

A REASSESSMENT OF NUCLEIC ACID MELTING

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Abstract – A brief critique of DNA and RNA melting, particularly from a physico-chemical perspective, is presented. These melting phenomena have been employed to obtain quantitative estimates of the stability of putative base pairs, thus apparently bolstering the double helical structure of DNA. It is argued herein that the titled phenomena may not be what they seem: in particular, the strategy based on the van't Hoff equation may not be valid, and alternative interpretations of the results merit serious consideration. These arguments cast a shadow on current views about nucleic acid structure and stability.

Keywords – base pairing; DNA melting; double helix; RNA melting; van't Hoff equation;

A BRIEF INTRODUCTION

The melting of nucleic acids refers to the heating of a solution of DNA or RNA with the simultaneous monitoring of the UV absorbance of the sample. Generally, this leads to an increase in the absorbance (A) at around 260 nm, a sigmoid plot of A vs. the temperature (T) being obtained. A very large number of such studies have been reported over the past half-century or so, with a variety of nucleic acid substrates, both synthetic and natural [1-6].

Among these, rigorous and extensive studies have attempted to extract thermodynamic data pertaining to the interactions between the purine and pyrimidine bases comprising the nucleic acids. These are based on the supposition that DNA oligomers exist at normal temperatures in

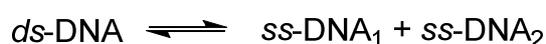
the duplex form, which can be separated into the component single strands by heating (Scheme 1). Furthermore, an ingenious adaptation of the classical van't Hoff equation apparently leads to an estimate of the stability of the complementary base pairs. The fundamental significance of these studies is clearly obvious.

All the same, the phenomenon *per se* and its applications as above are not without ambiguities, brought to the fore by recent assertions that the DNA double helix may not be the dominant form in the solution state [7]. As studies of DNA melting have all along assumed the duplex as the exclusive ground state structure, it seems opportune to review the salient features of nucleic acid melting, with a particular focus on problems and uncertainties that seem to have been overlooked.

A CRITICAL APPRAISAL OF THE OBSERVATIONS

Evidence of duplex-monomer equilibrium at low temperatures

Nucleic acid melting is performed in a wide temperature range, between 0-10 °C at the lower end and ~ 70 °C at the upper, at μM concentrations varying five- to ten-fold (Fig. 1). Remarkably, in the case of DNA oligomers, there is clear evidence of an equilibrium that is concentration dependent, even at the lowest temperature studied [3-6]. Thus, higher concentrations decrease the absorbance perceptibly, which implies a shift towards the duplex form. In fact, these can be of the order of 10% of the total absorbance change for ~ a five-fold change in concentration. (The entire exercise is based on the premise of a hyperchromic shift in the single stranded monomer relative to the duplex form.)



Scheme 1. The reversible thermal conversion of double-stranded DNA (*ds*-DNA) into the complementary single-stranded components *ss*-DNA₁ and *ss*-DNA₂

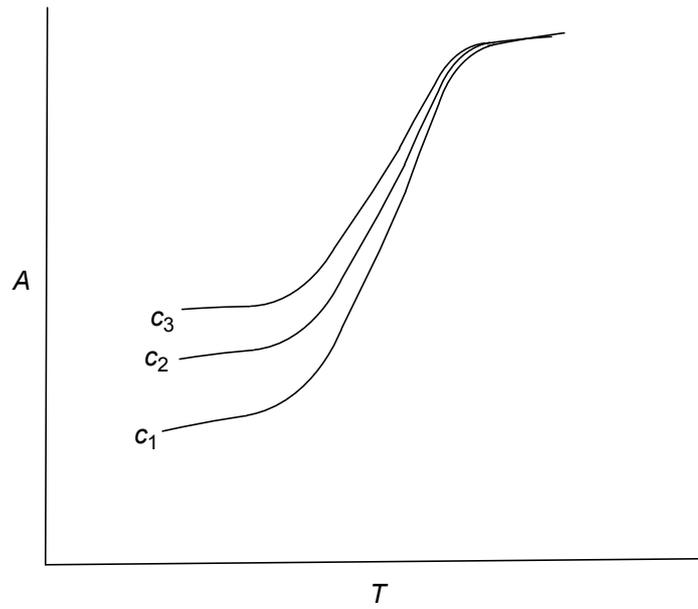


Fig. 1. Experimentally determined DNA melting curves obtained at different concentrations ($c_1 > c_2 > c_3$)

