The energetic basis of the DNA double helix: a combined microcalorimetric approach

Paulius Vaitiekunas, Colyn Crane-Robinson and Peter L. Privalov*

Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA

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ABSTRACT

Microcalorimetric studies of DNA duplexes and their component single strands showed that association enthalpies of unfolded complementary strands into completely folded duplexes increase linearly with temperature and do not depend on salt concentration, i.e. duplex formation results in a constant heat capacity decrement, identical for CG and AT pairs. Although duplex thermostability increases with CG content, the enthalpic and entropic contributions of an AT pair to duplex formation exceed that of a CG pair when compared at the same temperature. The reduced contribution of AT pairs to duplex stabilization comes not from their lower enthalpy, as previously supposed, but from their larger entropy contribution. This larger enthalpy and particularly the greater entropic results from water fixed by the AT pair in the minor groove. As the increased entropy of an AT pair exceeds that of melting ice, the water molecule fixed by this pair must affect those of its neighbors. Water in the minor groove is, thus, orchestrated by the arrangement of AT groups, i.e. is context dependent. In contrast, water hydrating exposed nonpolar surfaces of bases is responsible for the heat capacity increment on dissociation and, therefore, for the temperature dependence of all thermodynamic characteristics of the double helix.

INTRODUCTION

Understanding that two complementary DNA strands are wound into a double helix and that their separation and copying is the key process in replication of the genetic information (1) immediately raised interest in the energetic basis of this molecular construction, that is the forces between the complementary strands and the work needed for their separation. Solution of this problem has required development of two special calorimetric techniques, Differential Scanning and Isothermal Titration Calorimetry (DSC and ITC). With the appearance of these instruments (for microcalorimetry evolution see (2)) the literature has been flooded with papers on double helix thermodynamics (for reviews see (3–8)). The published results were, however, rather controversial in a number of aspects.

The first DSC studies of natural DNA suggested that their unfolding/dissociation proceeds without noticeable heat capacity increment, $\Delta C_p$ (9). Since according to Kirchhoff’s relation, $\Delta C_p = \alpha (\Delta H)/\alpha T$, it was assumed, and widely accepted, that the enthalpy of DNA duplex dissociation/association does not depend on temperature (10–13). On the other hand, the enthalpy of DNA dissociation at elevated temperatures, determined by DSC, was found to be in conflict with the enthalpy of association of the complementary strands measured by ITC at lower temperatures: the strand association enthalpies at room temperatures were found to be much smaller in magnitude than the melting enthalpies at higher temperatures (14–16). This suggested, therefore, that the enthalpy of double helix formation should be temperature dependent, i.e. unfolding of the double helix should result in a heat capacity increment. According to (16) the heat capacity effect of base pairing varies from 130 to 423 J/K·mol-bp depending on the DNA sequence; according to (17) it is in the range 160–400 J/K·mol-bp; however (18) suggested values between 280 and 380 J/K·mol-bp, depending on the salt concentration. Thus, it was concluded that the ‘error in determining the heat capacity increment of DNA duplex melting is so big that it prevents any rigorous thermodynamic analysis of the stability of the nucleic acid duplexes’ (19).

The situation as regards the enthalpy of base pairing in DNA was no less confusing: various authors gave very different numbers in the range between 35 and 60 kJ/mol-bp, but all authors agreed that the enthalpy of CG base pairing significantly exceeds that of AT base pairing (8,12,20–23). Although the spread of published values did not render this conclusion particularly convincing, it was widely accepted as it did accord with the Watson-Crick DNA model in which the AT base pair is linked by only two hydrogen bonds but the CG pair by three. This paper considers the difficulties which are faced in studying the energetic basis of the DNA duplex and suggests a methodology for overcoming these problems by the...
combined use of ITC and DSC microcalorimetry. The idea of using the combination of these two microcalorimetric techniques was originally suggested in 1999 (15,24) and has since been considerably developed (2). Here, we demonstrate its efficiency using DNA duplexes of various lengths and compositions:

9-CG duplex consisting of three CGC/GCG triplets:

5′-CGC-GGC-GGC-3′
3′-GGG-CCG-GGC-5′

12-CG duplex consisting of four such triplets:

5′-CGC-GGC-GGC-GGC-3′
3′-GGG-CCG-GGC-GGC-5′

15-CG duplex consisting of five such triplets:

5′-CGC-GGC-GGC-GGC-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

and duplexes in which the central CGC/GCG triplets are replaced by AAA/TTT triplets:

9-AT

5′-CGA-AAA-GGC-3′
3′-GGG-CCG-GGC-5′

12-AT/AT

5′-CGA-AAA-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-5′

15-AT/TA/AT

5′-CGA-AAA-AAG-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

15-AT/TA/AT

5′-CGA-AAA-AAG-TTT-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

15-AT/TA/AT

5′-CGA-AAA-AAG-TTT-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

and duplexes in which the central CGC/GCG triplets are replaced by AAA/TTT triplets:

9-AT

5′-CGA-AAA-GGC-3′
3′-GGG-CCG-GGC-5′

12-AT/AT

5′-CGA-AAA-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-5′

12-AT/TA

5′-CGA-AAA-TTT-GGC-3′
3′-GGG-CCG-GGC-GGC-5′

12-TA/AT

5′-CGA-AAA-TTT-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

12-A/T

5′-CGA-ATATAT-GGC-3′
3′-GGG-CCG-GGC-5′

15-AT/TA/AT

5′-CGA-AAA-TTT-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

15-AT/TA/AT

5′-CGA-AAA-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

and duplexes in which the central CGC/GCG triplets are replaced by AAA/TTT triplets:

