Energy—structure correlations of plasmid DNA in different topological forms

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ABSTRACT
Differential scanning microcalorimetry (DSC), UV absorption and circular dichroism (CD) have been used to study structure and stability of linear (lin), open circular (oc), supercoiled (cd) and relaxed circular duplex (rd) DNA and calf thymus (CT) DNA. Investigations were made in low salt buffer and in the presence of 7.2 M NaClO₄. The chaotropic action of perchlorate promotes a reduction of the overall stability of DNA, which permits a direct determination of the transition enthalpies of all four DNA configurations. The stabilities against thermal denaturation have been found to increase in the series lin~oc<cd<rd. These relative stabilities can be rationalized on the basis of the linkage between supercoiling and secondary structural changes in topologically constrained duplex DNA. On the basis of these studies, a model of the melting process could be suggested that is consistent with the energetic and spectroscopic data.

INTRODUCTION
Supercoiled DNA is an example for the linkage between energy, structure, and function in biological macromolecules (1). The topological state of DNA affects its replication (2-4), recombination (5-8), transcription (9-11), nucleosome assembly (12-14), and repair (15, 16). The unique structural features, which characterize naturally occurring supercoiled molecules and determine their increased biological reactivity are single strand regions (17-19), cruciform structures (2, 20-25) and stretches of left handed Z-form (26-30).

Though all these secondary structure adjustments of circular DNA achieve a partial decrease of the unfavorable Gibbs energy originating from the linking deficiency, the resulting molecule is still under strain and thermodynamically unstable relative to its relaxed circular form. Recently we have shown for Col El amp (31) and other DNAs (paper in preparation) that the unfavorable Gibbs energy of supercoiling is predominantly determined by the enthalpic contribution. Thus the torsional and bending deformations of supercoiled DNA and the local transitions to alternative secondary structures mentioned before are mainly the result of enthalpy partitioning.

It is of considerable interest to determine the enthalpy required to dissociate base pairs in supercoiled (cd), relaxed (rd) and open circular (oc) DNA and to compare them with corresponding values for linear (lin) DNA. Introduction of an operational enthalpy criterion
would considerably facilitate theoretical estimates of the equilibrium structure, which are usually complicated or even precluded by the requirement of an exact evaluation of the entropy terms in the expression for the free energy.

High sensitivity scanning microcalorimetry has been successfully applied to thermal studies on linear DNAs (32) and it is highly desirable to perform analogous investigations on circular DNAs. However, the stability of circular DNAs involves serious experimental problems. Melting of base pairs in supercoiled and relaxed DNA extends over a large temperature range and is finished only well above 100°C (33). This prevented precise microcalorimetric measurements in the past, most instrumentation being limited to scanning up to 100°C. The advent of new microcalorimeters that can measure up to 130°C solved the instrumental problem. A more serious principle obstacle is, however, the occurrence of single strand breaks above approximately 90°C, which can only be circumvented by decreasing the transition temperature to below 90°C.

An appropriate procedure to promote the overall decrease in melting temperature is to use chaotropic salts such as sodium perchlorate (34), rubidium trichloroacetate (35, 36) and tetraethylammonium bromide (37). We have employed 7.2 M sodium perchlorate in the buffer to complete melting of both supercoiled and relaxed DNA below 100°C.

Since microcalorimetry can only monitor the enthalpy changes involved in base unstacking and breaking hydrogen bonds, subtle structural changes such as premelting (38) might escape detection by this method. Therefore we employed CD and UV techniques to follow these phenomena in more detail. In general good correspondence with respect to the overall shape and transition temperature has been found between the UV and scanning microcalorimetric studies suggesting that both techniques monitor the same processes. CD spectroscopy provided additional information on structural changes that were not detected by UV absorption and microcalorimetry.

MATERIALS AND METHODS

Restriction endonuclease Eco RI was purchased from Renner GmbH, Mutterstadt, DNAse I from Sigma, Munich, FRG.

E. coli Strains and Plasmids

E. coli strain M 609 harboring Col El amp (11 000 bp) was a gift of Dr. R. Mattes (Boehringer, Tutzing, FRG). Plasmid pWH931 (3 823 bp) in E. coli strain RR1 was a gift from W. Hillen (University of Erlangen, FRG). Plasmid pUC19 (2 686 bp) was transformed into E. coli HB101. Plasmid pOE 101 (5 700 bp) was a gift of K. Beyreuther, University of Köln.

Isolation of Plasmid DNA

E. coli strains were grown in LB-medium, chloramphenicol treated and lysed by NaOH/SDS treatment as described by Maniatis et al. (39). In the case of Col El amp plasmid cell lysis followed the procedure of Voordouw et al. (40). Plasmid DNA was purified by centrifugation in a cesiumchloride gradient in the presence of 0.5 μg/ml ethidium bromide.

Preparation of DNAs with Different Configurations

Linear DNAs were prepared from cd DNA by digestion with restriction endonuclease Eco RI. Open circular Col El amp DNA was prepared from cd DNA by limited digestion with DNAse I (40). Relaxed duplex plasmid DNA was prepared by incubating cd DNA with
topoisomerase I from *E. coli* in 10 mM Tris, 2 mM MgCl₂, 1 mM EDTA, pH 8, for 30 min at 37°C.

**Preparation of Topoisomerase I from *E. coli***

Topoisomerase I from *E. coli* was isolated from *E. coli* cells according to the procedure of Wang (41) with modifications introduced by H.P. Vosberg (MPI Heidelberg unpublished results). The modifications comprise chromatography steps using phosphocellulose, hydroxylapatite and DNA cellulose. DNA cellulose was prepared as described by Litman (42).