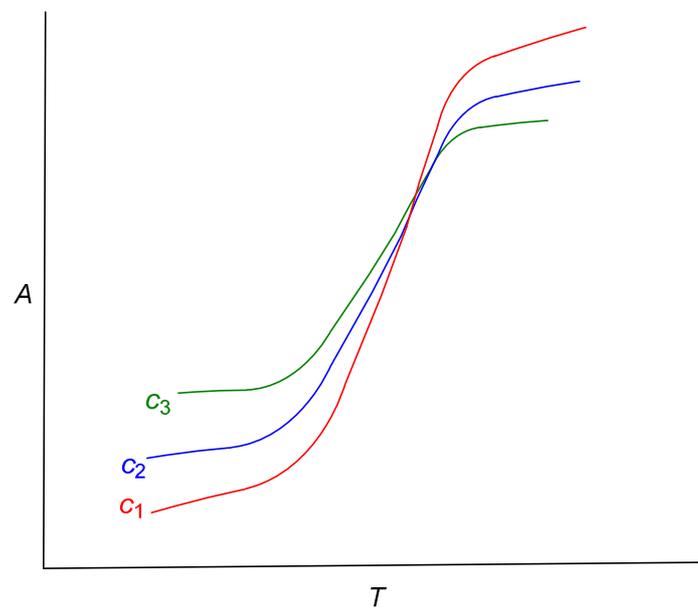


Fig. 2. Expected DNA melting curves at different concentrations ($c_1 > c_2 > c_3$)

It is particularly noteworthy that the observed absorbance changes are the opposite of those expected from purely concentration effects. Thus, higher concentrations would generally lead to higher absorbance values, in the absence of the shifts in equilibrium: these shifts, therefore, are clearly underestimated by the observed absorbance changes! (The nearly five-fold change in concentration would lead to corresponding changes in absorbance, in the absence of the shifts in equilibrium.)

Furthermore, the coalescence of the curves at the higher temperature ranges, again, is inexplicable, as upon full strand separation the more concentrated samples should show correspondingly higher absorbance values! This implies that the curves should cross at an intermediate stage in the melting process (Fig. 2): And, indeed, this is a possible explanation for the coalescence region, indicating that the melting process is perhaps incomplete at that stage. (This is interestingly reminiscent of an isosbestic point sometimes observed in the electronic spectra of samples undergoing a chemical change [8] – *vide infra* for a fuller discussion. Although there is no apparent reason why all the curves should cross at a single point, the closely spaced lines would tend to bunch up around the intersection regions.)

Of course, it is (presumably) impractical to heat the samples beyond ~ 70 °C, for reasons of stability and the onset of evaporation. All the same, the above arguments invalidate the melting process as a technique of any accuracy in the study of nucleic acid stability. Therefore, not only the assumption that the duplex is the sole form at the beginning of the melting process, but also the exercise as a whole appears dubious and unreliable.

The relative dissociation does not vary with temperature

The melting experiment, as described above, leads to a family of sigmoid curves that are closely spaced (Fig. 1). Remarkably, the spacings between the curves are nearly constant for the greater part of the temperature range studied, except for the gradual coalescence observed

beyond ~ 60 °C. The constancy of the spacings apparently implies that the extent of dissociation varies similarly with temperature across all concentrations.

$$\Delta H^\circ = 6RT_m^2(df/dT)_{T=T(m)} \quad (1)$$

Interestingly, this is explicable in the two-state model (Scheme 1) as it can be shown that the rate of change of the extent of dissociation is constant at each temperature, say at the melting temperature T_m (Eqn. 1) [6], for the particular system under study. (In Eqn. 1 f is the fraction of bases paired, ΔH° the standard enthalpy change and T_m the melting temperature, *vide infra*.)