9-AT

5′-CGA-AAA-GGC-3′
3′-GGG-CCG-GGC-5′

12-AT/AT

5′-CGA-AAA-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-5′

12-AT/TA

5′-CGA-AAA-TTT-GGC-3′
3′-GGG-CCG-GGC-GGC-5′

12-TA/AT

5′-CGA-AAA-TTT-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

12-A/T

5′-CGA-ATATAT-GGC-3′
3′-GGG-CCG-GGC-5′

15-AT/TA/AT

5′-CGA-AAA-TTT-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

15-AT/TA/AT

5′-CGA-AAA-AAG-GGC-3′
3′-GGG-CCG-GGC-GGC-GGC-5′

MATERIALS AND METHODS

DNA duplex preparation

Custom-synthesized DNA oligonucleotides were purchased from Integrated DNA Technologies Incorporated and additionally purified by anion exchange FPLC on a Mono-Q column using a linear 0.1–1 M gradient of NaCl in 10 mM Tris–HCl, 1mM EDTA, 20% (v/v) acetonitrile (pH 7.4). After precipitation with ethanol, the samples were dried at room temperature, dissolved in water and dialyzed against working buffer. To prepare duplex, equimolar amounts of complementary strands were mixed, placed in boiling water and slowly cooled. Novex 4–20% TBE gels and the nucleic acid gel stain Sybr Gold were used to check the absence of any excess single strands. The molar DNA concentration was determined from the absorbance at 260 nm \( \lambda_{260} \) using the following equation (25,26):

\[
[\text{DNA}] = \frac{\lambda_{260} (12010 \cdot G + 15200 \cdot A + 8400 \cdot T - 7050 \cdot C)}{10^{5.4}}
\]

where \( G, A, T \) and \( C \) are the number of dG, dA, dT and dC nucleotides, respectively.

For calorimetric studies, solutions of single-stranded oligonucleotides were dialyzed for 30 h with three changes of buffer using a 500–1000 molecular mass cut-off membrane (Spectra/Por Biotech). Solutions of duplexes for calorimetric studies were dialyzed under the same conditions using 3500–5000 molecular mass cut-off membranes. The buffer used in the experiments was 5 mM Na phosphate (pH 7.4) with added 150 mM NaCl.

Isothermal titration calorimetry

ITC was performed on a Nano-ITC titration calorimeter (TA instruments Inc., New Castle, DE, USA). The calorimeter was calibrated with electrically generated heat impulses and by the heat of protonation of Tris base. All solutions were degassed by placing under vacuum for a short time immediately prior to use. Samples of DNA duplexes were prepared with the same batch of buffer to minimize artifacts due to minor differences in buffer composition. Calorimetric experiments used solutions of DNA duplexes of about 50 μM in the reactor cell and two orders higher for the syringe. Since the oligonucleotides differ in their ability to aggregate, the less aggregated strands were placed in the syringe because this solution is at a significantly higher concentration than that in the cell. The data were corrected for the dilution heat effect determined by control injections of DNA into buffer and analyzed using the program provided by TA Instruments, as described elsewhere (2).

Differential scanning calorimetry

Scanning calorimetric experiments were carried out on a capillary DSC instrument build at Johns Hopkins University, a prototype of the Nano DSC of TA Instruments. Details of the instrument’s performance and data acquisition are given elsewhere (2,27). The heating and cooling rate was 1 K/min at a constant over-pressure of 2 atm: this was required to prevent appearance of bubbles upon heating and to expand the heating range of aqueous solutions up to 110°C, required for studying the thermostable DNA duplexes. DNA duplexes and the separated strands were studied over concentrations from 0.5 to 3.5 mg/ml. Partial specific volumes for duplexes and single strands were taken as 0.54 cm³/g. Since the heat effects observed in heating and cooling experiments were highly reversible, the enthalpy of formation of residual structure in the strands was determined mostly from the cooling experiments as these permit heat capacity measurements down to 0°C. Calorimetrically determined heat capacity profiles of the DNA duplexes were analyzed using the CpCalc program, which was developed by Dr George Privalov at Johns Hopkins University and is now provided with the Nano DSC from TA Instruments.
**Spectroscopy**

UV absorption was measured using a Perkin Elmer Lambda 25 UV/VIS spectrophotometer equipped with the PTP-6 Peltier System.

**RESULTS**

**DSC versus ITC**

Figure 1 presents DSC recordings of the heat effects observed upon heating and subsequent cooling of the 12-CG DNA duplex, demonstrating the excellent reversibility of the temperature induced processes of complementary strand dissociation/association: the excess heats of these two processes appear as mirror images. Correspondingly, the enthalpies of association of the complementary strands of this duplex upon cooling should be of the same magnitude but opposite in sign to the enthalpy of dissociation measured by the DSC in the heating experiment. It is notable that linear extrapolation of the initial apparent heat capacity function to the higher temperatures suggests that, although dissociation of strands proceeds with extensive excess heat absorption, it is not accompanied by a noticeable heat capacity increment, i.e. \( \Delta C_p = 0 \). It appears, therefore, that the excess heat effect of duplex unfolding/dissociation, determined as the area above the extrapolated initial heat capacity line, equals about 420 kJ/mol and, according to Kirchoff’s relation, \( \Delta C_p = \partial \Delta H/\partial T \), it does not depend on temperature. Thus, the enthalpy of formation of this duplex at room temperature should be of the same magnitude but opposite in sign, \(-420 \) kJ/mol.

Figure 1 also presents an original ITC-recording of the heat effects on titration of one of the strands of the 12-CG DNA duplex by its complementary strand at 30°C. According to this experiment the enthalpy of duplex formation at that temperature is only \(-160 \) kJ/mol, a value in sharp contrast to the DSC-measured enthalpy of temperature induced dissociation upon heating, or to its association upon consequent cooling, taking place at around 85°C.

There could be several reasons for the observed discrepancy between the DSC and the ITC measured enthalpies: (i) the melting enthalpy of the duplex does in fact depend on temperature, i.e. the assumption that DNA melting proceeds without heat capacity increment is incorrect; (ii) the duplex formed at 30°C is not completely folded; (iii) the separated strands have residual structure, so that in order to associate they must first unfold and the heat of their unfolding contributes significantly to the observed heat effect of duplex formation.