**Agarose gel electrophoresis**

Electrophoresis was used for qualitative and quantitative characterization of the plasmid configuration. 19x19x0.5 cm³ horizontal slab gels were employed, containing 0.7% agarose and 0.4 μg/ml ethidium bromide. The electrophoresis buffer consisted of 89 mM Tris, 89 mM boric acid, 2,5 mM EDTA, 0.4 μg/ml ethidium bromide pH 8. A constant current of 45 mA was applied for 14 h.

**Determination of the Percentage of cd DNA**

DNA concentration was determined by UV absorption measurements at 260 nm, using an extinction coefficient of $E_{260 \text{ nm}} = 20 \text{ cm}^2 \text{ mg}^{-1}$. Dilution experiments of plasmid DNA from 7.2 M NaClO₄ to salt free buffer showed that the extinction coefficient was independent of the presence of the salt. The percentage of cd DNA was obtained from scans at 560 nm of negatives of polaroid photos taken from the agarose gels under UV illumination.

**Heat Capacity Scanning Microcalorimetry**

These measurements were made employing an electronically modified scanning microcalorimeter DASM (43) at various scanning rates (0.2 to 1°C/min), though most frequently at 1°C/min. The instrument has been interfaced to an Aljos microcomputer for automatic data collection and processing. The calorimetric data are digitized and stored in floppy disks at 0.1 or 0.01°C intervals for subsequent analysis. The DNA solutions were dialyzed at 6°C to equilibrium with 4 changes of the buffer (11). Typical DNA concentrations employed were in the order of 1 mg/ml. For each DNA measurement a calibration run was performed with reference and sample cell filled with equilibrium dialysis buffer. Base pair molecular weights of 654 and 658 were used for Col El amp DNA and calf thymus DNA, for pUC 19 and pWH931 a molecular weight of 660 was employed. The partial specific volume was taken to be $v = 0.547 \text{ cm}^3 \text{ g}^{-1}$ (40).

**CD-Studies**

Circular dichroism was measured using thermostated cells of 1 cm or 0.1 cm optical path length in a Jasco J-500 A spectropolarimeter. The cell was heated in steps of 5 to 10°C outside the transition range and 2 to 5°C in the transition range. The temperature of the solution was measured inside the cell using a YSI temperature probe. At each temperature a CD spectrum (320 nm to 215 nm) was recorded and corrected for the baseline. For calculation of molar ellipticities the same base pair molecular weights were used as for the enthalpy calculations.

**UV-Absorption Studies**

Variation of the absorbance at 260 nm with temperature was measured using a thermostated cell (1 cm optical path length) in a Gilford spectrophotometer 2400. The heating rates were 0.5 to 1°C per minute. Differentiation of the OD vs. temperature profiles was done manually at 0.5 deg. intervals. Optical densities of the solutions used did not exceed a value of 1.5 at room temperature.

**RESULTS**

**CD-Spectra at 25°C**

Typical CD spectra at room temperature are shown in Fig. 1. In low salt buffer (Fig. 1a) the absorption bands are conservative exhibiting the short wavelength negative and long wavelength positive ellipticity with a minimum at 245 nm and a maximum at about 270 to 280 nm. In 7.2 M NaClO₄ solution (Fig. 1b) the positive absorption is greatly reduced.
and the CD spectra are characteristic of what has often been referred to as "C-type DNA" conformation by having a practically unchanged negative and a greatly reduced positive band (44). There is, however, no appreciable shift of either the maximum or minimum position. In both buffers there are characteristic differences in the negative and positive dichroic bands of the various plasmid DNA configurations. For calf thymus DNA which we measured to have a reference sample of known parameters, there is quantitative agreement with literature data (45).

Melting studies in low salt buffer

a) UV and CD Measurements

The optical studies on calf thymus and Col El amp DNA using 5 mM cacodylate, 1 mM EDTA, pH 7 gave the results summarized in Figs. 2a, b and 3d-i. Ellipticity at 270 and 245 nm (left ordinates, solid curves) and ΔOD/ΔT at 260 nm; (right ordinates, dashed curves) have been plotted as a function of temperature. The UV transition curves of Col El amp DNA in both its linear and oc configuration show a higher degree of fine structure than those of calf thymus DNA, indicating the occurrence of highly cooperative subtransitions (46, 47). Due to its stability, cd DNA cannot be dissociated within the experimentally accessible temperature range. Therefore UV absorption monitors only the small peak around 68°C which results from melting of oc- and lin DNA impurities and the beginning of the main melting peak which extends beyond 100°C.

The temperature dependence of circular dichroism is characteristically different for the negative ellipticity at 245 nm and the positive ellipticity at 270 nm. The negative ellipticity at 245 nm of calf thymus, lin, oc and cd Col El amp DNA varies only insignificantly in the premelting region but shows a strong increase to less negative values during the main transition. The similarity of the temperature dependence of ellipticity at 245 nm for all four types of DNA renders observation at that wavelength not very useful for detection of the subtle predenaturational conformational changes.

However, it is worth noting that negative CD and UV absorption do not monitor the

Figure 1:

Circular dichroism spectra of calf thymus DNA (CT) ----, and various conformations of Col El amp plasmid DNA: linear (lin) ---; open circular (oc) ----; superhelical (cd) ---

A: low salt buffer solution (5 mM cacodylate, 1 mM EDTA, pH 7, 20°C):
CT-DNA: 0.057 mg/ml
cd Col El amp DNA: 0.054 mg/ml, 96% pure
oc Col El amp DNA: 0.051 mg/ml, 94% pure
lin Col El amp DNA: 0.060 mg/ml, purity >98%

B: High salt buffer solution (15 mM sodium citrate, 7.2 M NaClO₄, pH 7.0, 20°C):
CT-DNA: 0.029 mg/ml
cd Col El amp DNA: 0.051 mg/ml, 96% pure
oc Col El amp DNA: 0.056 mg/ml, 94% pure
lin Col El amp DNA: 0.045 mg/ml, purity >98%
Figure 2:

Variation with temperature of ultraviolet absorption (260 nm) (differential curves shown as dashed lines have ordinates on the right) and circular dichroism (245 nm and 270 nm) (solid curves) of calf thymus DNA (CT).

