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KINETICS OF DNA THERMAL STABILITY IN THE PRESENCE OF CISPLATIN

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Antitumor drug cisplatin forms in DNA intermediate monofunctional and final bifunctional adducts that produces strong structural distortions to the double helix. To study the influence of both distortions on DNA thermal properties, we have monitored $\delta T_m(t)$ that is the time dependence of a change in DNA melting temperature under incubation with cisplatin. Cisplatin/nucleotide molar ratio was 0.01 to 0.1, incubation was carried out in 0.01 M $NaClO_4$. Previously found alkaline conditions were used for melting experiments. The alkaline medium makes the thermal effect of cisplatin much stronger and prevents further transformation of intermediate monofunctional adducts under heating. Processing of the experimental curves $\delta T_m(t)$, modeling of adduct formation and of the dependence $\delta T_m(t)$ were carried out. It has been found that, at t<8 min, only monofunctional adducts originated from initially monoaquated cisplatin influence $\delta T_m(t)$. Their concentration is highest and impact on thermal stability is maximal at t=8 min. For 8 min<t<10 h, a change in $\delta T_m(t)$ is mainly caused by accumulation of bifunctional adducts, although there is some influence of intermediate monofunctional adducts arisen from initially non-dissociated cisplatin. Only bifunctional adducts influence $\delta T_m(t)$ at t>10 hours. Kinetic and thermal characteristics of both intermediate monofunctional and final bifunctional adducts were determined. Among other things, we have demonstrated a two-fold stronger thermal destabilization of the double helix by a bifunctional adduct relative to a monofunctional one and a strong influence of initial state of cisplatin in incubation medium on the kinetics of DNA thermal stability under incubation with cisplatin.

DNA-complexes with platinum compounds – optical melting studies – kinetics of DNA platination

Հակաուռուցքային դեղանյութ հանդիսացող ցիսպլատինը կապվում է ԴՆԹ-ի հետ՝ առաջացնելով միջանկյալ մոնոֆունկցիոնալ և վերջնական բիֆունկցիոնալ ադուկտներ, որոնք բերում են երկպարույրի կառուցվածքային խախտմանը: ԴՆԹ-ի ջերմային հատկությունների վրա այդպիսի խախտումների ազդեցությունն ուսումնասիրելու նպատակով, դիտարկել ենք ($T_m(t)$ ֆունկցիան, որը ԴՆԹ-ի հալման ջերմաստիճանի փոփոխության կախվածությունն է ցիսպլատինի հետ ինկուբացիայի ժամանակից: Օգտագործվել է ցիսպլատին/նուկլեոտիդ մոլյար հարաբերությունը 0,01-ից 0.1, ինկուբացիան կատարվել է 0.01 M $NaClO_4$-ում: Հալման փորձերը կատարվել են հիմնային պայմաններում, որը մեծացնում է ցիսպլատինի ազդեցությունը ԴՆԹ-ի ջերմակայունության վրա, ինչպես նաև խոչընդոտում է մոնոֆունկցիոնալ ադուկտների հետագա վերափոխմանը, ինչը տեղի է ունենում չեզոք միջավայրում տաքացման հետևանքով: Աշխատանքում կատարվել է փորձնական կորերի մաթեմատիկական մշակում, ադուկտների առաջացման մոդելավորում և ($T_m(t)$ կախվածության ուսումնասիրություն: Հայտնաբերվել է, որ t<8 րոպե դեպքում ($T_m(t)$-ի վրա ազդում են միայն մոնոֆունկցիոնալ ադուկտները, որոնք առաջացել են սկզբնական մոնոհիդրատ ցիսպլատինից: t=8 րոպե դեպքում մոնոֆունկցիոնալ ադուկտների կոնցենտրացիան ամենամեծն է և նրանց ազդեցությունը ջերմակայունության վրա առավելագույնն է: 8 ր<t<10 ժ ընթացքում ($T_m(t)$-ի փոփոխությունը հիմնականում պայմանավորված է բիֆունկցիոնալ ադուկտների կուտակմամբ, թեև որոշակի ազդեցություն ունեն նաև միջանկյալ մոնոֆունկցիոնալ ադուկտները, որոնք առաջանում են սկզբնական չդիսոցված ցիսպլատինից: t>10 ժ դեպքում ($T_m(t)$-ի վրա ազդում են միայն բիֆունկցիոնալ ադուկտները: Որոշվել են պարամետրեր,
բնութագրող կինետիկան և ջերմակայունությունը ինչպես միջանկյալ մոնո ֆունկցիոնալ, այն-