**DNA duplex melting**

Considering the apparent heat capacity function of the DNA duplex (Figure 1, left panel), one notes that it starts to increase from the very beginning of heating, namely in the temperature range from 0 to 45°C, over which the duplex is generally regarded as still being fully folded. The observed rise in the heat capacity of the duplex on heating might result from increased fraying of its ends, in which case one would expect that the specific heat capacities calculated per base pair should depend on the length of the duplex, increasing with reduction in the number of base pairs.

As can be seen from Figure 2, at low temperatures the initial partial molar heat capacities of the three CG duplexes are different: with increase in the number of base-pairs their initial values increase, as does the temperature and area of the heat absorption peak. However, recalculated per mole of base pair the specific partial molar heat capacity functions of these three duplexes at temperatures below and above the heat absorption peaks are very similar. It follows, therefore, that the contributions of the base pairs to the DNA heat capacities are additive. Thus, one can conclude that the increase of heat capacity of the duplex upon heating results from intensified fluctuations over the whole of its length and not only of its ends. This might, for example, be a torsional
oscillations of the double helix that intensify with temperature rise.

Figure 2 shows also that duplex stability increases with the number of base pairs: longer duplexes dissociate at higher temperatures and with larger excess heat effects. The Inset in Figure 2 shows the specific excess heat effects plotted against the transition temperatures; the averaged slope of this temperature dependence, which represents the contribution of a single base pair to the heat capacity increment, appears to be \( \frac{\Delta H}{\Delta T} = (150 \pm 40) \text{ J/K-mol-bp} \) for these CG duplexes. Bearing in mind Kirchoff’s relation, \( \frac{\Delta H}{\Delta T} = \Delta C_p \), one can conclude that unfolding of these DNA duplexes proceeds with a heat capacity increment of about this magnitude.

A similar situation is observed with the DNA duplexes containing AT base pairs in the central part (Figure 3). Comparison of their heat capacity profiles with that of CG duplexes of similar length shows that the initial and final partial molar heat capacities of all duplexes containing the same number of base pairs are indistinguishable, notwithstanding their very different thermostabilities. It follows that the AT and GC base pair contributions to the total heat capacity of the duplex are similar and additive. As expected, the presence of AT base pairs decreases duplex stability significantly, however, absolutely unexpected is the observation that unfolding/dissociation of the less thermostable AT duplexes appears to proceed with a larger heat effect.

The standard heat capacity of the fully folded duplex

While the heat absorption peaks of DNA duplex unfolding in Figure 2 appear to be temperature dependent, meaning that dissociation proceeds with a heat capacity increment, this increment is not apparent in the original DSC recording shown in Figure 1, nor in the partial molar heat capacity profiles of these duplexes in Figures 2 and 3. It appears that the heat capacity increment of unfolding is somehow screened by the gradual effect of thermal energy accumulation by the duplex upon heating. If this is the case, the apparent heat capacity function of the duplex at temperatures below the extensive heat absorption peak (associated with the cooperative separation of its strands) cannot be simply regarded as the intrinsic heat capacity of the fully folded duplex. However, precise determination of the excess enthalpy of duplex melting requires knowledge of the partial
**heat capacity of the fully folded duplex over the whole considered temperature range. Such a standard heat capacity function can be constructed using the heat capacity increment, \( \Delta C_p \), calculated for the considered duplex. For the 12-CG duplex the molar heat capacity increment is: \( \Delta C_p = 12 \times 0.15 \text{ kJ/K-mol-bp} = 1.80 \text{ kJ/K-mol} \). Subtracting this heat capacity increment from the heat capacity of the 12-CG duplex above 110°C, where it is completely unfolded and connecting this point with the heat capacity at 0°C, where it might be regarded as being completely folded, we obtain the hypothetical heat capacity function for the fully folded duplex (Figure 4).

It appears then that starting from 0°C the partial heat capacity functions of the DNA lie above this standard heat capacity function of fully folded duplex, i.e. excess heat absorption takes place from the very beginning of heating and culminates with the extensive heat absorption associated with the cooperative separation of the DNA strands. This excess heat effect therefore represents the total enthalpy of unfolding of the fully folded duplex into its fully unfolded complementary oligonucleotides. The total enthalpy and the enthalpy of the cooperative phase of temperature induced melting of all the studied duplexes are listed in Table 1.

Assuming that the cooperative process of DNA dissociation represents a two-state bimolecular reaction and the heat capacity increment takes place only at this stage, which proceeds with exposure of the DNA bases to water, one can deconvolute the total excess heat effect into its gradual and cooperative components using, for example, the CpCalc program (2):

\[
\Delta H^\text{tot} = \Delta H^\text{grad} + \Delta H^\text{coop}
\]

(2)

In accordance with the assumption that heat capacity increment takes place only at the cooperative stage of duplex dissociation the enthalpy of the cooperative phase is expressed as:

\[
\Delta H(T)^\text{coop} = \Delta H_i^\text{coop} - (T_i - T)\Delta C_p,
\]

(3)

while the enthalpy of the gradual phase is assumed to be temperature independent.