- a, b) [CT-DNA] = 0.057 mg/ml; 5 mM cacodylate, 1 mM EDTA, pH 7.0.
- c, d) [CT-DNA] = 0.029 mg/ml; 15 mM citrate buffer, 7.2 M NaClO₄ pH 7.0.

We monitored the positive ellipticity at 270 nm as a function of temperature to follow the predenaturational alterations of the secondary and tertiary structure. Calf thymus (Fig. 2b) and lin Col El amp DNA (Fig. 3g), exhibit ellipticity changes as a function of temperature which are qualitatively similar. The open circular (Fig. 3h) and closed duplex forms (Fig. 3i) of the plasmid DNA show different temperature dependences. oc DNA does not exhibit an increasing positive ellipticity in the premelting region as does lin DNA, while cd DNA shows a gradual decrease.

b) Calorimetric measurements

In low salt buffer (5 mM cacodylate, 1 mM EDTA, pH 7) only linear- and open circular DNA can be completely dissociated in the calorimetrically accessible temperature range.
Figure 3:
Variation with temperature of heat capacity (a-c), UV absorption (d-f) and CD (d-i) of lin, oc, and cd Col E1 amp DNA in low salt buffer (5 mM cacodylate, 1 mM EDTA, pH 7.0) (differential UV absorption curves shown as dashed lines have ordinates on the right).

a) lin DNA: concentration 0.663 mg/ml, purity >98%.
b) oc DNA: concentration 0.351 mg/ml, purity 94%, 6% lin.
c) cd DNA: concentration 0.472 mg/ml, purity 92%, 8% oc and lin DNA (note the different scale of the $c_p$-axis in c).
d) UV and CD (245 nm) melting curves of lin DNA (0.060 mg/ml), purity >98%.
e) UV and CD (245 nm) melting curves of oc DNA (0.051 mg/ml), 93% pure.
f) UV and CD (245 nm) melting curves of cd DNA (0.054 mg/ml), 94% pure.
g) CD (270 nm) melting curve of lin DNA (0.060 mg/ml).
h) CD (270 nm) melting curve of oc DNA (0.051 mg/ml).
i) CD (270 nm) melting curve of cd DNA (0.054 mg/ml).

(3 to 110°C); the high stability of cd and rd DNA prevents complete unfolding. This is illustrated by Figs. 3a-c which show the heat capacity vs. temperature profiles of lin, oc, and cd Col E1 amp DNA. It is worth noting that in Fig. 3c the heat capacity scale is one tenth of that in Figs. 3a and 3b. The peak around 68°C represents the oc and/or lin DNA impurity of approximately 8%.

The fine structure of lin DNA is slightly more pronounced than that of oc DNA. This may be due to the different ways of preparation, because digestion with DNAsel I in the course of preparation of oc DNA may result in nicks randomly distributed over the plasmid,
thus reducing the cooperativity of DNA stretches observable in our measurements. It is, however, also possible that it reflects a slightly different mechanism of unfolding, since also the temperature dependence of CD was found to be different for lin and oc DNA. (Figs. 3g,h).

However, major subtransitions are very similar and the maximum of apparent heat capacity occurs at approximately 68°C in both configurations. Integration of the transition peaks yields very similar stabilization enthalpies of $\Delta H_{st} = -36.4 \pm 2.0 \text{kJ/mol of base pair}^{-1}$ and $\Delta H_{st} = -37.2 \pm 1.3 \text{kJ/mol of base pair}^{-1}$ for oc DNA and linear DNA, respectively. For purposes of comparison with known values of transition enthalpies, analogous measurements were performed with calf thymus DNA in the cacodylate buffer (line 4 of Table 1). Thermodynamic parameters for lin, oc and CT DNA are listed in Table 1.

**Melting Studies in 7.2 M NaClO₄**

**a) UV and CD measurements**

Complete transition curves for all configurations of Col El amp DNA could be observed in 7.2 M NaClO₄, 15 mM citrate buffer pH 7.0 (34). Cacodylate buffer was replaced by 0.15 M citrate to allow for concentration measurements by phosphate analysis (48) in addition to absorption measurements. It has been demonstrated that both methods give identical results in 7.2 M sodium perchlorate, when employing the same extinction coefficient as in diluted buffer solution.

To illustrate the typical variations with increasing temperature of the circular dichroism, spectra of cd (4a) and rd pOE 101 DNA (4b) are presented in Fig. 4. It is evident that there are alterations of the shape and height of the negative and positive bands of the spectra. However, it is also obvious from the solid lines which connect ellipticities at 270 nm

<table>
<thead>
<tr>
<th>DNA species</th>
<th>$T_{0.5}, \degree C, (K)$</th>
<th>$\Delta G_{st} (298 \text{ K})$ [kJ/mol bp]</th>
<th>$\Delta H_{st}$ [kJ/mol bp]</th>
<th>$\Delta S_{st}$ [J/mol bp-K]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col El amp lin</td>
<td>68 (341.15)</td>
<td>-4.7</td>
<td>-37.2 ± 1.3</td>
<td>-109</td>
</tr>
<tr>
<td>Col El amp oc</td>
<td>67 (340.15)</td>
<td>-4.5</td>
<td>-36.4 ± 1.3</td>
<td>-107</td>
</tr>
<tr>
<td>Calf thymus⁰</td>
<td>64 (337.15)</td>
<td>-3.7</td>
<td>-31.8*</td>
<td>-94.3</td>
</tr>
<tr>
<td>Calf thymus*</td>
<td>75.5 (348.65)</td>
<td>-5.8</td>
<td>-40.0 ± 2.0</td>
<td>-115</td>
</tr>
</tbody>
</table>