Alternatively, the constancy of the spacing may also reflect the insensitivity of the equilibrium constant to changes in temperature. This would happen if the standard enthalpy change is vanishingly small (~ 0), as can be seen from the van't Hoff equation (*vide infra*). If so, a much larger temperature range would be required to perceptibly shift the equilibrium towards products (*cf.* 'isosbestic point' above). (The extent of dissociation would also remain constant, along with the equilibrium constant and the concentration by Eqn. 3, *vide infra*.)

In another scenario, the observed changes in absorbance could arise from a process other than the breakdown of the duplex. In any case, in view of the dubious nature of the melting exercise as a whole (*vide supra*) the significance of these findings is unclear, although a fuller discussion is attempted further below.

The melting temperature is concentration dependent

A key characteristic of the melting phenomenon is the so-called melting temperature (T_m), referring to the temperature at which the presumed dissociation is exactly half-complete (taken to be the mid-point of the sigmoid curve). The curves are laterally displaced in their vertical parts along the x axis (representing the temperature), so the more concentrated the solution the higher is the T_m (Fig. 1). This is apparently in accord with the presumed

equilibrium between the duplex and the monomers (Scheme 1), which shifts towards the duplex at higher concentrations (by Le Chatelier's principle [9]).

However, in view of the arguments presented above, the significance of the T_m is in doubt, and clearly needs to be reassessed (*vide infra*).

The extent of dissociation, but not the equilibrium constant, is concentration dependent

The equilibrium constant (K) for the dissociation of the double-stranded form (ds -DNA) into the single strands (ss -DNA) is shown in Eqn. 2 (*cf.* Scheme 1). Thus, K possesses the dimensions of concentration (M). However, although (of course) K itself is invariant with the concentration (c), the fraction of dissociation (α) increases with increasing dilution at a given temperature (*vide supra*). Hence, K is related to both α and c in a somewhat complex way (Eqn. 3). However, K is related only to c at an assumed value of α , say 0.5 as at the T_m .
[Note: $\alpha = (1-f)$.]

$$K = [ss\text{-DNA}_1][ss\text{-DNA}_2]/[ds\text{-DNA}] \quad (2)$$

$$(1/K) = 2(1-\alpha)/(\alpha^2 c) \quad (3)$$

These considerations form the basis of the calculation of K at various T_m values from the observed sigmoid plots (Fig. 1) [4-6]. Thence, the standard enthalpy change (ΔH°) for the equilibrium (Scheme 1) is calculated from the variation of K with temperature *via* the van't Hoff equation (Eqn. 4) [9]. As K is related to the standard Gibbs free energy change (ΔG°), the standard entropy change (ΔS°) may also be calculated.

$$(d\ln K)/d(1/T) = -\Delta H^\circ/RT^2 \quad (4)$$

The exercise thus depends on the assumption that the T_m represents a convenient stage in the reaction at which the absorbance represents exactly half the overall change (in A). However, again, as the exercise is riven by uncertainties the validity of these thermodynamic calculations needs to be reassessed (*vide infra*).

CAVEATS AND ALTERNATIVE EXPLANATIONS

Current views of DNA melting and its flaws

To reiterate, a serious problem with the current analysis of DNA melting is the observation that a perceptible equilibrium – presumably between *ds*-DNA and *ss*-DNA – exists even at low temperatures. In fact, the extent of this equilibrium is not easy to judge from the available data. Thus, around a five-fold change in concentration apparently leads to around a ten percent change in the *A* at constant temperature. (This is relative to the total change in *A* over the entire temperature range, and is almost certainly an underestimation, *vide supra*.)

The relationship between the extent of dissociation (α), the equilibrium constant (K) and the concentration is apparently complex. For the case of a reversible gas phase dissociation reaction (of the type: $X = 2Y$), the variation of α with the pressure at various values of K is shown in Fig. 3 [9]. It is seen that α generally changes marginally at most pressures for nearly all values of K (except at very low pressures): this implies that it is nearly impossible to estimate K from the changes in dissociation! (This is an excellent example for the case of a self-complementary DNA duplex, with the pressure being replaced by the concentration.)