պես էլ վերջնական բիֆունկցիոնալ ադուկտների համար:

Մասնավորապես ցույց է տրված, որ բիֆունկցիոնալ ադուկտի դեպքում ԴՆԹ-ի ջերմակայունությունը 2 անգամ ուժեղ է նվազում, ի տարբերություն մոնոֆունկցիոնալ ադուկտի, ինչպես նաև հաստատված է՝ ինկուբացիոն միջավայրում ցիսպլատինի սկզբնական վիճակի ուժեղ ազդեցությունը ԴՆԹ-ի ջերմակայունության կինետիկայի վրա:

ԴՆԹ կոմպլեքսներ պլատինային միացությունների հետ – ԴՆԹ-ի հալման սպեկտրալ ուսումնասիրություն – ԴՆԹ-ի պլատինացման կինետիկա

Противоопухолевый препарат цисплатин связывается с ДНК, образуя промежуточные монофункциональные и конечные бифункциональные аддукты, которые приводят к сильным структурным искажениям двойной спирали. Для изучения влияния таких искажений на термостабильность ДНК мы исследовали функцию $\delta T_m(t)$, являющуюся зависимостью изменения температуры плавления ДНК от времени инкубации с цисплатином. Использовалось молярное соотношение цисплатин/нуклеотид от 0.01 до 0.1, инкубацию проводили в 0.01 M $NaClO_4$. Эксперименты по плавлению проводили в щелочной среде, усиливающей эффект цисплатина на термостабильность ДНК и препятствующей дальнейшей трансформации промежуточных монофункциональных аддуктов, которая происходит при нагревании в нейтральной среде. Была проведена математическая обработка экспериментальных кривых $\delta T_m(t)$, моделирование образования аддуктов и соответствующего изменения термостабильности ДНК. Обнаружено, что при t<8 мин на термостабильность ДНК влияют только монофункциональные аддукты, образующиеся из исходно моногидратированного цисплатина. При t=8 мин концентрация монофункциональных аддуктов и их воздействие на термостабильность ДНК максимальны. В течение временного периода 8 мин<t<10 ч изменение $\delta T_m(t)$ в основном вызвано накоплением бифункциональных аддуктов, хотя промежуточные монофункциональные аддукты, которые образуются из первоначально недиссоциированного цисплатина, также оказывают некоторое влияние. При t>10 ч только бифункциональные аддукты оказывают влияние на $\delta T_m(t)$. Были определены кинетические параметры и изменение термостабильности, обусловленное как промежуточными монофункциональными, так и конечными бифункциональными аддуктами. В частности, показано, что бифункциональный аддукт в два раза сильнее снижает термостабильность двойной спирали по сравнению с монофункциональным аддуктом, а также продемонстрировано сильное влияние исходного состояния цисплатина в инкубационной среде на кинетику термостабильности ДНК.

Комплексы ДНК с соединениями платины – спектральное исследование плавления ДНК – кинетика платинирования ДНК

Cisplatin (cis-$Pt(NH_3)_2Cl_2$) is an effective antitumor drug that exerts its biological activity by direct strong coordination binding to DNA. It is used for treatment of testicular, ovarian, cervical, head, neck, esophageal, and lung cancer. Therefore various studies have been carried out to reveal its action mechanism [4, 9]. Cisplatin includes two cis-amine non-leaving ligands and two labile chloride leaving groups. The major product of DNA-cisplatin reaction is intra-strand cross-links between adjacent purines [4, 9]. Their formation is slow and hindered by chloride ions. Therefore DNA platination in vitro is usually carried out in $NaClO_4$. The ion ClO_4- does not stop platination in contrast to Cl^-. The half time ($t_{1/2}$) of the first chloride dissociation and substitution with a water molecule is ~2 hours [1]. The hydrolysis is followed by quick formation in DNA of intermediate monofunctional adduct mainly with N7 atom of guanine. The monofunctional adduct undergoes a slow chloride dissociation and hydrolysis characterized with approximately the same $t_{1/2}$ [1]. After that, the second coordination bond with N7 atom of a neighboring purine of the same strand arises quickly. These intra-strand cross-links account for 90% of products. Additionally, 6% of platinated sites form interstrand cross-links between neighboring guanines of different strands. Thus, almost all final adducts are bifunctional.