Enthalpy of melting for lin and oc Col El amp DNA in 5 mM cacodylate, 1 mM EDTA, pH 7.0 was determined by DSC. The Gibbs energy of stabilization $\Delta G_{st}$ was calculated from the equation $\Delta G_{st} = \Delta H_{st}(1 - T/T_{0.5})$, where $T = 298.15 \text{K}$ and $T_{0.5}$ is the respective melting temperature in K. $\Delta H_{st}$, the association enthalpy, is assumed to be independent of temperature. Entropy, $\Delta S_{st}$, was calculated using the relation $\Delta S_{st} = (\Delta H_{st} - \Delta G_{st})/T$. The thermodynamic parameters of calf thymus DNA labeled by the superscript ⁰ refer to one measurement in the cacodylate buffer. Therefore no error limits are given. The CT DNA results labeled by a * refer to studies in 15 mM citrate buffer pH 7.0. The larger transition parameters reflect the higher ionic strength of the citrate relative to the cacodylate buffer. Both experiments yielded transition enthalpies that are very similar to data reported in the literature which were obtained under comparable ionic strength conditions. The 2 digits after the point reported for the absolute temperatures are not meant to imply higher accuracy than for the °C. They just result from addition of 273.15 K in the conversion process. All energy and entropy parameters refer to one mole of base pairs.
Figure 4:
CD spectra of cd and rd pOE 101 DNA at different temperatures in 15 mM citrate, 7.2 M NaClO₄, pH 7.0 (temperatures are given in °C).

a) cd DNA: (0.051 mg/ml), purity >94%
b) rd DNA: (0.046 mg/ml), purity 96%

The solid curves connect ellipticities at 270 nm at the various temperatures to demonstrate that the temperature courses of the ellipticities of the two topological forms of pOE 101 DNA are similar to those given for cd and rd ColE1 amp DNA in Figs. 5k and 5l.
at the various temperatures, that the dependence on temperature of the ellipticity is very similar to that observed for cd and rd Col E1 amp DNA (Figs. 5k,l) in the same buffer.

Fig. 5 shows the variations with temperature of heat capacity, UV and CD. Dashed curves in Figs. 5e-h refer to ΔOD/ΔT versus temperature plots, solid lines to CD vs. temperature graphs. Calorimetric transition profiles are shown in Figs. 5a-d.

The variation with temperature of the negative ellipticities at 245 nm are again qualitatively similar for calf thymus DNA (Fig. 2b) and all configurations of Col E1 amp DNA. (Solid lines in Fig. 5e-h). The positive ellipticities at 270 nm display configuration specific differences (Fig. 5i-l). Particularly the topologically constrained cd and rd DNAs show temperature variations of molar ellipticity which are different from those of the other DNA configurations (Figs. 5k,l).

![Figure 5:](image-url)

**Figure 5:**
Variation with temperature of heat capacity (a-d), UV absorption (e-h) and CD (e-l) of lin, oc, cd and rd Col E1 amp DNA in 15 mM sodium citrate, 7.2 M sodium perchlorate, pH 7.0 solution. Dashed differential UV melting curves (e-h) have ordinates on the right and CD melting curves (solid curves) have ordinates on the left.

- a) lin DNA: 0.828 mg/ml, purity >98%
- b) oc DNA: 0.385 mg/ml, purity 95%, 5% lin
- c) cd DNA: 1.565 mg/ml, purity 94%, 6% lin and oc
- d) rd DNA: 1.300 mg/ml, purity >98%
- e, i) lin DNA: 0.045 mg/ml for UV and CD studies, purity >98%
- f, j) oc DNA: 0.051 mg/ml for UV and CD studies, purity >98%
- g, k) cd DNA: 0.056 mg/ml for UV and CD studies purity >98%
- h, l) rd DNA: 0.055 mg/ml for UV and CD studies, purity 96%
The changes in the premelting region of cd DNA (Fig. 5k) are opposite to those of lin and oc DNA. Ellipticity decreases between 20 and 40°C, where UV absorption and heat capacity remain constant. Between 50 and 70°C ellipticity increases again. In this temperature range, both UV and heat capacity transition curves clearly indicate partial base pair dissociation. Above 75°C, where the majority of base pairs is disrupted, ellipticity decreases slightly but considerably less than for lin and oc DNA.

It is worth noting that at 20°C the absolute value of the molar ellipticity at 270 nm of cd DNA is clearly higher than that of lin, oc, and rd DNA.

The temperature course of the ellipticity at both 270 and 245 nm was also determined for rd DNA as shown in Figs. 5h,l. In contrast to cd DNA there is no change in the positive ellipticity at 270 nm up to 40°C and the absolute value for the positive ellipticity in this range is somewhat lower than that for cd DNA. From 40°C to 75°C there is an increase in the positive ellipticity, and the maximum value reaches that of cd DNA. Above 75°C it is again decreasing.

b) **Calorimetric studies on Col El amp DNA, pUC 19 and pWH 931**

The decrease in stability resulting from the presence of 7.2 M sodium perchlorate in 0.15 M citrate buffer pH7 is illustrated in Figs. 5a-d. Apparent heat capacity has been plotted vs. temperature for linear (5a), oc DNA (5b), cd DNA (5c) and rd Col El amp DNA (5d). Typically in 7.2 M sodium perchlorate the fine structure of the transition peak is less pronounced, the apparent heat capacity maximum is decreased by approximately 18°C, and the stabilization enthalpies are reduced.

