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EVALUATION OF ERRORS IN DETERMINATION OF DNA MELTING CURVE REGISTERED WITH DIFFERENTIAL SCANNING CALORIMETRY

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The differential scanning calorimetry (DSC) is more sensitive than UV absorption spectrophotometry as a tool for the measurement of DNA melting curves. The advantage of DSC is a direct determination of differential melting curves (DMC) obtained without numerical differentiation. However, the difference between the helix-coil transition enthalpies of AT and GC base pairs can cause distortions in the shape of melting curve. Up to date, the errors caused by those distortions were not evaluated. In this study, a simple procedure of recalculation of a calorimetric DMC into a real DMC is developed. It demonstrates that the "real" melting curve and differential melting curve deviate very slightly from the same two curves calculated from DSC data. The melting temperature and the temperature melting range are usually the same even if the difference in the enthalpies is several times higher than a real one.

Differential scanning calorimetry – high-resolution melting profiles – DNA plasmids – calf thymus DNA

ԴՆԹ-ի հալման կորերի ստացումը դիֆերենցիալ սկանավորող կալորիմետրի (ԴՍԿ) միջոցով ավելի ճշգրիտ է քան՝ ՈւՄ սպեկտրոֆոտոմետրի միջոցով: ԴՍԿ-ի առավելությունը կայանում է հալման դիֆերենցիալ կորերի (ՀԴԿ) ուղղակիորեն որոշման մեջ, առանց թվային դիֆերենցման: Այնուհանդերձ, ԱԹ և ԳՑ-հիմքերի զույգերի էնթալպիաների տարբերությունը կարող է հանգեցնել հալման կորի տեսքի խախտման: Մինչ օրս այդ տարբերությամբ պայմանավորված սխալանքի արժեքները որոշված չեն: Աշխատանքում առաջարկվում է կալորմետրիկ ՀԴԿ – «իսկական» ՀԴԿ վերահաշվարկի ընթացք: Վերահաշվարկի կիրառումը ցույց է տվել, որ «իսկական» հալման կորը և հալման դիֆերենցիալ կորը աննշան են տարբերվում ԴՍԿ-ի տվյալներով որոշված նմանատիպ կորերից: Վերջինս ճիշտ է նաև հալման ջերմաստիճանի և հալման ջերմաստիճանային ինտերվալի համար նույնիսկ եթե ԱԹ և ԳՑ-հիմքերի զույգերի էնթալպիաների տարբերությունը մի քանի անգամ մեծ է իրական արժեքից:

Դիֆերենցիալ սկանավորող կալորիմետր – բարձր լուծողականության հալման պրոֆիլներ – պլազմիդային ԴՆԹ – հորթի ուրցագեղձի ԴՆԹ

Измерение кривых плавления ДНК методом дифференциальной сканирующей калориметрии (ДСК) является более точным, чем при использовании УФ-спектрофотометрии. Преимуществом ДСК является прямое определение дифференциальных кривых плавления (ДКП), получаемых без численного дифференцирования. Тем не менее, разница в энтальпиях АТ и ГЦ-пар оснований может вызвать искажения в форме кривой плавления. До настоящего времени величины ошибки, вызванной этим различием, не было определено.

В этой работе предлагается простая процедура пересчета калориметрических ДКП в "истинные" ДКП. Ее применение показало, что "истинная" кривая плавления и дифферен-

циальная кривая плавления незначительно отличаются от аналогичных кривых, рассчитанных по данным ДСК.

Последнее справедливо для температуры плавления и температурного интервала плавления даже в случае, если разница в энтальпии АТ и ГЦ-пар в несколько раз выше ее реальной величины.

Дифференциальная сканирующая калориметрия – профили плавления высокого-разрешения – плазмидная ДНК – ДНК тимуса теленка

The differential scanning calorimetry (DSC) gives more accurate melting profiles than UV absorption spectrophotometry as a tool for the measurement of DNA melting. The advantage of DSC is a direct determination of differential melting curves (DMC) obtained from thermograms without numerical differentiation. However, there is a widely spread opinion that calorimetry has shortcomings that hinder exact determination of the melting curve, differential melting curve (DMC), melting temperature and temperature melting range. The most important of them is the difference between the helix coil-transition enthalpies of AT and GC base pairs. Because of lower enthalpy of AT base pairs, the low temperature part of calorimetric DMC that corresponds to melting of AT-rich regions is lower than real DMC that is the first temperature derivative of the fraction of melted base pairs (fig. 1A and 2). The high temperature part corresponding to melting of GC-rich regions is located above the real DMC. The calorimetric melting curve is shifted towards higher temperature (fig. 1B). At the same time, there is no exact evaluation of the distortion value caused by this effect.