As we have previously found, alkaline medium (pH~10.5) strengthens a change in DNA melting temperature (δTm) caused by cisplatin binding [7]. Moreover, intermediate monofunctional adducts formed by cisplatin become stable against heating and can be studied in melting experiments [7].

These findings allowed us to measure kinetics of the shift in melting temperature under platination, i.e., to determine the dependence $\delta T_m(t)$. The formation of intermediate monofunctional and final bifunctional adducts of platination are well resolved in the plot $\delta T_m(t)$ if melting experiments are carried out in alkaline medium. It allows separate measurement of the rate of their formation and their influence on DNA thermal stability. Such measurements, their processing and modeling have been carried out in this study to determine kinetic and thermal parameters of platination.

Materials and methods. Ultra pure calf thymus DNA that we prepared was used (protein<0.1%, RNA<0.1%, molecular mass ~30 MDa). The properties of this DNA have been previously described (6). DNA at a concentration of 1.2 mg/ml was incubated with cisplatin of Sigma-Aldrich Chemical Company. For measurements of melting temperature kinetics under platination, Pt/nucleotide molar ratio (r) was 0.01 to 0.1.

Cisplatin was added to DNA from a stock solution in distilled water (1 mg/ml) after several day of incubation at 25^0C. This stock solution contains 35 % of undissociated, 65 % of monoaquated and less 1% of diaquated cisplatin [10]. The equilibrium is reached during 25 hours after cisplatin's dissolution [2, 10].

The DNA-cisplatin mixture was incubated in 0,01 M $NaClO_4$ at 37^0C in the dark (pH~6). At time intervals from 1 min to 48 h, aliquots of the reaction mixture were withdrawn. Platination in the aliquots was stopped by adjusting NaCl concentration to 0,1 M. This chloride concentration stops cisplatin binding and transformation of intermediate monofunctional adducts at temperature lower 37^0C, but there is a well pronounced further development of this reaction if the temperature is higher 50^0C (not shown). Then the samples of DNA solution were diluted to concentration 0,075 mg/ml and frozen at -28^0C [3, 7, 8, 11].

For melting experiments, DNA was thawed and diluted to 0.04 mg/ml. The melting was carried out in 0.1 M NaCl, 0.005 M Na_2CO_3, 0.001M $NaClO_4$, 5.10^{-5}M EDTA, pH 10,5. In this buffer, the melting temperature of unmodified DNA is $65\pm0,3^0$C. DNA helix-coil transition was registered by measuring the optical density at 260 nm as a function of temperature using a SF-26 spectrophotometer (LOMO, Russia) or Perkin-Elmer Lambda 800 UV/VIS (USA).

The results of the time dependences obtained under incubation and further melting of platinated DNA were exhibited as a relative change in melting temperature $\theta(t)= \delta T_m(t)/\delta T_{max}$ where $\delta T_m(t)$ is the time dependence of a change in melting temperature, i.e., $\delta T_m(t)=T_m(t)-T_m(t=0)$, and $\delta T_{max}=\delta T_m(t\geq 10$ h) is the maximal shift in melting temperature that is reached during 10 hours of incubation. The dependences θ(t) are almost the same for the Pt/nucleotide molar ratio r from interval 0,01 to 0,1. For these r values, almost all cisplatin binds to DNA during incubation [5].

In all calculations, the fractions of primary products (monoaquated and non-dissociated cisplatin), intermediate monofunctional and final bifunctional adducts are given in regard to total cisplatin in incubation medium.