The enthalpy values in sodium perchlorate buffer are listed in Table 2. For oc DNA and linear DNA they are again similar within experimental uncertainty, but by $-15(-18$ respectively) kJ/(mol base pair)$^{-1}$ less negative than the corresponding $\Delta H_{st}$ values in low salt buffer.

The minor peak at 50°C in Figs. 5c and 5d results from the presence of oc DNA.

<table>
<thead>
<tr>
<th>DNA species</th>
<th>$T_{0.5}$, °C, (K)</th>
<th>$\Delta G_{st}$ (298 K) [kJ/mol bp]</th>
<th>$\Delta H_{st}$ [kJ/mol bp]</th>
<th>$\Delta S_{st}$ [J/mol bp·K]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col El amp lin</td>
<td>50.5 (323.65)</td>
<td>$-1.7$</td>
<td>$-22.1 \pm 0.9$</td>
<td>$-68$</td>
</tr>
<tr>
<td>Col El amp oc</td>
<td>51 (324.15)</td>
<td>$-1.7$</td>
<td>$-21.8 \pm 0.7$</td>
<td>$-67$</td>
</tr>
<tr>
<td>Col El amp cd</td>
<td>75 (348.15)</td>
<td>$-3.8$</td>
<td>$-26.8 \pm 0.8$</td>
<td>$-77$</td>
</tr>
<tr>
<td>Col El amp rd</td>
<td>75.6 (348.15)</td>
<td>$-4.1$</td>
<td>$-27.9 \pm 0.9$</td>
<td>$-80$</td>
</tr>
<tr>
<td>Calf thymus$^{U}$</td>
<td>46 (319.15)</td>
<td>$-1.5$</td>
<td>$-22.6^{*}$</td>
<td>$-71$</td>
</tr>
</tbody>
</table>

Thermodynamic parameters of various configurations of Col El amp DNA in 15 mM citrate buffer, pH 7.0 containing 7.2 M sodium perchlorate have been derived from DSC measurements using the equations given in Table 1; superscript $^{U}$ denotes that 1 control experiment in the same buffer was performed.
impurities. This is particularly evident from Fig. 6 where the original calorimetric recordings of the melting curves of cd pWH 931 DNA and lin pWH 931 DNA are superimposed. These curves demonstrate also clearly that no detectable Δc_p is associated with melting of either DNA species. This finding is typical for all calorimetric studies presented here. It is worth noting, since it implies temperature independent transition enthalpies and entropies.

Integration of the heat capacity vs. temperature peak in Fig. 5c gives a stabilization enthalpy of \( \Delta H_{st} = -26.8 \pm 0.8 \text{kJ(mol of base pair)}^{-1} \). This \( \Delta H_{st} \) value is more negative than the stabilization enthalpy observed for oc- and linear DNA in the same buffer.

Analogous heat capacity measurements were performed on relaxed circular \( ColE1 \) amp DNA. The shape of the transition profile of relaxed DNA (Fig. 5d) is very similar to that of supercoiled cd DNA. However, the stabilization enthalpy \( \Delta H_{st} = -27.9 \pm 0.9 \text{kJ(mol of base pair)}^{-1} \) is even more negative than that of supercoiled cd DNA.

In order to have a reference \( \Delta H \) value in 15 mM citrate, 7.2 M sodium perchlorate solution pH 7.0, the transition enthalpy of calf thymus DNA, which is well known for a variety of solvent conditions, has also been determined. A stabilization enthalpy of \( \Delta H_{st} = -22.6 \text{kJ(mol of base pair)}^{-1} \) was found for CT DNA which is very similar to the \( \Delta H \) values determined for linear and oc \( ColE1 \) amp DNA.

A summary of the thermodynamic parameters of the various topological forms of \( ColE1 \) amp DNA and of CT DNA is given in Table 2.
Table 3: Thermodynamic Stability Parameters of the Plasmids pUC19 and pWH 931 in 7.2 M Sodium Perchlorate

<table>
<thead>
<tr>
<th>DNA species</th>
<th>$T_{0.5}$, °C, (K)</th>
<th>$\Delta G_{st}$ (298 K) [kJ/mol bp]</th>
<th>$\Delta H_{st}$ [kJ/mol bp]</th>
<th>$\Delta S_{st}$ [J/mol bp-K]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19 lin</td>
<td>51.5 (324.65)</td>
<td>-1.9</td>
<td>-22.7 ± 0.3</td>
<td>-70</td>
</tr>
<tr>
<td>pUC19 oc</td>
<td>51.5 (324.65)</td>
<td>-1.9</td>
<td>-22.7 ± 0.3</td>
<td>-70</td>
</tr>
<tr>
<td>pUC19 cd</td>
<td>79.7 (352.85)</td>
<td>-4.1</td>
<td>-26.3 ± 0.5</td>
<td>-75</td>
</tr>
<tr>
<td>pUC19 rd</td>
<td>80.2 (353.35)</td>
<td>-4.3</td>
<td>-27.4 ± 0.9</td>
<td>-78</td>
</tr>
<tr>
<td>pWH 931 lin</td>
<td>52.8 (325.95)</td>
<td>-2.0</td>
<td>-24.0 ± 0.2</td>
<td>-74</td>
</tr>
<tr>
<td>pWH 931 cd</td>
<td>79.7 (352.45)</td>
<td>-4.2</td>
<td>-27.2 ± 0.7</td>
<td>-77</td>
</tr>
<tr>
<td>pWH 931 rd</td>
<td>80.4 (353.55)</td>
<td>-4.5</td>
<td>-28.4 ± 0.9</td>
<td>-80</td>
</tr>
</tbody>
</table>

Thermodynamic parameters of various configurations of plasmids pUC19 and pWH 931 in 15 mM citrate buffer, pH 7.0 containing 7.2 M sodium perchlorate have been derived from DSC measurements using the equations described in Table 1.