**DNA duplex formation**

The total enthalpy of duplex formation could, in principle, also be obtained using ITC by measuring the heat effect of association of complementary oligonucleotides. It is known, however, that at the modest temperatures used in ITC experiments, single-stranded oligonucleotides are not completely unfolded and may form both intra- and intermolecular structures (13,15,24). Such residual structure can be revealed either by changes in the UV spectrum at 260 nm (i.e. by the hypochromic effect), or by the excess heat absorption of their solutions upon heating. An advantage of DSC for such experiments is that it gives direct information on the heat involved in unfolding the residual structures in the separated oligonucleotides. Furthermore, from the dependence of observed heat effects on the concentration of

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**Table 1.** The melting characteristics of the studied DNA duplexes at 283 \( \mu \)M concentration in 150 mM NaCl, 5 mM Na-Phosphate pH 7.4

| DNA duplexes | \( T_i (\degree C) \) | \( \Delta H^\text{tot} (\text{kJ/mol}) \) | \( \Delta H^\text{coop} (\text{kJ/mol}) \) |
|--------------|------------------|------------------|--------------|
| 9-CG         |                  |                  |              |
| 5’-CGCGCGCGG-3’ |                  |                  |              |
| 3’-CGCGCGCGG-5’ |                  |                  |              |
| 12-CG        |                  |                  |              |
| 5’-CGCGCGCGGCGG-3’ |                  |                  |              |
| 3’-CGCGCGCGGCGG-5’ |                  |                  |              |
| 15-CG        |                  |                  |              |
| 5’-CGCGCGCGCGCGG-3’ |                  |                  |              |
| 3’-CGCGCGCGCGGCGG-5’ |                  |                  |              |
| AT duplexes  |                  |                  |              |
| 9-AT         |                  |                  |              |
| 5’-GCGAAAAACGC-3’ |                  |                  |              |
| 3’-CGTTTTGCGG-5’ |                  |                  |              |
| 12-AT/AT     |                  |                  |              |
| 5’-GCGAAAAACGC-3’ |                  |                  |              |
| 3’-CGTTTTGCGG-5’ |                  |                  |              |
| 12-AT/TA     |                  |                  |              |
| 5’-GCGAAAAACGC-3’ |                  |                  |              |
| 3’-CGTTTTGCGG-5’ |                  |                  |              |
| 12-TA/AT     |                  |                  |              |
| 5’-GCGAAAAACGC-3’ |                  |                  |              |
| 3’-CGTTTTGCGG-5’ |                  |                  |              |
| 12-A/T       |                  |                  |              |
| 5’-GCGAAAAACGC-3’ |                  |                  |              |
| 3’-CGTTTTGCGG-5’ |                  |                  |              |
| 15-AT/TA/AT  |                  |                  |              |
| 5’-GCGAAAAACGC-3’ |                  |                  |              |
| 3’-CGTTTTGCGG-5’ |                  |                  |              |
| 15-AT/AT/AT  |                  |                  |              |
| 5’-GCGAAAAACGC-3’ |                  |                  |              |
| 3’-CGTTTTGCGG-5’ |                  |                  |              |
| Errors       | ±0.3             | ±15              | ±10          |
oligonucleotides one can judge if the residual structure is of intra- or inter-molecular origin.

DSC studies of the separated oligonucleotides showed that their heating indeed proceeds with significant excess heat absorption, indicating that they all possess a substantial amount of residual structure, which melts upon heating (Figure 5). It is remarkable that the melting profiles of complementary strands are typically very different. Some oligonucleotides melt over two separate temperature ranges with different concentration dependence, showing that they form also an intermolecular structures (Figure 6).

The question now is how to bring the DSC and ITC data on the heats of DNA duplex dissociation/association into correspondence. Using the 12-CG DNA duplex as an example, Panel (a) of Figure 7 shows an ITC determination of the enthalpy of association of the two complementary strands at 40°C. Panel (b) shows the DSC-determined partial molar heat capacity function of the 12-CG duplex and the heat capacity function expected for the fully folded duplex: the hatched area of this heat capacity profile shows the heat of duplex premelting for the temperature of 40°C, that at which the ITC experiment was carried out. Panels (c) and (d) show the heat capacity profiles of the separated complementary strands; here the hatched areas correspond to the enthalpies of residual structure in these strands at the temperature of the ITC experiment, i.e. at 40°C. These three enthalpies must all be added to the ITC-measured value so as to obtain the total enthalpy required to form a fully folded duplex at 40°C from totally unfolded single strands.

The results of such combined calorimetric experiments for the 12-CG DNA duplex are presented in Figure 8: triangles show the ITC-measured enthalpies of association of the complementary strands at different fixed tempera-

Figure 5. The excess heat capacity profiles observed upon heating/cooling of the individual complementary oligonucleotides that make up the 9-CG, 12-CG and 15-CG duplexes (left hand panels) and the 9-AT, 12-AT/TA and 15-AT/TA/AT duplexes (right hand panels), all in 150 mM NaCl, 5 mM Na-phosphate, pH 7.4.

Figure 6. The excess heat capacity DSC profiles observed upon heating/cooling of the two 12 nucleotide complementary oligonucleotides, 5′-CGCCGCCGCCGC-3′ and 3′-GCGGCCGCCGCGC-5′, at different concentrations (indicated in micromolar) all in 150 mM NaCl, 5 mM Na-phosphate, pH 7.4. Dashed lines show temperature derivatives of the extinction coefficients of the considered oligonucleotides at 260 nm, reflecting changes in hypochromic effect upon heating/cooling at oligonucleotide concentrations too low to allow DSC measurements.

Figure 7. (a) ITC titration of the 5′-CGCCGCCGCCGC-3′ strand by the complementary 3′–GCGGCCGCCGCGC–5′ strand at 40°C. (b) The DSC-measured partial molar heat capacity of the 12-CG duplex; the hatched area represents the enthalpy of the duplex premelting upon heating to 40°C. (c) and (d) are the partial heat capacities of the two isolated oligonucleotides; the hatched areas show the enthalpy of the residual structures in the single strands remaining at 40°C. All in 150 mM NaCl, 5 mM Na-phosphate, pH 7.4.
Figure 8. Molar enthalpies of formation of the 12-CG duplex in the presence of 150 mM NaCl in 5 mM Na-phosphate pH 7.4 measured by ITC (triangles) and corrected for residual structure in the complementary oligonucleotides (squares) and also for duplex premelting (circles).

