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DETERMINATION OF MELTING TEMPERATURE FOR MULTI-PEAK DIFFERENTIAL MELTING CURVES OF DNA

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This study was carried out because of a widely spread mistaken viewpoint that melting temperature (T_m) cannot be used as a reasonable parameter in the case of multi-peak differential melting curves (DMC) of DNA. However, there were no theoretical or experimental evidences or disclaimers of this viewpoint. In this work such study has been carried out. We have tested various definitions of T_m. Indeed, some of them give unreasonable dependences of T_m on relative concentration of modifications (r_b). At same time, the average temperature of the helix-coil transition calculated as the integral over the temperature of the product of DMC and temperature gives reasonable smooth dependences $T_m(r_b)$. The same definition is the best for the dependence of T_m on the average *GC*-content.

***Keywords*:** differential scanning calorimetry, high-resolution melting profiles, DNA plasmids, Calf Thymus DNA.

Introduction. This study was carried out because of a widely spread mistaken viewpoint that melting temperature (T_m) cannot be used as a correct parameter in the case of multi-peak differential melting curves (DMC). Indeed, theoretical and experimental studies demonstrate that the increasing concentration of DNA chemical modifications strongly changes the shape of multi-peak DMC [1–4]. Therefore, a change in T_m additionally reflects this change in shape besides a change in the DNA thermal stability in itself. However, there were no theoretical or experimental evidences or disclaimers of this viewpoint. In this work such study has been carried out.

When medium used for DNA melting experiments is changed or DNA structure is chemically modified, the shift of the melting temperature (δT_m) characterizes the thermal impact on the double helix. In general, T_m corresponds to the middle of the temperature melting range of DNA helix-coil transition. The T_m value can be calculated in different ways considered below. Here we have selected the definition of T_m that is dependent only on DNA *GC*-content, independent of DNA sequence and give the most smooth dependence of T_m on DNA *GC*-content or on the per nucleotide concentration of randomly distributed DNA chemical

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modifications (r_b). It is shown that the average temperature of the helix-coil transition (the integral over the temperature of the product of the DMC and temperature) is the most suitable for various studies.

Materials and Methods. We examine a DNA molecule of N base pairs (*bp*), which can be unmodified or include ω chemically modified sites located at base pairs with numbers $n_1,n_2,...,n_\omega$. It is supposed that each modification locally changes the free energy of the helix-coil transition by $\delta G(T)$. If chemical modifications destroy the double helix, they usually increase the free energy of the helical state, decrease the free energy of the helix-coil transition $\Delta G_{h\text{-}c}$ (i.e. $\delta G(T)<0$) and lower the melting temperature ($\delta T_m<0$). The conventional Poland–Fixman–Freire procedure [5–8] was used for calculation of DNA melting curves of unmodified and chemically modified DNA.

The following DNA parameter values are used for calculation: total number of base pairs, N=10 *kbp*, the fraction of *GC* base pairs, GC_{av}=0 or GC_{av}=0.25÷0.75. For $GC_{\text{av}} \neq 0$, DNA sequences of *AT* and *GC*-base pairs were produced with a random number generator. After generation of a random sequence of base pairs, a random sequence of chemical modifications for a given per nucleotide concentration r_b was also generated.

As an example of a real sequence plasmid pBR 322 (4361 *bp*) linearised with EcoRI was used in calculations.

The loop entropy factor for a loop of L base pairs formed between boundaries of internal melted region, $f(L)=(L+1)^{-1.7}$ [7, 8]; the factor of cooperativity or statistical weight assigned to the boundaries of an internal melted region bordered by helical ones, $\sigma = 5\cdot10^{-5}$ [7]; the strand association parameter, $\beta = \sigma$ [9]; the enthalpy, entropy of the helix-coil transition and melting temperatures of *AT* and *GC* base pairs, ΔH_{AT}=8.5 *kcal*/(*mol*·*bp*), ΔH_{GC}=9.4 *kcal*/(*mol*·*bp*), $\Delta S=\Delta S_{AT}=\Delta S_{GC}$=24.85 *cal* /deg., T_{AT}=64.3 ^0C and T_{GC}=107.2 ^0C [7].