To confirm the generality of our results for the different configurations of circular DNA in sodium perchlorate buffer additional measurements with two other plasmids, pUC 19 and pWH 931 were made. The remarkable result of these studies is the finding that the thermodynamic parameters observed for pUC 19 and pWH 931 are rather similar to those measured for identical configurations of Col El amp DNA and, even more importantly, that the same rank order of stability lin~oc<cd<rd is followed. Thus we can conclude confidently that the differences in the thermodynamic stability parameters summarized in Tables 2 and 3 reflect general energy differences determined by the different superstructure and are not an accidental result for one plasmid. Again the $\Delta H$ values for the rd configurations are more negative by about 1 kJ/(mol of base pairs) than those of the cd configurations.

DISCUSSION

Melting of the various configurations of plasmid DNA in low and high salt buffer is a complex phenomenon that requires different techniques for its analysis. Each of the three methods applied in these investigations, scanning microcalorimetry, UV-, and CD absorption, provides characteristic information and the three together yield a satisfactory description of the energetic and structural events involved in the temperature induced changes of secondary and tertiary structure of DNA.

The three techniques monitor different aspects of the transitions: the variation with temperature of heat capacity seen by microcalorimetry reflects the additive energetic effects of changes in hydrogen bonding, base stacking, hydration, ion binding, and torsional and bending rigidities; ultraviolet absorption measures changes in hyperchromicity which primarily reflect base stacking interactions; CD spectroscopy monitors the difference in absorption between left and right circularly polarized light by an asymmetric molecule and is therefore sensitive to alterations in the relative orientation of neighboring chromophores.
Although the three methods characterize the transition via different windows, common features can be recognized. In general there is good correspondence between the melting curves obtained by UV absorption and scanning microcalorimetry. This pertains to the transition range, the midpoint temperature of the transition, and to the fine structure of the transition curves. Therefore the major effects seen by UV and calorimetric studies are associated with base pair dissociation. CD seems to react sensitively to minor reorientations of the secondary and tertiary structure of DNA that need not involve base pair unstacking. These premelting phenomena of changes of the positive band of CD (around 270 nm) have been studied before but primarily on linear DNAs (45, 49-53).

Melting in Low Salt Buffer

a) Optical studies

The data for calf thymus DNA in low salt buffer (Fig. 2a) are in good agreement with published CD transition curves (45) at 278 nm. The shape of the 270 nm melting curve of linearized Col El amp DNA is similar to that of calf thymus DNA but it differs somewhat from the shape of the 270 nm melting curve of the oc species; the latter does not exhibit the premelting increase of the positive CD with temperature. There is, nevertheless, a difference between the CD-premelting behaviour of lin and oc DNA that remains, just as there is a difference between lin and oc DNA in the differentiated UV melting curves with regard to resolution of subtransitions. The 270 nm CD-band of cd Col El amp DNA (Fig. 3i) continuously decreases with increasing temperature as one would expect from the temperature induced removal of negative superhelical turns with increasing temperature. According to (54, 55) the increased positive CD of supercoiled DNA relative to that of the same DNA in linear form is proportional to the number of superhelical turns. Using a temperature coefficient of $1.2 \times 10^{-2}$ deg/(K-base pair) (56) an increase in temperature from 20 to 80°C is expected to remove 22 negative superhelical turns.

Around 80°C cd DNA commences to melt as can be seen in Fig. 3f. The small peak around 70°C results from the 8% oc and lin DNA impurity that was detected in cd DNA.

Although the positive CD at 270 nm is particularly sensitive to predenaturational structural changes, the negative CD at 245 nm too reflects to some extent structural changes before complete base pair dissociation. This is already apparent from its variation with temperature at low salt (Figs. 3d-f) but it becomes quite obvious in 7.2 M perchlorate (Fig. 5e-h), where transition temperatures as derived from CD curves would always be lower than those derived from UV melting curves.

b) Calorimetric melting studies of lin and oc Col El amp DNA

Within experimental error the low salt $\Delta H_{st}$ and $\Delta G_{st}$ values of oc and lin DNA are the same (ca. $\Delta H_{st} = -37$ and $\Delta G_{st} = -4.6$ kJ/(mol of base pair)$^{-1}$), which suggests that the circular single strand remaining after oc DNA strand separation does not base stack to any
detectably higher extent than the linear strands. Under the same conditions the parameters of calf thymus DNA are $\Delta G^\circ_t = -3.7 \text{kJ/(mol base pair)}^{-1}$ and $\Delta H^\circ_t = -31 \text{kJ/(mol base pair)}^{-1}$. This latter value is in excellent agreement with enthalpies reported for calf thymus DNA in various other comparable buffer systems (57).

Inspection of the DSC transition curves of lin and oc DNA reveals a remarkable degree of fine structure. Occurrence of fine structure has been noticed before in UV transition curves for native linear $Col\ El$ DNA (58) and for a variety of other DNAs (46,47). We did not attempt to perform a quantitative analysis of the DSC curves in terms of cooperative subtransitions, since it would exceed the scope of the present study. Qualitatively these transitions reflect the highly heterogeneous G-C and A-T base pair distribution, which probably plays an important functional role. It has been demonstrated that the origin of replication of $Col\ El$ DNA is particularly rich in G-C base pairs (58). In a recent DSC study it has been shown that the fine structure of transition curves is correlated to gene organisation of $Col\ El$ amp plasmid DNA (47).