Results and Discussion. *The kinetics of DNA thermal stability under platination*

In our previous study [7], we have found that alkaline medium (pH~10.5) makes the shift in the melting temperature caused by cisplatin (δT_m) much stronger relative to melting carried out at pH~7. Additionally, intermediate monofunctional adducts become stable against heating in alkaline conditions. Therefore the properties of both monofunctional and bifunctional adducts can be studied in melting experiments if registration of melting curves is carried out at pH~10.5 after various time intervals of DNA incubation with cisplatin in 0,01 M $NaClO_4$ at pH 6. A monotonous decrease of melting temperature with time ($T_m(t)$ occurs from the first minutes of incubation of DNA with cisplatin, and the maximal destabilization shift (δT_{max}) is reached during 10 hours. Later $T_m(t)$ and $\delta T_m(t)=T_m(t)-T_m(t=0)$ do

not change. The maximal destabilization corresponds to the shift δT_{max}= -12±1,5^{0}C for Pt/nucleotide molar ratio r=0,05.

In our present study, the shift $\delta T_m(t)$ is normalized to $\delta T_{max}=\delta T_m(t \geq 10$ h). For this relative shift in melting temperature $\theta(t)= \delta T_m(t)/\delta T_{max}$, all kinetic curves θ(t) are very close if r≤0.1. All dependences $\theta(t)$ are monotonous and change with time from zero to unity.

In fig. 1, the time dependence $\theta(t)= \delta T_m(t)/\delta T_{max}$ for r=0.05 is exhibited (curve 1 exp). The parameter $t_{1/2}$ determined as $\theta(t_{1/2})$=0.5 is equal to 70 min. A two-step change in $\theta(t)$ is well seen. To carry out separation of slow and quick steps, the curve 1 exp was differentiated and dissolved into the two constituents. Then the constituents of the differential curve were separately integrated to obtain corresponding constituents 1a and 1b of curve 1 exp (fig. 1). Constituent 1a is characterized with $t_{1/2a}$=1.2 min and its height is equal to 0.29. For constituent 1b, the value of $t_{1/2b}$ is 120 min and the height is 0.71.

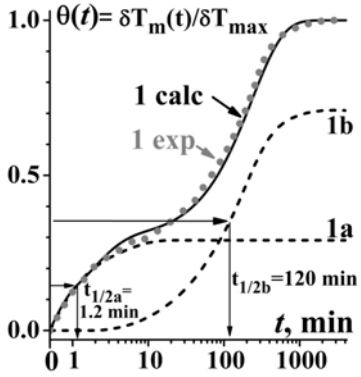

Fig. 1. The time dependence of a relative change in DNA melting temperature caused by binding and transformation of cisplatin: $\theta(t)=\delta T_m(t)/\delta T_{max}$; $\delta T_m(t)=T_m(t)-T_m(t=0)$; $\delta T_{max}=T_m(t\geq 10$ h)$-T_m(t=0)$ is the maximal shift in melting temperature reached at t >10 h. The experimental curve (**1 exp**) and the curve calculated using Eqs.(3)-(14) (**1 cal**) are in good agreement. The curves **1a** and **1b** are the constituents of the experimental curve $\theta(t)$ (**1 exp**). At t<8 min, constituent **1a** reflects a relative change in melting temperature caused the formation and accumulation of monofunctional adducts formed from monoaquated cisplatin. The growth of θ (t) above constituent **1a** at t>8 min is depicted with constituent **1b** that mainly reflects accumulation of bifunctional adducts.

Binding to DNA and further transformation of cisplatin. In this study, the relative concentration (fraction) of initial monoaquated (*ma*) and non-dissociated (*c*) cisplatin, intermediate monofunctional (*m*) and final bifunctional adducts (b) are given in regard to the total concentration of cisplatin in incubation medium.

In cisplatin stock solution we use (concentration 1 mg/ml and pH 5, 6), the fraction of monoaquated cisplatin (θ_{ma}) is equal to 0,65 and the fraction of unaquated (non-dissociated) cisplatin (θ_c) is 0,35 (10). The value of $t_{1/2}$mma for formation of monofunctional adducts from monoaquated cisplatin evaluated in various studies is 1÷6 minutes ((1) and references therein). There are no other types of adducts that can influence DNA thermal stability during the first minutes of incubation, because parallel formation of monofunctional adducts from initially unaquated (non-dissociated) cisplatin (θc=0.35) is too slow ($t_{1/2}$mc~2h). Although inter-strand and intra-strand cross-links can be quickly formed from diaquated cisplatin, its fraction in the cisplatin stock solution is less 0.01 (10). Therefore these DNA modifications do not influence δTm(t) and θ(t) if t is low. Thus, the first step of the kinetic curve 1 exp in fig. 1 is caused by quick formation of monofunctional adducts from monoaquated cisplatin (*ma*) that arises in stock solution of