The melting curve $\vartheta(T)$ that is the fraction of melted base pairs and DMC $\vartheta_T'(T)$ were calculated. Then T_m was obtained using its different definitions.

Ultra pure Calf Thymus DNA was used (protein<0.1%, RNA<0.1%, molecular mass ~30 *MDa*). The properties of this DNA have been previously described [10]. High-resolution melting profiles were obtained using a model of differential scanning microcalorimeter DASM 4 ("BIOPRIBOR", Russia) with a cell volume 0.5 *mL*. In the differential scanning calorimetry (DSC) experiments, we followed standard procedures [11]. The melting was carried in 0.1 *M* NaCl, 5 *mM* Na_2CO_3, 0.05 *mM* EDTA, pH 7. DNA concentration was 1 *mg*/*mL*.

Experimental Part.

Various Definitions of the DNA Melting Temperature. The illustration of different definitions of T_m shown at melting curve $\vartheta(T)$ and DMC $\vartheta_T'(T)$ is exhibited in Fig. 1. If a calorimetric thermogram or UV differential melting curve demonstrates a single peak, then the position of the maximum can be taken as the melting temperature ($T_m=T_{\max}$) [12, 13].

If DMC includes several peaks [11, 14–16] and they do not change their relative position under the influence of a factor that shifts T_m [14–16], then the position the highest peak can also be considered as melting temperature ($T_m=T_{\max}$), which is determined by equation

$$\vartheta'_T\left(T_{\max}\right) = \max\left[\vartheta'_T\left(T\right)\right]. \quad (1)$$

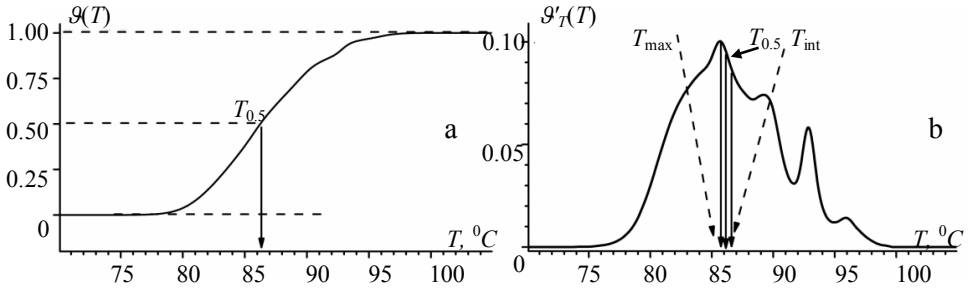

Fig. 1. Various definitions of the melting temperature ($T_{0.5}$, $T_{\max}$, T_{int}) illustrated for melting curve $\vartheta(T)$ (a) and differential melting curve $\vartheta'_T(T)$ (b) of ultra pure DNA from Calf Thymus.

A strong changes in T_m without a change in the shape of DMC occurs, for example, under alteration of Na^+ ion [14, 15] or formamide concentration [16].

For some melting experiments, it is even more convenient to determine T_m as the temperature position of the highest point of DMC or DSC curves, if the end of melting occurs at high temperature and cannot be recorded because of instrumental restrictions. In this case, other definitions of T_m cannot be used, because the whole melting curve is required for their determination.

The most popular definition of DNA melting temperature is the temperature that corresponds to the half of melted base pairs (T_m=$T_{0.5}$) or to a half-change of the parameter used for registration of the helix-coil transition:

$$\vartheta\left(T_{0.5}\right) = 0.5\,. \quad (2)$$

The melting temperature determined as the average temperature of the helix-coil transition (T_{int}) is used to characterize asymmetric DMC and DSC curves, especially when T_m is used to evaluate the average *GC*-content of DNA [17]. The value of T_{int} is given by equation

$$T_{\text{int}} = \int_{T_s}^{T_e} T \cdot \vartheta'_T\left(T\right) dT\,, \quad (3)$$

where $\vartheta'_T(T)\,dT$ is considered as the fraction of base pairs melted out in the temperature interval (T, T+dT); T_s and T_e are the temperatures that correspond to the start and to the end of DNA melting respectively.

One of the advantages of Eq. (3) is its closer correspondence to the average *GC*-content of DNA in comparison with $T_{\max}$ and $T_{0.5}$ [17].