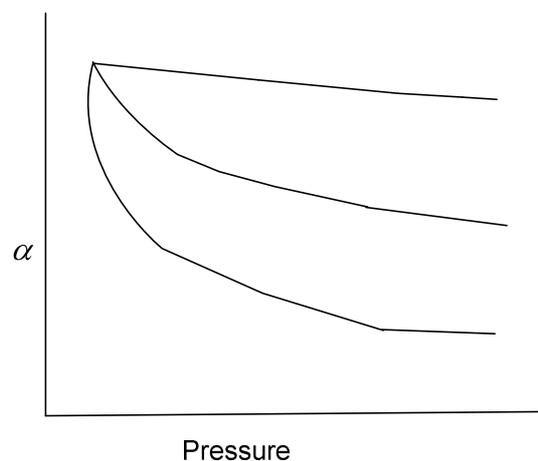


Fig. 3. The variation of α with the pressure for three values of K .

These arguments indicate that the equilibrium constant (K) for the reaction in Scheme 1 can be high, low or moderate, even at low temperatures. The analysis normally presented assumes an insignificantly low value for K (*i.e.* negligible amounts of *ss*-DNA present) at low temperatures. It now appears this assumption needs to be treated with caution.

The alternative possibility would imply that substantial amounts of *ss*-DNA are present even prior to the commencement of heating, which obviously raises the question of the origin of the observed changes in absorbance. As argued in a recent paper [7], this likely would represent the uncoiling of *ss*-DNA itself, with the melting of local duplex structures, formed internally rather than intermolecularly. (It is also noteworthy that a negative slope in the plot of Eqn. 4 implies that $\Delta H^\circ > 0$, although negative values for ΔH° , ΔS° and ΔG° have been reported, apparently without comment!)

These arguments indicate that DNA melting is a dubious technique for estimating base-pair stabilities. The possibility that what is being observed in these experiments is likely the breakdown of hairpin and other looped structures in *ss*-DNA is apparently supported by reports on the melting of (single stranded) RNA [7,10,11]. Also noteworthy is the assertion that the dismantling of the *ds*-DNA duplex would require prohibitive amounts of energy, certainly unavailable not only *in vivo* but also *in vitro* in aqueous media [7].

Alternative models of the DNA melting phenomenon

There are three possible alternative models, which explain most of the observations and the above objections to the current model to varying extents, as discussed below (*cf.* Fig.1).

(1) In the first model, the melting phenomenon is viewed as an incomplete conversion of *ds*-DNA to *ss*-DNA (Scheme 1), with the coalescence region at high temperature being an ‘isosbestic point’, as discussed above. However, this model is based on the assumption that the equilibrium constant K (Eqn. 2) is ~ 0 at the lower temperature ranges. This seems

dubious in view of the perceptible equilibrium shifts observed (changing α though not K). In other words, a very low value of K would imply an insignificant level of *ss*-DNA, perhaps below detection limits.

This model is also not compatible with the possibility that $\Delta H^0 \sim 0$ as then K would be nearly invariant with T , so no change would be observed. However, the nearly constant spacing between the sigmoid curves may arise from the constancy in the variation of α [Eqn. 1, noting $f = (1-\alpha)$].

(2) In the second model, the melting phenomenon is viewed as representing only the uncoiling of *ss*-DNA. It is likely that *ds*-DNA is present in equilibrium to an unknown extent, but does not break down under the experimental conditions. The breakdown would perhaps occur beyond the coalescence region ('isosbestic point', *vide supra*), in a hypothetical experiment in which the heating is continued further.

According to this model, the various sigmoid curves at different concentrations represent different mixtures of *ss*-DNA and *ds*-DNA, with only the former undergoing transitions to a relatively uncoiled form upon heating. The coalescence region possibly represents the beginning of the breakdown of the *ds*-DNA in the mixture, which is not observed further for the above discussed reasons.

This model assumes that the equilibrium in Scheme 1 is shifted by changes in the concentration, but not so much by heating. This can be justified by assuming that $\Delta H^0 \sim 0$ as argued above. (Note that both α and K are assumed to be largely invariant under the conditions employed. This also implies that the equilibrium between *ds*-DNA and *ss*-DNA (Scheme 1) is determined essentially by entropic effects.)

However, a problem is that the sigmoid plots are not expected to merge, *i.e.* there should be no coalescence, as shown in Fig. 4. This is because the absorbance values should reflect only

the concentrations throughout the temperature range, as only the (monomolecular) uncoiling of *ss*-DNA is presumed to occur. The observation, of course, is that the absorbance reaches a constant value for all the concentrations (Fig. 1).