Figure 9. (a) and (c) are the ITC-measured molar enthalpies of formation of the 9-CG, 12-CG and 15-CG and 9-AT, 12-AT/TA and 15-AT/TA/AT duplexes, corrected for residual structures in the separated strands and for premelting. (b) and (d) are the specific molar (i.e. per base pair) enthalpies of these duplexes. Crosses indicate the total enthalpy values of formation of the considered duplexes obtained from the DSC-measured excess heat of duplex melting and attributed to the transition temperatures, \( T_t \). All in 150 mM NaCl, 5 mM Na-phosphate, pH 7.4 solutions.

DISCUSSION

The enthalpy of DNA duplex formation

One of the first important achievements in studying the physical properties of the DNA double helix was the observation that its stability increases with CG base pair content (28,29). This was considered a strong argument for the correctness of the Watson–Crick DNA model (1), according to which the DNA duplex is stabilized by hydrogen bonding between the complementary bases: two between A and T and three between C and G bases. This explanation of the rise in DNA stability with increase of CG content became conventional in all textbooks of Biochemistry and Molecular Biology. Thus, in Watson et al. (30) we read: ‘Because each CG base pair is held together by 3 hydrogen bonds rather than the two holding each AT base pair, higher temperatures are necessary to separate CG-rich strands than to break apart AT-rich molecules’. Similar statements are made in more recent editions of the biochemistry textbooks (see e.g. (31,32)). The most unexpected result of this calori-
metric study of DNA duplexes is, therefore, that the specific enthalpies of their dissociation/association (i.e. calculated per base pair) appear to be larger in magnitude for the duplexes containing several AT base pairs (see Figures 3 and 9).

Since in duplexes consisting only of CG pairs, contributions of individual base pairs to the enthalpy of duplex unfolding appear to be additive, dividing the total enthalpy of dissociation by the number of bases pairs in the duplex gives the enthalpic contribution of a single base pair. To extract the contribution of AT pairs from duplexes having mixed composition, one should first subtract the expected contribution of the two terminal (CGC/GCG) triplets, i.e. six CG base pairs, from the measured total enthalpy of dissociation and then divide the remaining enthalpy by the number of AT base pairs in the duplex:

$$\Delta H_{AT}(T) = \frac{\Delta H_{\text{dualplex}}(T) - N_{CG}\Delta H_{CG}(T)}{N_{AT}} \quad (4)$$

The contributions of AT and CG base pairs to the total enthalpy of double helix formation obtained by this approach from the ITC data are listed in Table 2. As expected, the spread between the specific enthalpy values is particularly low for the CG pair derived from the 9-, 12- and 15-CG duplexes for which it is close to the expected experimental error. For the AT base pairs derived from duplexes containing both CG and AT pairs the spread of values is larger. Nevertheless, it is clear that the enthalpic contribution of an AT base pair exceeds that of a CG base pair over the whole considered temperature range.

In contrast to the ITC experiment, which provides only the total enthalpies of DNA duplex formation at fixed temperatures, DSC experiments permit determination of the enthalpy of the gradual and cooperative phases of the temperature-induced DNA unfolding (Table 1). It appears that the cooperative phase of unfolding starts when a certain enthalpy level is reached in the duplex upon heating. Therefore, at least to a first approximation, one can assume that this starting enthalpy level is specific for the considered duplex, i.e. it does not depend on temperature and is equally distributed between the CG and AT pairs. Thus, in this approximation only the enthalpy of the cooperative phase of duplex dissociation, resulting in exposure of all its groups to water, is taken to be temperature dependent. Therefore, the contribution of an AT base pair to the total enthalpy of duplex unfolding at 25°C is determined as:

$$\Delta H_{AT}^{\text{tot}}(25^\circ C) = \Delta H_{AT}^{\text{coop}}(25^\circ C) + \Delta H_{\text{rad}}^{\text{grad}} / N, \quad (5)$$

where $N$ is the total number of base pairs in the considered duplex.

The total and cooperative enthalpies of the CG and AT base pairs obtained from DSC measurements on all the considered duplexes, extrapolated to 25°C, are listed in Table 3. It is remarkable that the total enthalpy values extrapolated to 25°C are in good correspondence with those directly measured by ITC at this temperature and listed in Table 2. The correspondence between the data obtained by two very different approaches shows that they represent reliable thermodynamic characteristics of the AT and CG base pairings. It is also apparent that these enthalpy values differ from most of those published (e.g. (12, 21)). This is because in previous studies the contribution of residual structure in the complementary oligonucleotides forming the DNA duplex was not taken into account and the temperature dependence of all thermodynamic parameters specifying formation of the double helix was neglected.

The most notable feature of the data presented in Tables 2 and 3 is that the enthalpic contributions of the CG and AT base pair differ significantly: both experiments, the ITC and DSC, show that the contribution of the AT base pair to the total and cooperative enthalpies of duplex dissociation at standard temperature 25°C are larger than that of the CG base pair. The question is then: why is the CG-rich DNA duplex more stable? Could it be because the entropy of CG base pair dissociation is lower than that of the AT base pair?