**Melting in 7.2 M Sodium Perchlorate**

Molecular interpretation of the present melting studies rests largely on the knowledge of the actual structure that the various DNA species assume at room temperature. The characteristic change from a conservative to a nonconservative CD spectrum shown in Fig. 1a,b for low and high salt, respectively, has frequently been interpreted as indicating a structural change from a B-type structure to a "C-type" structure (44, 45, 51). Although this interpretation seemed plausible for linear DNAs, since increasing salt concentration and lowering temperature both increase the average helix rotation angle (51), the structure responsible for the CD spectra in Figure 1b cannot be the standard C-type structure of DNA (59) derived from fiber studies. The average changes of the rotation angle caused by the addition of salt in solution are much smaller than those observed for the C-type structure in fibers. An increase of only 0.8° in rotation angle is obtained when the salt concentration is increased from 0.05 M NaCl to 5.4 M NH$_4$Cl (60), whereas C-DNA differs from B-DNA among other features by a 2.8° larger rotation angle.

For the interpretation of the melting studies we assume that the same change in rotation angle, i.e. 0.8°, occurs when the plasmids are transferred from low salt buffer to 7.2 M sodium perchlorate solution. This assumption appears to be justified in view of the results by Anderson and Bauer (61) which demonstrated that ammonium ions were most, sodium ions least effective in promoting the change towards a larger rotation angle.

**Number of Superhelical Turns of cd and rd $Col\ El$ amp DNA in 7.2 M NaClO$_4$**

At 20°C in low salt buffer the $Col\ El$ amp DNA used in our studies had a superhelix density of 0.057, which is equivalent to a formal number of 60 titratable negative superhelical turns (N = 11 000 bp). Transfer into 7.2 M sodium perchlorate solution will produce
approximately 24 additional negative superturns in the cd DNA, using the above value of 0.8° for the increase in rotation angle. Covalently closed plasmid DNA should thus have 84 negative superhelical turns in 7.2 M sodium perchlorate solution, if the stability of the DNA were the same as in low salt buffer.

7.2 M NaClO₄ unwinds approximately 3.9% of PM2 cd DNA at room temperature (62). Since at high salt concentrations changes in CD (51) as well as in stability (Fig. 5) were found to be independent of base composition, we use the same percentage for calculation of open base pairs in Col El amp DNA. This yields 429 open base pairs that remove 429/10.4 = 41 negative superhelical turns. The actual number of negative superhelical turns that cd DNA assumes at room temperature is therefore approximately 84 — 41 = 43.

rd DNA on the other hand can for all practical purposes be assumed to be relaxed under these buffer conditions. This is supported by the close similarity of the magnitude of the 270 nm CD signal for rd and oc DNA at room temperature. The corresponding 270 nm CD signal of cd DNA in perchlorate is decidedly more positive, which in view of the results of Maestre and Wang (54) and Gray et al. (55), is indicative of the existence of negative supercoiling.

Thus the CD data at 25°C of cd and rd DNA in 7.2 M NaClO₄ (Fig. 5k,l) can be interpreted as showing the existence of negative supercoiling in cd DNA and essentially no supercoiling in rd DNA. This conclusion is important for the assignment of the ΔH values to the structural changes associated with melting.

Variation with Temperature of the Number of Superhelical Turns of cd and rd DNA

Temperature increase causes a decrease of the duplex winding angle of 0.012 deg/(K·bp) (56, 61), thereby removing negative superhelical turns or, alternatively, introducing positive turns. A temperature increase of 30°C thus removes 11 negative superhelical turns in cd DNA. From the calorimetric melting curves one can estimate the percentage of 3.2% dissociated base pairs at 55°C. This corresponds to 35 superhelical turns removed by base pair dissociation. Overall this results in a formal number of (—43) — (—46) = 3 superhelical turns, reflecting the relaxation of cd DNA at this temperature. The CD for cd DNA reaches a minimum in the vicinity of 55°C, taken as support of the previous estimates.

Further increase in temperature with concomitant decrease in duplex winding angle should result in the introduction of positive superhelical turns due to the invariance of the linking number. However, increase in temperature promotes also further base pair dissociation that would relax positive supercoiling. Superposition of these effects is suggested as being responsible for the observed dependence on temperature of the positive CD signal. The CD melting curves of oc- and lin Col El amp and calf thymus DNA in 7.2 M sodium perchlorate demonstrate that base pair dissociation corresponds to a decrease of the 270 nm CD. Increase of CD with increasing temperature above 55°C cannot be explained by single
strand formation. We tentatively imply that the increase in CD of the cd DNA up to approximately 75°C is suggestive of the formation of positive supercoiling.

The interpretation gains some support from the temperature course of the positive CD signal of rd DNA in the same buffer. We have reasoned that at room temperature rd DNA is close to being relaxed. The appearance of positive superhelical turns should therefore start at a lower temperature than for cd DNA. Fig. 51 shows that the 270 nm CD of the rd DNA commences to increase around 40°C where no base pair dissociation can be detected yet by UV absorption or microcalorimetry.

Stability of CT, lin and oc Plasmid DNA in 7.2 M NaClO₄ Buffer

Addition of 7.2 M perchlorate to the buffer solution leads to a substantial decrease in stability of all configurations of DNA. The lower stability is reflected in both a lower transition temperature and a lower transition enthalpy. An intriguing phenomenon is the similarity of the transition enthalpies of calf thymus DNA, and lin- and oc DNA in 7.2 M NaClO₄, although at low ionic strength there are significant differences between the ΔH values. (Table 1: -31.8 compared to -37.2 kJ/(mol of bp)). The magnitude of the ΔH value is conspicuously similar to twice the value that has been found for unstacking enthalpies of single stranded synthetic polynucleotides in neutral aqueous solution (32).