cisplatin. Corresponding constituent 1a reflects a relative change in melting temperature caused by the accumulation of monofunctional adducts formed from monoaquated cisplatin from stock solution (*mma*). This process overlaps the time interval $0<t<8$ min and $t_{1/2mma}$=1,2 min. At $t>8$ min, constituent 1a does not change. Additional increase in $\theta(t)$ above constituent 1a is exhibited by constituent 1b caused by all other types of adducts. This second step of curve 1 exp in fig. 1 corresponds to the two slow processes: transformation monofunctional adducts into final bifunctional ones and formation of monofunctional adducts (*mc*) from unaquated (non-dissociated) cisplatin (*c*) that accounts for 35 % in stock solution ($\theta_c(0)$=0,35).

Modeling of the time dependences of thermal stability and of the fractions of monofunctional and bifunctional adducts. As it was done before (1), let us consider cisplatin transformation as parallel successive pseudo-first-order reactions. Initially monoaquated cisplatin (ma) undergoes the following transformation during incubation with DNA:

$$\textit{monoaquated cisplatin}\ (\mathbf{ma}) \rightarrow \textit{monoadduct}\ (\mathbf{mma}) \rightarrow \textit{biadduct}\ (\mathbf{bma}) \qquad (1)$$

Initially undissociated cisplatin (c) transforms in the following way:

$$\textit{cisplatin}\ (\mathbf{c}) \xrightarrow{k_{mc}} \textit{monoadduct}\ (\mathbf{mc}) \xrightarrow{k_b} \textit{biadduct}\ (\mathbf{bc}) \qquad (2)$$

The first stage in Eq.(1) is quick (1÷6 min) and the second one is slow (~2 h) (1). In Equations (2), both stages are slow (~2 h). Equations (1)-(2) are simplified by omission of short-lived intermediates of very low relative concentration. Those are monoaquated cisplatin originated from non-dissociated cisplatin (Equations (2)) and aquated monofunctional adducts (Equations (1), (2)).

The time dependences of the fractions of all compounds involved in reactions given with Equations (1), (2) are the following:

$$\theta_c(t) = \theta_c(0) \cdot \exp(-k_{mma} \cdot t) \qquad (3)$$

$$\theta_{ma}(t) = \theta_{ma}(0) \cdot \exp(-k_{mc} \cdot t) \qquad (4)$$

$$\theta_{mc}(t) = \theta_c(0) \cdot \frac{k_{mc}}{k_{mc} + k_b} \cdot [\exp(-t \cdot k_{mc}) - \exp(-t \cdot k_b)] \qquad (5)$$

$$\theta_{mma}(t) = \theta_{ma}(0) \cdot \frac{k_{mma}}{k_{mma} + k_b} \cdot [\exp(-t \cdot k_{mma}) - \exp(-t \cdot k_b)] \qquad (6)$$

$$\theta_{bc}(t) = \theta_c(0) \cdot \left[1 - \frac{k_b}{k_b - k_{mc}} \cdot \exp(-t \cdot k_{mc}) + \frac{k_{cm}}{k_b - k_{mc}} \cdot \exp(-t \cdot k_b)\right] \qquad (7)$$

$$\theta_{bma}(t) = \theta_{ma}(0) \cdot \left[1 - \frac{k_b}{k_b - k_{mma}} \cdot \exp(-t \cdot k_{mma}) + \frac{k_{mma}}{k_b - k_{mc}} \cdot \exp(-t \cdot k_b)\right] \qquad (8)$$