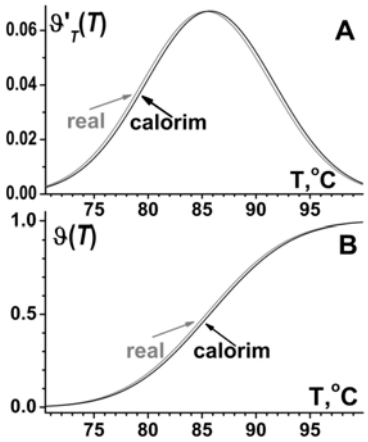

Fig. 1 A) Calorimetric differential melting curve (DMC) simulated with the Gaussian function characterized with the temperature melting interval ΔT=15°C and the results of its recalculation into the real DMC for H_{AT}= 8500 cal/(mol bp), H_{GC}=12500 cal/(mol bp). **B)** Calorimetric and real melting curves, which correspond with the differential melting curves depicted in fig. 1A.

As the differential scanning calorimetry, the UV registration of DNA absorption also does not directly measure the fraction of melted base pairs, and various methods are required to escape experimental errors [1-3]. However, the contribution to the total change of absorbance is approximately the same for AT and GC base pairs at λ=270 nm [1, 2].

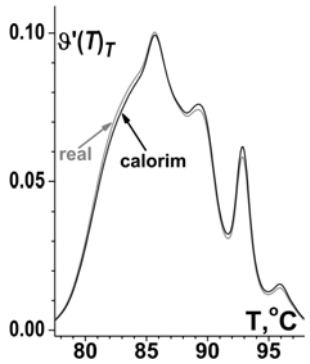

Fig. 2 Calorimetric DMC for calf thymus DNA and results of its recalculation into the real DMC for H_{AT}=8500 cal/(mol bp), H_{GC}=12500 cal/(mol bp).

In this study, a simple procedure of recalculation of a calorimetric DMC into a "real" DMC is proposed. Its use demonstrates that the method of differential scanning calorimetry causes negligible error in the determination of the melting curve, differential melting curve (DMC), melting temperature and temperature melting range.

Materials and methods. 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 [6]. 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 DSC experiments, we followed standard procedures [9]. The melting was carried in 0,1 M NaCl, 5 mM Na_2CO_3, 0,05 mM EDTA, pH 7.

In this study, the first derivative of the temperature dependence of the fraction of melted base pairs is called a "real differential melting curve" ($\vartheta'T(T)$) to distinguish it from the calorimetric differential melting curve ($\vartheta'_{cT}(T)$) obtained from thermograms by subtraction of buffer baseline, sample baseline and normalization. The calorimetric melting curve $\vartheta_c(T)$ is calculated by integration of the calorimetric differential melting curve $\vartheta'_{cT}(T)$, i.e., it is the temperature dependence of heat absorption normalized to the total heat absorption caused by the DNA helix-coil transition. Both calorimetric dependences can be expressed through the additional heat capacity caused by the helix-coil transition ($\Delta C_p(T)$):

$$\vartheta'_{c_T}(T) = \Delta C_p(T) / \int_{Ts}^{Te} \Delta C_p(t)dt \qquad (1),$$

$$\vartheta_c(T) = \int_{Ts}^{T} \vartheta'_{c_T}(t)dt = \int_{Ts}^{T} \Delta C_p(t)dt / \int_{Ts}^{Te} \Delta C_p(t)dt = H(T) / \bar{H} \qquad (2)$$

where T_s and T_e are the start and end of the temperature interval of the DNA helix-coil transition, $H(T)$ is the heat absorption at a given temperature and $\bar{H}$ is the total heat absorption caused by the DNA helix-coil transition that is equal to its enthalpy.

Real melting temperature (T_m) and calorimetric melting temperature (T_{mc}) correspond to the half of melted base pairs and to the half of additional heat absorption caused by the helix-coil transition, respectively. The temperature melting range for both cases (ΔT=1/$\vartheta'_T(T_m)$ and ΔT_c=1/$\vartheta'_{cT}(T_{mc})$) were determined as the inverse of the real or calorimetric DMC at melting temperature.

The study was carried out for calf thymus DNA, EcoRI-cut pBR322 DNA, and two calorimetric DMC curves simulated with the Gaussian function. The latter curves correspond to low and high DNA heterogeneity with the temperature melting range ΔT=3 or 15°C, respectively. The two values of the difference in enthalpies of GC and AT base pairs were considered: 1.1 and 4 kcal per mole of base pairs. The first value is used in different studies [10], and is closer to the experiment carried out for natural DNAs of various GC content [5]. The second difference occurs between enthalpies of the most [GC (CG)] and the least [TA (AT)] stable duplets of the nearest neighbors of base pairs [1]. It is the highest possible limit of the enthalpy difference.