If DMC includes a single symmetric peak or the asymmetry is negligible, than all T_m definitions give very close values. The question is which of these definitions is the most suitable for the study of the dependence of T_m on the *GC*-content or on the relative concentration of DNA chemical modifications in the case of multi-peak fine structure of DMC.

Melting Temperature of DNA with Different GC-Content. Since the dependence of $T_m(GC)$ is widely used in various DNA thermodynamic studies, let us consider which of definitions gives correct dependence $T_m(GC)$. In part, this issue was considered earlier [17]. In our present study we analyze suitability of

various definitions for much more complex fine structure of DMC (Fig. 2). Such structure is produced by DNA of N=10^4 *bp*, which includes two regions of random sequences with the lengths m_{GC_1} and m_{GC_2}. The regions distinguish in their average *GC*-content (GC_1 and GC_2). The total average *GC*-content (GC_{av}) of whole DNA is calculated using equation

$$GC_{\text{av}} = \frac{m_{GC_1} \cdot GC_1 + m_{GC_2} \cdot GC_2}{N}. \quad (4)$$

If GC_{av} is specified instead of m_{GC_i}, then following equations are used to calculate m_{GC_1} and m_{GC_2} :

$$m_{GC_1} = N\left(\left(GC_2 - GC_{\text{av}}\right)/\left(GC_2 - GC_1\right)\right),\ m_{GC_2} = N\left(\left(GC_{\text{av}} - GC_1\right)/\left(GC_2 - GC_1\right)\right). \quad (5)$$

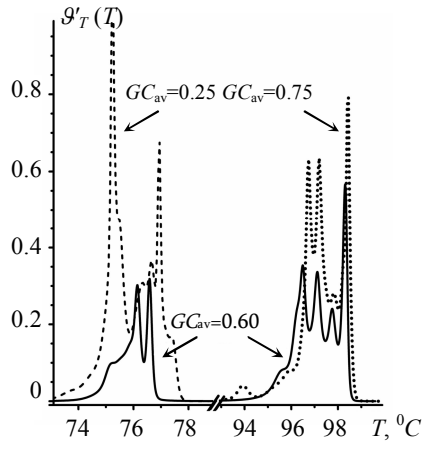

Fig. 2. Three DMC for DNA with sequences of 10^4 *bp* obtained with random number generator. The curves GC_{av}=0.25 and GC_{av}=0.75 correspond to a single random block of 10 *kbp*. The sequence corresponding to the curve with GC_{av}=0.6 consists of two random blocks with GC_1 = 0.25 (3 *kbp*) and GC_2 = 0.75 (7 *kbp*).

Every sequence is generated with the random number generator for different initializing values. For the first m_{GC_1} base pairs that form *AT*-rich block, the condition of *GC* base pair at the *i*-th base pair is $0 < X_i \le GC_1$. For the residual $m_{GC_2} = N - m_{GC_1}$ base pairs that form *GC*-rich block, the condition for *GC* base pair is $0 < X_i \le GC_2$ where X_i is the number produced by the random number generator at the *i*-th call. DMC were obtained for two sequences of 10 *kbp* that included one random block with GC_{av}=0.25 or GC_{av}= 0.75. All other sequences include the two blocks with GC_1=0.25 and GC_2=0.75. Three of the calculated DMC with the average *GC* values 0.25 (m_{25}=10 *kbp*), 0.60 (m_{25}=3 *kbp*) and 0.75 (m_{25}=0 *bp*) are shown in Fig. 2.

As follows from Fig. 2, it is difficult to assess T_m in the considered case, because of a very large melting range and complex multi-peak shape of the DMC. The sequences that include two blocks demonstrate a two-step character, and each step includes several narrow peaks (curve GC_{av}=0.6).

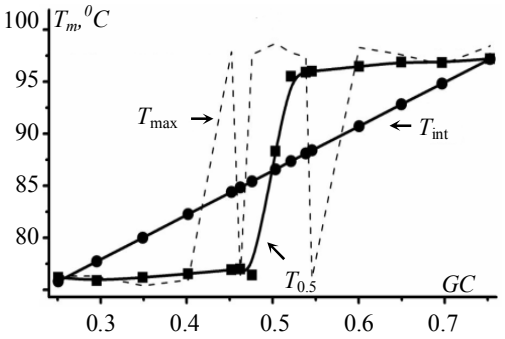

Fig. 3. The *GC* dependences of the melting temperature, defined as position of thermogram maximum ($T_{\max}$), as temperatures corresponding to 50% of melted base pairs ($T_{0.5}$) and as an average temperature of the helix-coiltransition (T_{int}).