### The entropic contribution to base pairing

The entropy of molecular components associating into a complex is usually determined by analyzing the isotherm of this reaction if it is reversible and simple. Formation of the DNA duplex is a reversible reaction but is not simple since all the oligonucleotides form inter and intramolecular aggregates (see Figure 6), which must first unfold in order to associate. Thus, to determine the net entropy of duplex formation from ITC experiments one should take into account the entropies of unfolding of the oligonucleotides, as we did in determining the net enthalpy of duplex formation. The entropy correction is, however, more complicated than that of the enthalpy, as it assumes integration of the $\Delta C_V^{\text{exc}} / T$ term over all the considered temperature range. Therefore, it is easier to determine the entropies of duplex unfolding from

| Table 2. The contributions of AT and CG base pairs to the total enthalpy of the DNA duplex formation derived from the corrected ITC data shown in Figure 9 |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| $T$ (°C)        | 9-CG            | 12-CG           | 15-CG           |
| 15              | 23.9            | 26.7            | 26.0            |
| 20              | 24.8            | 27.5            | 26.7            |
| 25              | 25.2            | 28.2            | 27.2            |
| 30              | 25.6            | 28.7            | 28.0            |
| 35              | 26.7            | 29.2            | 28.7            |
| Averaged for 25°C | 26.8 ± 1.0      |                 |                 |
| $\Delta H_{AT}^{\text{tot}}$(kJ/mol-bp) |
| 9-AT            | 29.0            | 29.5            | 29.1            |
| 12-AT/TA        | 29.1            | 30.4            | 29.4            |
| 15-AT/TA/AT     | 29.1            | 31.0            | 29.3            |
| 30.1            | 31.7            | 30.6            |
| 28.9            | 32.0            | 30.8            |

The contributions of AT and CG base pairs to the total enthalpy of association (i.e. calculated per base pair) appear to be larger in magnitude for the duplexes containing several AT base pairs (see Figures 3 and 9). Since in duplexes consisting only of CG pairs, contributions of individual base pairs to the enthalpy of duplex unfolding appear to be additive, dividing the total enthalpy of dissociation by the number of bases pairs in the duplex gives the enthalpic contribution of a single base pair. To extract the contribution of AT pairs from duplexes having mixed composition, one should first subtract the expected contribution of the two terminal (CGC/GCG) triplets, i.e. six CG base pairs, from the measured total enthalpy of dissociation and then divide the remaining enthalpy by the number of AT base pairs in the duplex:
Table 3. The DSC-measured total and cooperative enthalpy, entropy and Gibbs energy of association of CG and AT base pairs, extrapolated to the standard temperature of 25°C

| CG duplexes | $-\Delta H_{CG}$ (kJ/mol-bp) | $-\Delta S_{CG}$ (J/K·mol-bp) | $-\Delta G_{CG}$ (J/K·mol-bp) |
|-------------|-------------------------------|-------------------------------|-------------------------------|
|             | coop | total | coop | total | coop |
| 9-CG        |      |       |      |       |      |
| 5′-CCCGCGCCGC-3′ 3′-GGGGGGCGGG-5′ | 18.4 | 25.8 | 39.7 | 62.5 | 6.6 |
| 12-CG       |      |       |      |       |      |
| 5′-CCCGCGCCCGGC-3′ 3′-GGGGGGCGGGG-5′ | 19.2 | 27.4 | 40.4 | 63.4 | 6.3 |
| 15-CG       |      |       |      |       |      |
| 5′-CCCGCGCGCCCGGGC-3′ 3′-GGGGGGCGGGGGGG-5′ | 18.7 | 26.3 | 41.8 | 66.1 | 6.2 |
| Averaged    |      |       |      |       |      |
|             | 18.8±0.5 | 26.5±1.0 | 40.6±1.0 | 64.0±1.5 | 6.4±0.2 |

| AT duplexes | $-\Delta H_{AT}$ | $-\Delta S_{AT}$ | $-\Delta G_{AT}$ |
|-------------|------------------|------------------|------------------|
|             | coop | total | coop | total | coop |
| 9-AT        |      |       |      |       |      |
| 5′-CGCAAAACGC-3′ 3′-GGTTTTCGG-5′ | 31.5 | 35.3 | 86 | 98 | 5.8 |
| 12-AT/AT    |      |       |      |       |      |
| 5′-CGCAAAAAAACGC-3′ 3′-GGTTTTTTTGCG-5′ | 30.1 | 34.3 | 86 | 98 | 4.5 |
| 12-AT/TA    |      |       |      |       |      |
| 5′-CGCTTTAAACGC-3′ 3′-GGCTTTATGGC-5′ | 29.0 | 32.5 | 82 | 93 | 4.6 |
| 12-A/T      |      |       |      |       |      |
| 5′-CGCATATATGC-3′ 3′-GGTATATAGCG-5′ | 25.1 | 28.8 | 70 | 80 | 4.2 |
| 15-AT/TA/AT |      |       |      |       |      |
| 5′-CGCAATTTAACGC-3′ 3′-GGTTTTTTTGCG-5′ | 25.1 | 28.8 | 68 | 80 | 4.8 |
| Averaged    |      |       |      |       |      |
|             | 28.1±1.8 | 32.0±2.0 | 78±5.0 | 90±6.0 | 4.7±0.3 |

DSC experiments since at the temperatures of duplex dissociation the separated strands are completely unfolded because these temperatures are too high for oligonucleotides to form residual structure.

The standard entropy of cooperative dissociation of a heterodimer can be determined by dividing the DSC-measured heat of this cooperative processes by the absolute temperature and correcting that for the concentration:

$$\Delta S_{coop}(T_t) = \frac{\Delta H_{coop}}{T_t} + R \ln \left( \frac{[N]}{2} \right)$$  \hspace{1cm} (6)

where $[N]$ is the initial concentration of dimer (2). Extrapolation of this entropy to the standard temperature of 25°C is carried out using the known heat capacity increment, $\Delta C_p$:

$$\Delta S_{coop}(25^\circ) = \Delta S_{coop}(T_t) - \Delta C_p \times \ln(T_t/289.2)$$  \hspace{1cm} (7)

The entropy of the gradual process is determined by integration of the excess heat effect divided by the absolute temperature:

$$\Delta S_{grad} = \int_{T_0}^{T} \frac{C_p(T) - C_p(T_0)}{T} dT$$  \hspace{1cm} (8)

and is assumed not to depend on temperature (see previous section).