A possible explanation for this is that the end products of the process – presumably uncoiled *ss*-DNA – possess enormously high extinction coefficients, so that the differences in the absorbance in terms of concentration are beyond the detection limits of the spectrometer. Thus, the difference in the *A* values (Δ_A) at two different concentrations would be given by Eqn. 5 from the Beer-Lamberts law (ϵ being the extinction coefficient, Δc the difference in concentrations and l the path length) [12].

$$\Delta_A = \epsilon l \Delta c \quad (5)$$

If $\epsilon \gg \Delta c$, $\Delta_A \sim \epsilon l$ (essentially a constant value), assuming a certain limiting sensitivity of the spectrometer being employed. This also implies that the value of *A* then has little quantitative significance!

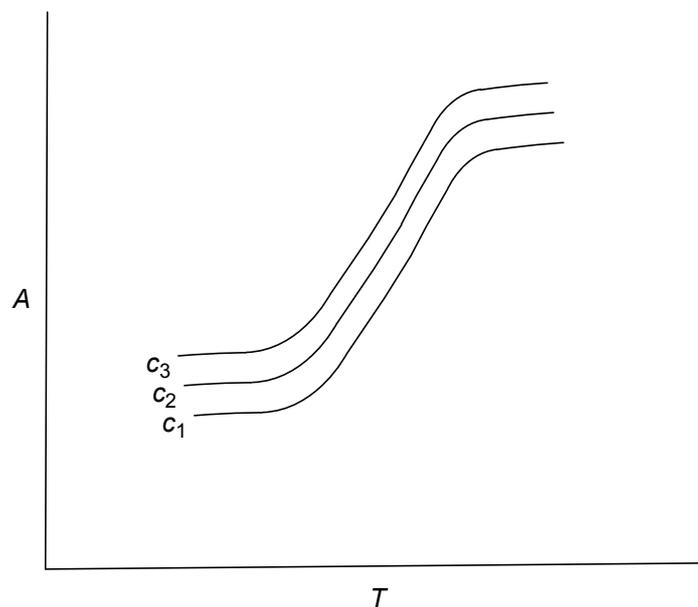


Fig. 4. Expected DNA melting curves involving only the uncoiling of *ss*-DNA at different concentrations ($c_1 > c_2 > c_3$)

(3) In the third and last model, the melting phenomenon is viewed as representing both the uncoiling of *ss*-DNA (major component) and the breakdown of *ds*-DNA (minor component). The nearly constant spacing maintained between the sigmoid curves (*vide supra*) thus indicates constant variation in α (Eqn. 1), rather than $\Delta H^0 \sim 0$ for the breakdown of *ds*-DNA. It seems reasonable to assume that the lower part of each curve (Fig. 1) represents the uncoiling of *ss*-DNA, with the upper parts also including the dissociation of *ds*-DNA (Scheme 1).

In other words, the increase in absorbance observed in Fig. 1 represents largely the uncoiling of *ss*-DNA, although at the end of the melting process only uncoiled *ss*-DNA is present. Thus, this model is a variation of the previous one, with the coalescence of the plots being explained as above.

CONCLUSIONS

Despite intensive experimental efforts spanning several decades, considerable ambiguity surrounds the phenomenon of nucleic acid melting, in terms of both the technique *per se* and the interpretation of the results garnered. In particular, perceptible concentration effects on the duplex-monomer equilibrium raise doubts about the assumption that the duplex is the sole form at low temperatures. The coalescence of the sigmoid plots observed in the melting experiments at the higher temperature ranges is an unexpected and intriguing feature that is not easily explained. Thus, the melting temperature (T_m) now seems of dubious validity, as also the application of the van't Hof equation to obtain thermodynamic data from the T_m .

Several alternative scenarios apparently emerge from these shadows, although a clear-cut picture remains elusive. All the same, the possibility that DNA melting essentially represents the uncoiling of single-stranded DNA – present in equilibrium with the double-stranded form to an unknown extent – seems the most likely. Ironically, the only viable conclusion is that

DNA melting is too dubious a technique for the quantitative estimation of the stability of the purine-pyrimidine base pairs composing the nucleic acids!

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