The results of calculation for three definitions of T_m ($T_{0.5}$, $T_{\max}$, T_{int}) are shown as their dependence on GC_{av} (Fig. 3). It is seen that only T_{int} gives reasonable linear dependence on GC_{av} and can be used for thermograms and DMC of complex multi-peak shape. Other definitions ($T_{\max}$, $T_{0.5}$) do not demonstrate linear behavior and do not correspond the average *GC*-content.

Melting Temperature of Chemically Modified DNA. A similar problem in determination of melting temperature arises, if various factors change T_m along with the fine structure of DSC thermograms and/or DMC. One of such factor is DNA chemical modifications caused by some antitumor drugs such as platinum compounds [18]. These compounds strongly change the fine structure of DMC [1–4].

As in previous case, computer modeling allows us to test the suitability of different definitions of the T_m for DNA that includes chemical modifications. We have considered the dependence of T_m on relative concentration of chemical modifications randomly distributed along DNA with a given primary structure. If a given definition of T_m (Eqs. (1)–(3)) gives monotonous dependence $T_m(r_b)$ that is characterized with law deviation from a linear approximation $T_{m\_\text{apr}}(r_b)$, then it can be used as a parameter that correctly describes a change in DNA thermal stability. For quantitative evaluation of the smoothness of the functions $T_m(r_b)$, the root mean square error of approximation (RMSEA) is used:

$$\text{RMSEA}\left[\delta T_m\left(r_b\right)\right] = \left[\left(\sum_{j=1}^{N_p}[\delta T_m(r_{bj}) - \delta T_{m\_\text{apr}}(r_{bj})]^2\right)/N_p\right]^{1/2}, \qquad (6)$$

where N_p is number of points used in calculation; $\delta T_m(r_b) = T_m(r_b) - T_m(r_b = 0)$.

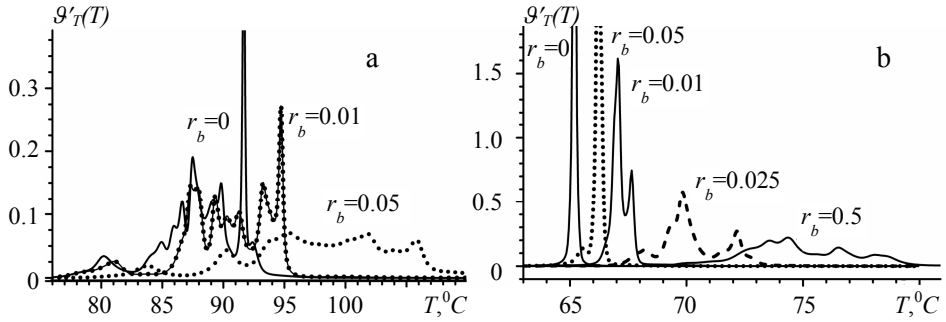

Fig. 4. DMC ($\vartheta'_T(T)$) for unmodified (r_b=0) and chemically modified DNA. Calculation was carried out for EcoRI-cut pBR322 DNA (a) and poly(dA)·poly(dT) (b).

For the modeling of the impact of chemical modifications on DNA melting, sequence of EcoRI-cut pBR322 plasmid DNA and homopolymer poly(dA)·poly(dT) were taken. The location of chemically modified sites was given with a random number generator. Chemical modification increases the free energy of the helix-coil transition by 2.5 *kcal* per mole of modifications. The results of calculation are shown in Fig. 4.

It is seen that a change in the fine structure of pBR322 and an increase in the temperature melting range occur as r_b increases. For poly(dA)·poly(dT), those changes are much stronger relative initial DMC of unmodified polymer.