In analyzing the entropy data obtained for duplexes differing in length one faces a serious problem: duplex thermostability rises with increase in the number of base pairs (Figure 2), showing that, in contrast to the enthalpy, the entropy of duplex dissociation is not an additive function of its size. This is because dissociation of the complementary strands results not only in an increase in their conformational freedom, which does depend on the number of base pairs and temperature, but also includes a translational entropy associated with the appearance of a new kinetic unit, a contribution which does not depend on the number of bases or temperature. However, the magnitude of the translational entropy, which should be taken into account, presents a problem.
Originally the value of the translational entropy was proposed by Gurney (33), who considered it as an entropy of dissolution of solute into the solvent: for 1 M standard aqueous solutions (containing 55 mol of water per mol of solute) this so-called ‘cratic’ entropy appears to be 8586 J/K-mol and is expected to be independent of the molecular weight of the solute and temperature. This ‘cratic’ entropy was adopted by Kauzmann and Tanford (34,35). Later this cratic entropy became a target of severe criticism by proponents of the statistical mechanics of gases as being physically ungrounded (36–40) and values of gases as being physically ungrounded (36–40) and values of these theoretical estimates are not realistic and the translational entropy.

The present analysis of the entropy of DNA duplex formation will use the cratic entropy, \( \Delta S^\text{cratic} = 34.5 \text{J/K-mol} \), as the translational entropy. To obtain the contribution of a single CG base pair to the conformational entropy one must exclude this translational entropy (34.5 J/K-mol) from the total conformational entropy of a duplex consisting only of CG base pairs and divide the remainder by the number of base pairs in the duplex, assuming their contributions are additive:

\[
\Delta S^\text{conf}_{\text{CG}}(T) = \frac{\Delta S^\text{tot}_{\text{CG}}(T) - \Delta S^\text{trans}_{\text{CG}}}{N_{\text{CG}}}
\]

The entropic contribution of AT base pairs can be determined from the total entropy of dissociation of the AT-containing duplexes by first excluding the translational entropy and then the entropy contribution of the terminal CG base pairs, before dividing by the number of AT pairs:

\[
\Delta S^\text{conf}_{\text{AT}}(T) = \frac{\Delta S^\text{tot}_{\text{duplex}}(T) - \Delta S^\text{trans}_{\text{CG}} - N_{\text{CG}} \Delta S^\text{conf}_{\text{CG}}(T)}{N_{\text{AT}}}
\]

As in the case of enthalpy, the contribution of the AT base pair to the total entropy is determined as:

\[
\Delta S^\text{tot}_{\text{AT}}(T) = \Delta S^\text{conf}_{\text{AT}}(T) + \Delta S^\text{grad}_{\text{AT}} / N
\]

The contributions of CG and AT base pairs to the dissociation entropy of the considered DNA duplexes, extrapulated to 25 °C, are listed in Table 3. Most remarkable is that the entropic contribution of the AT base pair substantially exceeds that of the CG pair.

For the temperature dependence of the base pair contributions to duplex stabilization see Supplementary Data 2.

**Contribution of the base pairs to double helix formation**

The two different calorimetric methods, ITC and DSC, both show that the total enthalpic and entropic contributions of the AT base pair exceeds that of the CG base pair (Tables 2 and 3). This difference between the contributions of the CG and AT base pairs is especially clear for the cooperative phase: it appears that while the cooperative enthalpic contribution of the CG base pair varies from 18.4 to 19.2 kJ/mol-bp, for the AT base pair in the considered seven duplexes they vary between 25.1 and 31.5 kJ/mol-bp. It is notable that these two ranges do not overlap: their mean values differ by about 10 kJ/mol-bp. Even larger is the difference between the entropic contributions of the AT and CG base pairs to the cooperative phase of duplex dissociation: for the CG base pair it varies in the range 40–42 J/K-mol-bp with a mean of 41 J/K-mol-bp, while for the AT it varies from 68 to 86 J/K-mol-bp with a mean of 78 J/K-mol-bp. Thus, the difference between the mean entropy values amounts to 37 J/K-mol-bp, i.e. 10 times exceeding the possible experimental error!

It is notable that the enthalpy and entropy contributions of the CG base pair to the cooperative phase (determined using three duplexes differing in the number of the CGC/GCG triplets) spread over a rather narrow range, while the enthalpy/entropy contributions of the AT base pair (determined from 7 duplexes differing in the arrangement of AT base pairs) vary in a 10 times larger range. These context dependent variations in the enthalpic and entropic contributions of the base pairs are often described in terms of interactions between near neighbors (12,21).

The most unexpected feature of the thermodynamic characteristics obtained for the DNA duplexes is, however, that in all cases the enthalpic and entropic contributions of AT base pairs significantly exceed those of CG base pairs and this difference is especially clear for the cooperative phase. Since duplex stability is determined by the base pair contributions to the Gibbs energy, \( \Delta G = \Delta H - T \Delta S \), it now follows that the CG-rich DNA duplex is more stable than the AT-rich duplex not because the enthalpy of CG dissociation is larger than that of ATs but because the entropy of its dissociation is lower. Alternatively this could be stated as: the AT-rich duplex is less stable than the CG-rich duplex because the entropy of AT dissociation is larger than the entropy of CG dissociation.