The set of the thermodynamic parameters corresponding to the low (1.1 kcal) enthalpy difference is the following: T_{AT}=65,2°C, T_{GC}=107,8°C, H_{AT}=8.4 kcal/(mol bp), H_{GC}=9.5 kcal/(mol bp), S_{AT}=S_{GC}=24.8 cal/(mol bp·K^{-1}) [10]. It corresponds to case of entropies that are equal for AT and GC base pairs. The second set was used as an illustration of the extreme case of a very high difference in enthalpies (4 kcal/(mol bp)): T_{AT}=65,2°C, T_{GC}=107,8°C, H_{AT}= 8,5 kcal/(mol bp), H_{GC}=12,5 kcal/(mol bp), S_{AT}=25,12 cal/(mol·bp·K^{-1}), S_{GC}=32.81 cal/(mol bp·K^{-1}).

For EcoRI-cut pBR322 DNA, the Poland-Fixman-Freire approach [8, 9] was used for direct calculation of real and calorimetric DMC for both sets of parameters. Then the method of recalculation developed in this study was applied to obtain a real DMC from a calorimetric one. Both approaches give very close real melting curves.

Results and Discussion.

Calculation of real melting curve from calorimetric melting curve.

Let the temperature T_l correspond to the melting out of the DNA regions with the average GC composition x_l. At that temperature, the majority of regions with $x<x_l$ are almost fully melted. If $x>x_l$, the regions are almost fully helical. It is obvious that

$$x_l = (T_l - T_{AT})/(T_{GC} - T_{AT}) \qquad (3)$$

The average per base pair enthalpy for those regions ($H_l(T_l)$) is given by Eq.(4):

$$H_l(T_l)=(1- x_l)\cdot H_{AT} + x_l \cdot H_{GC} = H_{AT} + x_l\cdot(H_{GC}-H_{AT}) \qquad (4)$$

$H_l(T_l)$ can be represented in the following way:

$$H_l(T_l)= H_{AT} + \frac{T_l - T_{AT}}{T_{GC} - T_{AT}}\cdot(H_{GC}-H_{AT}) \qquad (5)$$

Using $H_l(T_l)$, one can obtain the heat absorption at a given temperature ($H(T)$) and the total heat absorption (average enthalpy) caused by the DNA helix-coil transition ($\overline{H}$), and then calculate calorimetric melting curve as $\vartheta_c(T)= H(T)/\overline{H}$. Let us represent Eq.(5) in the following way:

$$H_l(T_l)= A + B\cdot T_l \qquad (6)$$

where

$$A=(T_{GC}\cdot H_{AT}-T_{AT}\cdot H_{GC}) / (T_{GC} - T_{AT}) \qquad (7)$$

$$B=(H_{GC}-H_{AT}) / (T_{GC} - T_{AT}) \qquad (8)$$

Then the additional heat absorption that is caused by partial helix-coil transition under heating to temperature T is given by Eq.(9):

$$H(T) = \int_{Ts}^{T} H_l(t)\cdot \vartheta_t'(t)\cdot \mathrm{d}t = A\cdot\vartheta(T) + B\cdot\int_{Ts}^{T} t\cdot\vartheta_t'(t)\cdot dt \qquad (9)$$

If $T \geq T_e$ (*i.e.*, for full DNA melting), $H(T)$ is equal to the average per base pair enthalpy ($\overline{H}$):

$$\overline{H} = H(T_e) = A + B\overline{T} \qquad (10)$$

where

$$\overline{T} = \int_{Ts}^{Te} t \cdot \vartheta_t'(t) \cdot \mathrm{d}t \qquad (11)$$

The calorimetric melting curve, which is the temperature dependence of the fraction of heat absorption caused by the helix-coil transition, can be represented in the following way:

$$\vartheta_c(T) = H(T)/\overline{H} = [A \cdot \vartheta(T) + B \cdot \int_{Ts}^{T} t \cdot \vartheta_t'(t) \cdot \mathrm{d}t]/(A + B\overline{T}) \qquad (12)$$

Differentiating Eq.(12), calorimetric DMC can be expressed in terms of real DMC (Eq.(13)):

$$\vartheta_{cT}'(T) = [(A + B \cdot T)/(A + B\overline{T})] \cdot \vartheta_T'(T) \qquad (13)$$

For our study, it is more required to obtain an expression for real DMC ($\vartheta'_T(T)$) in terms of calorimetric DMC ($\vartheta')_{CT}(T)$):

$$\vartheta_T'(T) = [(A + B \cdot \overline{T})/(A + B \cdot T)] \cdot \vartheta_{cT}'(T)] \qquad (14)$$