As follows from Fig. 5 and Table, $\delta T_{\text{int}}(r_b)$ demonstrates the most smooth dependence with the minimal RMSEA. An additional advantage of this represen-

tation is the closest values of a change in melting temperatures for homogeneous and heterogeneous DNA. The difference for other representations is larger.

The root mean square error of approximation (RMSEA, ^0C) or $\delta T_{max}(r_b)$, $\delta T_{0.5}(r_b)$ and $\delta T_{int}(r_b)$ for pBR322 and poly(dA)·poly(dT) (linear approximation)

RMSEA, ^0C	$\delta T_{\max}$	$\delta T_{0.5}$	δT_{int}
pBR322	2.854	0.253	0.074
poly (dA)·poly(dT)	0.069	0.070	0.012

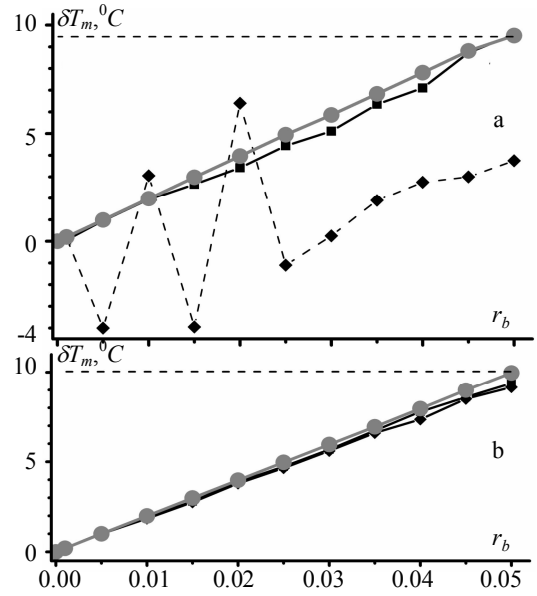

Fig. 5. The dependence of $\delta T_m(r_b)$. The curve $\delta T_{\text{int}}(r_b)$ (●) is the smoothest relative to $\delta T_{0.5}$ (■) and especially $\delta T_{\max}$ (◆).

The calculations were carried out for EcoRI-cut pBR322 DNA (a) and poly(dA)·poly(dT) (b).

The use of T_{int} also solves the problem of representation of the T_m in the case of irreversible binding of large ligands such as basic polypeptides and histones that cover long DNA regions, [19, 20]. They give rise to the two-step melting curve, because of formation of two types of blocks of different stability. Their DMC [19, 20] are similar to that shown in Fig. 2 (*GC*=0.6). In this case low and high temperature steps correspond to free DNA and DNA covered with irreversibly bound ligands. The dependence melting temperature $T_{0.5}(r_b)$ has a sigmoid shape as in Fig. 3. At the same time, T_{int} reflects total gradual linear increase in melting temperature and demonstrates linear dependence on r_b (Fig. 3).

We have found that independently of the shape of the DMC $\mathcal{G}'_T(T)$, the value of T_{int} is always very close to enthalpy/entropy ratio of the helix-coil transition (not shown).

Conclusion. In the article is proved the possibility of T_m calculating for multi-peak DMC and DSC thermograms and are analyzed various definitions of T_m and studied its dependences on the DNA *GC*-content and on the concentration of chemical modifications. It is shown that the average temperature of the helix-coil transition determined as the integral over the temperature of the product of the DMC and temperature is the best for various studies. This definition can be also used for other factors that change the thermal stability of the DNA double helix.