The general decrease in stabilizing enthalpy to a common value of about -23 kJ (mol base pair)⁻¹ may be rationalized in the following manner. The presence of 7.2 M NaClO₄ in the buffer causes an extensive breakdown of water structure (63-65), i.e. highly concentrated perchlorate solutions behave at room temperature like high-temperature aqueous systems. Walrafen (65) found that the Raman spectrum of water in 4 M NaClO₄ solutions at 25°C is equivalent to that of an aqueous solution without perchlorate at about 135°C. Such a loss of water structure results in a decrease in helix stabilizing hydrophobic interactions.

In addition to the decrease in hydrophobic interaction caused by the presence of 7.2 M NaClO₄, a second effect resulting from the salt is probably the competition with the DNA for hydration water. Following the results of Wolf and Hanlon (66), 36 mol H₂O (mol base pair)⁻¹ are required for maintaining the unperturbed B form of the double helix. In view of the sensitivity of DNA structure to changes in water activity the reduced water activity in the presence of 7.2 M NaClO₄ (67) is likely to promote destabilization (68). A decrease in the hydration would by itself lead to a significant increase in repulsion of the phosphate groups (68) as well as to a decrease in the stacking energy (66,69-71).

For 7.2 M NaClO₄ the cation concentration is high enough to shield DNA phosphate ions effectively and thereby keep repulsion low. Thus the reduction of the stabilizing enthalpy is probably not due to increased electrostatic repulsion but to reduced DNA hydration, resulting from the high ion concentration in solution. Destruction of hydration could also explain the decrease in cooperativity of the transition, since a hydration "spine" of the A-T base pairs is of vital importance for maintaining the intact B configuration (72,73).
Stability of topologically constrained DNA

Both supercoiled and relaxed circular DNAs are in general more stable than oc- or lin DNA against thermal denaturation. The higher stability of cd DNA has usually been attributed to the smaller configurational entropy gain on denaturation. This argument is, however, superficial, since the thermodynamic parameters relevant to stability are not the enthalpy and entropy changes of the macromolecule alone but those of the whole system, including hydration changes, ion redistribution etc. That this difference between configurational entropy changes of the macromolecule and entropy changes of the system is essential has been recently demonstrated by relaxation studies of Col El amp DNA in the presence of topoisomerase I (31). The present thermal unfolding studies support this view. The overall entropy changes (Tables 2 and 3) on unfolding (positive $\Delta H$ and $\Delta S$ values) of cd and rd DNA are larger than those of the corresponding linear or oc DNA. This is in contrast to what one would expect on the basis of theoretical calculations of the relative increase of the configurational entropy on unfolding of linear and cd DNA (74,75).

A straightforward method to establish relative Gibbs energies of stabilization, is to measure the enthalpies of denaturation, $\Delta H$, and the corresponding temperatures of 50% conversion, $T_{0.5}$. The Gibbs energies of stabilization, $\Delta G_{st}$, can then be calculated according to the relationship $\Delta G_{st} = \Delta H_{st}(1 - T/T_{0.5})$, which assumes a temperature independent $\Delta H$.

An intriguing result of this study is the dependence of the transition enthalpy and

![Figure 7: Schematic diagram of the sequence of molecular events involved in temperature induced melting of superhelical duplex DNA. From left to right: negatively supercoiled DNA with single strand regions ($r < 0$); relaxed DNA ($r = 0$); positively supercoiled DNA ($r > 0$); interwound coils.](image)
Gibbs energy on the topological state of the macromolecule. There are two questions, which need clarification a.) why are the transition enthalpies of both supercoiled and relaxed DNA higher than the corresponding $\Delta H$ values of the linear and oc forms? b.) why is the enthalpy of relaxed DNA larger than that of negatively supercoiled DNA?

The molecular interpretation, which answers both questions and which is consistent with the present studies is the following: We assume that the intrinsic stabilizing enthalpies per base pair in 7.2 M NaClO₄ solution are the same for all configurations of the plasmids. Therefore the different magnitude of the measured $\Delta H$ has to result from energy releasing or energy absorbing conformational changes occurring in the course of denaturation. Thus in addition to energy parameters DSC provides also information on the path of unfolding. cd DNA comprises negative superhelical turns in the presence of 7.2 M sodium perchlorate while rd DNA can be considered as being relaxed. The sequence of molecular events involved in melting of circular duplex DNA that is consistent with the optical and calorimetric studies presented here and with the known effects of sodium- and perchlorate ions on the structure and stability of DNA is schematically shown in Fig. 7. It provides a qualitative understanding why the transition enthalpies of cd and rd DNA are larger than those of lin and oc DNA: introduction of positive superhelical turns and coiling of the circular single strands requires the excess enthalpy. It also explains the different $\Delta H$ values for cd and rd.

Any increase in temperature will result in removal of negative superhelical turns (56). Since the linking number is unchanged by temperature increase, the reduction in the number of superhelical turns below the melting transition results in a reduction of torsional and bending stress and, concomitantly, in energy release (31). At some temperature a relaxed circular molecule occurs, which, on further temperature increase, will have to adopt positive superhelical turns in the residual undissociated duplex.

If melting is started with relaxed instead of negatively supercoiled DNA, there is no initial energy release associated with reduction in the number of negative supercoils, but temperature increase results immediately in introduction of positive supercoils and partial base pair dissociation. This must be the reason, why the transition enthalpy involved in melting of relaxed DNA is larger than that of negatively supercoiled DNA.

It is not possible at present to derive from these overall transition enthalpies a $\Delta H$ value for superhelix formation which could be compared to the thermodynamic parameters determined by isothermal microcalorimetry (31). One reason is that the two reference states are different, the relaxed circular DNA duplex at 37°C on the one hand and the coiled interwound, single strands at 75°C on the other hand.

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