits a variation of salt concentration at constant temperature. It is clear from these results (28) that closed DNAs undergo an early structural transition resulting in reduced supercoiling. The unique properties of buoyant RbTCA permit a more quantitative analysis of the early melting of a closed DNA. First, the chaotropic potency of the solvent reduces the melting

transition region to an experimentally accessible range at neutral pH. Second, the large buoyant density increment upon denaturation allows detection and precise determination of the extent of partial denaturation.

The melting transition for buoyant nicked DNA in RbTCA in the ultracentrifuge is hypersharp. As shown in Figure 3, the temperature span of the melt is about 0.5°C compared to a value of 5.6°C measured spectrophotometrically (28). The enhanced cooperativeness of the duplex disruption results from the denaturation gradient present in the ultracentrifuge, as pointed out above.

Comparison with Other Estimates of ϕ . The value of the EtdBr unwinding angle was long taken to be 12° based on model building (36). Experimental support for this value came from the determination (19) that the number of supercoils in polyoma DNA measured by buoyant alkaline titration (independent of ϕ) is approximately the same as that of SV40 DNA measured by titration in buoyant EtdBr/CsCl (37) taking ϕ to be 12°. Since these two viruses are similar in size and in many other characteristics, the experimental agreement was taken to be evidence for the 12° unwinding angle. This experimental approach was subject to several potential errors, which the authors carefully pointed out, and which in fact led to an underestimate of the actual extent of supercoiling. More recently the buoyant titration in alkaline CsCl was repeated (20) with the result $\phi = 26^\circ \pm 2.6^\circ$. This larger value is also supported by the EtdBr titration of closed DNAs of known unwinding produced by carbodiimide binding (38), $\phi = 24^\circ - 36^\circ$, and by electron microscopic length measurements of PM-2 DNA I hybridized with restriction fragments (39), $\phi = 28.5^\circ$. Keller (40) and Shure and Vinograd (41) have independently counted the number of bands on agarose gels for intermediates in the generation of relaxed, closed circles by topoisomerase to determine the number of supercoils in SV40 DNA. Comparison of this value to that obtained by EtdBr/CsCl buoyant banding results in estimates of 28° and 23°, respectively. Recently, X-ray crystallographic studies of iodinated dinucleoside monophosphate: dye complexes with ethidium (42, 43) yields a value of 10° for the angular orientation between the dinucleoside base pairs in a fragment of a DNA helix. Assuming that the intercalated dye: dinucleoside complex is on realistic model for an intercalated drug: DNA complex, this leads to a prediction of about 26° for the drug-induced unwinding angle in DNA (44). A similar estimate, 23°, was obtained by X-ray diffraction of a 2:1 complex of CpG and acridine orange (45). The magnitude of the intercalative unwinding angle determined here is $27.2^\circ \pm 0.5^\circ$ and is consistent with

these estimates. It should be emphasized that these values are averages, and no information is yet available concerning whether or not ϕ varies with dye occupancy or with base sequence.

Onset of the Salt-Induced Early Melting. The pitch of the DNA duplex may be increased by two mechanisms: a general unwinding in which base pairing remains intact but the number of base pairs per duplex turn, ζ , decreases; or a local denaturation in which single-stranded, unpaired regions are formed. For a closed DNA buoyant in RbTCA, the magnitudes of both the reduction in supercoiling and the buoyant density increase relative to ncDNA suggest that denaturation is the dominant effect. However, since the value of ζ in buoyant concentrations of RbTCA enters into the calculation of ϕ , it is necessary to estimate the extent to which ζ varies as conditions are changed from those of the standard state. The transfer of PM-2 DNA I from standard conditions (0.2 M NaCl, 37°C) to the experimental environment (3.4 M RbTCA, 35°C) may be considered to occur through a series of intermediate, elementary steps. The transfer of DNA I from 0.2 M NaCl to 0.2 M RbCl at constant temperature removes 5.4 supercoils (11). The subsequent transfer from 0.2 M RbCl to 0.2 M RbTCA removes one additional supercoil. This effect is not expected to arise from local denaturation, since the transfer occurs well below the melting region of ncDNA. The total duplex winding reduction of approximately 6.1 turns is probably due to a slight overwinding of the duplex in 0.2 M RbTCA relative to 0.2 M NaCl. In the next step the salt concentration is increased from 0.2 M to 3.4 M. For both NaCl and RbCl, non-denaturing solvents, the number of supercoils is relatively insensitive to changes in ionic strength in the range beyond 0.26 M (11). An equivalent increase in the chaotropic TCA⁻ concentration is, by contrast, expected to induce denaturation. The final transfer step, involving a shift in temperature from 37° to 35°C, introduces approximately 1 supercoil (11). We then estimate that in the absence of denaturation (TCA⁻ effect) the supercoiling of closed PM-2 DNA is reduced by 7 turns relative to its extent under standard conditions. This reduction in supercoiling would correspond to a change in duplex winding of less than 1%. Since PM-2 DNA contains approximately 94 supercoils, local denaturation would therefore appear to account for by far the greater part of the early melting observed in buoyant RbTCA.

Implications for DNA Structure in Solution. It has recently been suggested (46, 47 and see 48 for other references) that the duplex structure of DNA is not a double helix of the Watson-Crick type but that the two strands are in an essentially side-by-side configuration. The evidence and arguments

against this proposal are strong (10, 48) and, in our opinion, convincing.

Fundamentally, all such alternative models require that Lk_0 be either small or zero. In the case of PM-2 DNA, experiments based ultimately upon band counting in gel electrophoresis demonstrate absolutely that ΔLk for this molecule is close to 94.4 turns (12). Our present results have allowed us to calculate independently the extent of duplex denaturation required to remove the associated superhelical turns. The value of f at which this occurs for PM-2 DNA I is readily calculated from Figure 5 by drawing a line through the origin with the same slope as the bracketing lines. This line represents the value of f , denoted f_0 , associated with ΔLk for each DNA. For PM-2 DNA I, $f_0 = 0.102$. This value must, due to the nature of the buoyant experiments (see above), represent the fractional duplex winding associated with ΔLk . In general, then, $\Delta Lk/Lk_0 = f_0$. Combination of the above measurements results in the estimate $Lk_0 = 925$. The value of Lk_0 predicted by the original Watson-Crick model is 1000, assuming 10.0 base pairs per turn. In solution, however, the pitch appears to be about 10.4 base pairs per turn (10), leading to the expectation that $Lk_0 = 960$ for the double helix of PM-2 DNA in solution. It is therefore clear that the two strands of this DNA are in fact linked nearly exactly to the extent predicted by the classical double helical model. All proposed alternative structures which predict that Lk_0 is significantly different from ten base pairs per turn are therefore ruled out.

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- * Abbreviations used are: TCA, trichloroacetate; TFA, trifluoroacetate; Na₂EDTA, trisodium salt of ethylenediaminetetraacetic acid; Tris, tris (hydroxymethyl)aminomethane; EtdBr, ethidium bromide; DNA I, native covalently closed circular duplex DNA; cdDNA, closed duplex DNA; ncDNA or DNA II, nicked circular DNA.
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