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REFERENCES

1. **Kagemoto A., Takagi Y., Naruse K., Baba Y.** Thermodynamic Characterization of Binding of DNA with Cisplatin in Aqueous Solution by Calorimetry. // Thermochm. Acta, 1991, v. 190, № 2, p. 191–201.
2. **Nunomura K., Maeda Y., Ohtsubo E.** The Interaction of Platinum Complexes with DNA Studied by Differential Scanning Calorimetry. // J. Gen. Appl. Microbiol., 1991, v. 37, № 2, p. 207–214.
3. **Lando D.Y., Fridman A.S., Krot V.I., Akhrem A.A.** Melting of Cross-Linked DNA: III. Calculation of Differential Melting Curves. // J. Biomol. Struct. Dynam., 1998, v. 16, № 1, p. 59–67.
4. **Lando D.Y., Galyuk E.N., Chun-Ling Chang, Chin-Kun Hu** Temporal Behavior of DNA Thermal Stability in the Presence of Platinum Compounds. Role of Monofunctional and Bifunctional Adducts. // Journal of Inorganic Biochemistry, 2012, v. 117, № 1, p. 164–170.
5. **Poland D.** Recursion Relation Generation of Probability Profiles for Specific-Sequence Macromolecules with Long-Range Correlation. // Biopolymers, 1974, v. 13, № 9, p. 1859–1871.
6. **Fixman M., Freire J.J.** Theory of DNA Melting Curves. // Biopolymers, 1977, v. 16, № 12, p. 2693–2704.
7. **Wartell R.M., Benight A.S.** Thermal Denaturation of DNA Molecules: a Comparison of Theory with Experiment. // Physics Reports, 1985, v. 126, № 2, p. 67–107.
8. **Blake R.D., Bizzaro J.W., Blake J.D., Day G.R., Delcourt S.G., Knowles J., Marx K.A., SantaLucia J.** Statistical Mechanical Simulation of Polymeric DNA Melting with MELTSIM. // Jr. Bioinformatics, 1999, v. 15, № 5, p. 370–375.
9. **Manyanga F., Horne M.T., Brewood G.P., Fish D.J., Dickman R., Benight A.S.** Origins of the "Nucleation" Free Energy in the Hybridization Thermodynamics of Short Duplex DNA. // J. Phys. Chem. B, 2009, v. 113, № 9, p. 2556–2563.
10. **Lando D.Y., Egorova V.P., Krot V.I., Akhrem A.A.** Determination of Protein Contaminants in DNA Preparations of High Purity. // Molec. Biol., 1996, v. 30, № 3, p. 701–706.
11. **Volker J., Blake R.D., Delcourt S.G., Breslauer K.J.** High-Resolution Calorimetric and Optical Melting Profiles of DNA Plasmids: Resolving Contributions from Intrinsic Melting Domains and Specifically Designed Inserts. // Biopolymers, 1999, v. 50, № 3, p. 303–318.
12. **Marky L.A., Breslauer K.J.** Calculating Thermodynamic Data for Transitions of any Molecularity from Equilibrium Melting Curves. // Biopolymers, 1987, v. 26, № 9, p. 1601–1620.
13. **Breslauer K.J.** Extracting Thermodynamic Data from Equilibrium Melting Curves for Oligonucleotide Order-Disorder Transitions. // Methods in Enzymology, 1995, v. 259, p. 221–242.
14. **Blake R.D., Delcourt S.G.** Thermal Stability of DNA. // Nucleic Acids Research, 1998, v. 26, № 14, p. 3323–3332.
15. **Uedaira H., Kidokoro S.-i., Ohgiya S., Ishizaki K., Shinriki N.** Thermal Transition of Plasmid pBR322 Closed Circular, Open Circular and Linear DNA. // Thermochimica Acta, 194, v. 232, № 1, p. 7–18.
16. **Blake R.D., Delcourt S.G.** Thermodynamic Effects of Formamide on DNA Stability. // Nucleic Acids Res., 1996, v. 24, № 11, p. 2095–2103.
17. **Vardevanyan P.O., Babayan Yu.S., Vardapetyan R.R., Panosyan G.A., Aslanyan V.M. Karapetian A.T.** Simple Method of Definition of an Average DNA Nucleotide Composition from the Melting Curves. // Biophys., v. 28, № 1, 1983, p. 130–131.
18. **Wang D., Lippard S.J.** Cellular Processing of Platinum Anticancer Drugs. // Nat. Rev. Drug Discov., 2005, v. 4, № 4, p. 307–320.
19. **Yu S.S., Li H.J.** Helix-Coil Transition and Conformational Studies of Protamine-DNA Complexes. // Biopolymers, 1973, v. 12, № 12, p. 2777–2788.
20. **Li H.J., Brand B., Rotter A., Chang C., Weiskopf M.** Helix-Coil Transition in Nucleoprotein. Effect of Ionic Strength on Thermal Denaturation of Polylysine-DNA Complexes. // Biopolymers, 1974, v. 13, № 8, p. 1681–1697.