The expression for $\vartheta'_T(T)$ includes $\overline{T}$ that can be found from experimental function $\vartheta'_{cT}(T)$ by the following transformation of Eq.(14):

$$\vartheta_{cT}'(T)/(A + B \cdot T) = \vartheta_T'(T)/(A + B \cdot \overline{T}) \qquad (15)$$

Then both parts are integrated with respect to T between T_s to T_e. As a result, one obtains Eq.(16) for $\overline{T}$:

$$\overline{T} = (C^{-1} - A)/B \qquad (16)$$

where A and B are given by Eqs. (7),(8) and

$$C = \int_{Ts}^{Te} [\vartheta_{ct}'(t)/(A + B \cdot t)] \cdot dt \qquad (17)$$

Thus, Eqs.(14), (16), (17) give $\vartheta'_T(T)$ in terms of $\vartheta'_{cT}(T)$.

Results of calculation. As follows from Eq. (14), calorimetric DMC is located lower than real DMC at $T < \overline{T}$ and higher at $T > \overline{T}$. It is well seen from fig. 1A and 2. It is also obvious that a stronger difference of a calorimetric melting curve from the corresponding real melting curve occurs for a larger temperature melting range.

Recalculation of experimental calorimetric curve $\vartheta'_c(T)$ into a real one $\vartheta'_T(T)$ using Eqs.(14), (16), (17) was carried out for experimental DSC curve of calf thymus DNA and DSC curves simulated with the Gaussian function. Additionally, the same expressions were used for recalculation of calorimetric melting curve computed for of EcoRI-cut pBR322 DNA into a real one. The melting temperature and temperature melting range were also determined for all types of curves.

If the difference in enthalpy is equal to the value obtained for DNA with different GC content [5, 10], i.e., ~1 kcal per mole of base pairs, then the calorimetric curves (ϑ_c, ϑ'_{cT}) and real curves (ϑ, ϑ'_T) are very close in all considered cases (results of calculation are not shown). The difference in melting temperature and melting range for two types of curves also does not exceed 0.1°C. Unfortunately, the exact dependence of enthalpy on GC content, similar to that was obtained for melting temperature [1], has not been determined directly. Therefore we have done computation for a possible highest difference between enthalpies of AT and GC base pairs. Calorimetric melting studies of the heteropolymeric poly[d(AT)]-poly[d(AT)], poly[d(AC)]-poly[d(GT)], and poly[d(GC)]-poly[d(GC)] duplexes and the homopolymeric poly[d(A)]-poly[d(T)] duplex demonstrate that the maximal difference in enthalpies of AT and GC base pairs can not be higher 4 kcal per mole of base pairs [3]. The same difference occurs for enthalpies of the most GC (CG) and least TA (AT) stable nearest neighbors of base pairs [1]. However, the maximal difference between all other nearest neighbors is reliably less than 2 kcal per mole of base pairs [1], and the last value also exceeds the upper limit of the average difference between GC and AT-base pairs.

Our calculation demonstrates that the calorimetric and real curves are also very close for EcoRI-cut pBR322 DNA and for the Gaussian curve with $\Delta T=3^oC$ even in the case of these high 4 kcal difference (the results are not shown). As follows from these results and Eq.(13), high deviation between real and calorimetric melting curves can take place only when both the temperature melting range and the difference in the enthalpy of AT and GC base pairs are high. Therefore the difference between calorimetric and real curves (fig. 1 and 2) is seen only for the calorimetric DMC curve simulated with the Gaussian function characterized with a large temperature melting range ($\Delta T=15^oC$, fig. 1) and for DNA from calf thymus ($\Delta T=10.6^oC$, fig. 2). Because of lower enthalpy of AT base pairs, the low temperature part of calorimetric DMC that corresponds to melting of AT-rich regions is lower than real DMC (fig. 1A and 2). The high temperature part corresponding to melting of GC-rich regions is located above the real DMC. As follows from the fig. 1B, calorimetric melting curve is always shifted towards higher temperatures. However, the difference in melting temperature is less than 0.3°C. The difference in the temperature melting range measured for calorimetric and real melting curves is less than 0.05°C. However, it should be pointed that the real difference in enthalpies is 2-4 times less then the value of 4 kcal taken for demonstration of the enthalpy effect. The average value 1.1 kcal is rather closer to the real difference [5, 10] than the extreme case of 4 kcal difference. Therefore, a deviation of calorimetric curves from real ones is much lower than that demonstrated in the fig. 1 and 2.

Thus, the results of our work demonstrate that melting curve calculated as a relative heat absorbance caused by DNA helix-coil transition $\vartheta_c(T)$ is very close to the fraction of melted base pairs $\vartheta(T)$ and can be used without any recalculation. The closeness of the differential melting curves, melting temperatures and temperature melting ranges is also demonstrated.

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