The questions are therefore: why are the enthalpic and especially entropic contributions of AT pairs larger than those of CG pairs and why is this difference especially impressive for the cooperative phases of DNA duplex dissociation? The notable feature of the mean cooperative entropies of AT to CG base pairs is that their ratio amounts to 82/41 = 2.0, while for the total entropies it is 95/64 = 1.5, i.e. is much smaller. This implies that in the gradual phase the contributions of the AT and CG base pairs do not differ: their difference appears only in the cooperative phase associated with the separation of the complementary strands of DNA. How then can one explain such a dominance of the AT base pair contribution over the CG, especially in entropy, at the DNA dissociation phase? It certainly cannot be caused by differences in hydrogen bonding between complementary bases since the AT pair has fewer such bonds than CG. Nor can it be the difference in stacking interactions of the bases packed in the double helix, since these are quite similar for these two pairs. Could the difference be associated with the known ability of an AT base pair for local bending of DNA towards the minor groove, which results in the formation of a curved A-tract? Crystal structures of DNA fragments containing A-tracks showed that the base pairs are propeller twisted and that might provide improved base-stacking interactions between adjacent base pairs (43–45).
However, as seen in Table 3, the 15 bp duplexes with long AT sequences, which might form A-tracks, show enthalpy and entropy contributions of AT pairs similar to those from short sequences such as 9-AT with a single (AAA/TTT) triplet which hardly forms A-tract. It appears, therefore, that the excessive enthalpy and entropy of AT base pairing must be caused by a factor external to the DNA. This can only be the water specifically bound by the AT base pair.

The existence of such bound water molecules has been observed crystallographically and by NMR as a spine in the minor groove of AT-rich DNA (46–51). Furthermore, a secondary shell of water molecules runs along the groove in AT stretches, donating hydrogen bonds to the primary shell of oxygen atoms that assume the tetrahedral coordination characteristic of ice (52).

The presence of ordered water in the minor groove of the AT-rich DNA was demonstrated by calorimetric studies of Hoechst 33258 binding to such DNA. Hoechst 33258, a bisbenzimidazole compound the shape of which corresponds to the minor groove of AT-rich DNA, enters deep into this groove expelling water (53). Calorimetric studies of Hoechst 33258 binding to the AT-rich DNA duplex (CGAAATTTGCG)2 (very similar to our 12-AT/TA duplex) showed that this process is endothermic, i.e. entropy driven, which means that water in the minor groove of the AT rich DNA is in a more ordered state than the bulk water (54). Also, as we showed, while binding of various transcription factors to the major groove of DNA is enthalpy driven, binding to the minor groove is entropy driven (55,56). This experimental fact could be explained only by the different states of water in the minor and major grooves of DNA.

It appears, thus, that the water fixed in the minor groove of the AT rich DNA is responsible for the qualitative discrepancy in binding transcription factors to the minor and major grooves of DNA.

It should be noted that water ordering in the minor groove of the AT-rich DNA is provided not by apolar groups, as occurs in the case of proteins and provides the so called hydrophobic force (34,57). In contrast, in the AT minor groove water is fixed by the polar groups of the AT pair, namely by N3 of A and O2 of T (47,48) and is released upon dissociation of this pair. Judging by the excess entropy contribution of AT base pairing over CG base pairing which, according to our estimates, exceeds by almost two-fold that of melting ice (22 J/K mol), the AT fixed water molecule affects the state of a number of surrounding water molecules. Thus, one would expect that water ordering in the minor groove of DNA should depend on the mutual arrangement of the AT base pairs, and also on their orientations, i.e. the disposition of AT base pairs along the DNA and their mutual orientation might orchestrate water ordering in the minor groove.

An important feature of the enthalpy of DNA duplex unfolding/dissociation is that for all the considered DNA duplexes it increases linearly with temperature. This means that duplex unfolding proceeds with a defined heat capacity increment.

The origin of the heat capacity increment on DNA unfolding is a key question of DNA thermodynamics. It certainly does not result from an increase in the conformational freedom on dissociation of the complementary strands: this could be responsible only for a small part of the observed heat capacity effect (58). Neither can it be caused by exposure of polar groups on breaking the hydrogen bonds between complementary bases, because the heat capacity effect of the hydration of polar groups is negative (59,60). Thus, the increase of DNA heat capacity upon unfolding must result from some other mechanism. This can only be hydration of the exposed apolar surfaces of bases, as it is well known that transfer of apolar groups into water results in a considerable heat capacity increment (for review see (57)). This is explained by ordering of water around the nonpolar groups and the gradual ‘melting’ of this ordered water upon heating, which results in the apparent heat capacity increment. This thermodynamically unfavorable water ordering (i.e. an entropy decrease) by apolar groups is regarded, after Kauzmann (34), as a hydrophobic force. Thus, from the observed heat capacity increment of the DNA unfolding, one can conclude that hydrophobic forces contribute to the stabilization of the DNA double helical structure.

CONCLUSIONS

1. Heating of the DNA duplex leads to gradual intensification of its thermal fluctuations which culminates in the cooperative dissociation of its complementary strands. The temperature of DNA dissociation, i.e. its thermostability, increases with the content of the CG base pairs.
2. The enthalpy of cooperative dissociation/association of the duplex is a linear function of temperature and does not depend on the salt concentration. Thus, duplex unfolding proceeds with a heat capacity increment which does not depend on temperature or the presence of salt.
3. The contributions of the base pairs to the heat capacity increment of the DNA duplex are additive and equal for the AT and CG base pairs.
4. The enthalpy and entropy of AT-base pairing exceed those of CG base pairing. The increase in DNA duplex stability with CG base pair content is, therefore, provided not by the greater enthalpy of CG base pairing, as previously assumed, but by its lower entropy in comparison with that of AT base pairing.
5. The significantly larger enthalpic and especially entropic contributions of AT base pairing over that of the CG pair can result only from water fixed by the AT base pair in the minor groove and released upon dissociation of this group, i.e. on DNA unfolding.
6. The especially large excessive entropic contribution of the AT base pair, two-fold exceeding the entropy of melting ice, suggests that the water molecule fixed by this pair significantly affects the state of its neighbors. It appears, therefore, that the arrangement of AT pairs and their orientation orchestrates the state of water in the minor groove of DNA, resulting in context-dependent thermodynamic properties of DNA.
7. The results of this investigation significantly differ from those reported in the literature because in most previous studies of DNA duplexes no account was taken of the contribution of residual structure in the separated complementary strands and the temperature dependence of all the thermodynamic parameters specifying formation of the double helix was totally neglected.
SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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