In conditions of the cisplatin stock solution (1 mg/ml, pH 5.6), the sum of primary total fraction of non-dissociated $\theta_c(0)$ and monoaquated $\theta_{ma}(0)$ cisplatin is equal to unity:

$$\theta_c(0)+ \theta_{ma}(0)=1 \quad (9)$$

The fraction of total unbound cisplatin ($\theta_{cis\_tot}(t)$) is the sum of monoaquated and non-dissociated cisplatin:

$$\theta_{cis\_tot}(t)= \theta_{ma}(t) +\theta_c(t) \quad (10)$$

The total fractions of monofunctional and bifunctional adducts are the following:

$$\theta_m(t)= \theta_{mc}(t)+\theta_{mma}(t) \quad (11)$$

$$\theta_b(t)= \theta_{bc}(t)+\theta_{bma}(t) \quad (12)$$

The time dependences of all fractions were calculated for parallel successive pseudo-first-order reactions (Equations (1) and (2)) with primary conditions $\theta_c(0)=0.35$, $\theta_{ma}(0)=0.65$ where $\theta_c(t)$ and $\theta_{ma}(t)$ are the fractions of non-dissociated and monoaquated cisplatin, respectively (10). We also use the values of the pseudo-first-order rate constants $k_{mc}=10{,}2 \cdot 10^{-5}$ and $k_b=9{,}2 \cdot 10^{-5}$ s^{-1} for formation of monofunctional adducts from non-dissociated cisplatin and bifunctional adducts from monofunctional adducts determined by Bancroft et al. (1). Fig.1 demonstrates that, in 0.01 M $NaClO_4$, $t_{1/2mma}=1.2$ min for formation of monofunctional adducts from monoaquated cisplatin. It corresponds to pseudo-first-order rate constant $k_{mma}=9.7 \cdot 10^{-3} s^{-1}$. Using these parameters, we have calculated time dependences of fractions of two types of monofunctional ($\theta_{mc}(t)$, $\theta_{mma}(t)$) and bifunctional ($\theta_{bc}(t)$, $\theta_{bma}(t)$) adducts arisen from non-dissociated (c) and monoaquated (ma) cisplatin, respectively.

If all cisplatin in stock solution is monoaquated ($\theta_c(0)=0$, $\theta_{ma}(0)=1$), the height of the first step of curve 1 exp and the constituent 1a in fig. 1 would be maximal, i.e., $\delta T_{max\ m}/\delta T_{max}$ =0,45 (0,29/0,65=0,45), where δT_{max} m is a temperature shift caused by transformation of all cisplatin introduced in DNA solution into monofunctional adducts only.

At a studied cisplatin/nucleotide molar ratio (r=0.01-0.1), final cisplatin-DNA products are the same independently of cisplatin species in stock solution (1). These final products formed after 10 hours are mainly bifunctional ones (intra-strand and inter-strand cross-links account for 96 %) and their total per nucleotide amount is close to r. Therefore the final and maximal shift of melting temperature is almost fully caused by bifunctional adducts, i.e., $\delta T_{max\ b}= \delta T_{max}$, where δT_{max} is the maximal (final) shift in melting temperature, and $\delta T_{max\ b}$ is the contribution of bifunctional adducts in this final shift. Thus, $\delta T_{max\ b} /\delta T_{max}$ is equal to unity and $\delta T_{max\ m}/\delta T_{max}$=0,45 where $\delta T_{max\ m}$ is a temperature shift caused by transformation of all cisplatin introduced in DNA solution into monofunctional adducts only. It means that destabilizing effect of bifunctional adducts is 2,2-fold stronger than that of monofunctional ones (1/0,45). Taking into account these circumstances, the time dependence of a relative change in melting temperature can be represented in the following way:

$$\theta(t)= \delta T_m(t)/ \delta T_{max}=(\delta T_{max\ m}/\delta T_{max}).\theta_m(t) + (\delta T_{max\ b}/\delta T_{max}).\theta_b(t) \quad (13)$$

$$\theta(t)=0{,}45 \cdot \theta_m(t)+ \theta_b(t) \quad (14)$$

Kinetics of cisplatin transformation and its influence on temporal behavior of DNA thermal stability under platination. As follows from fig. 2A, the fractions of free monoaquated (θ_{ma}) and non-dissociated (θ_c) cisplatin decrease to zero during 8 min and 10 hours, respectively. Those time intervals correspond to the time of saturation for constituents 1a and 1b in fig. 1. During the first 8 min of incubation, only monofunctional adducts arisen from monoaquated cisplatin of stock solution are formed. Their maximal fraction

($\theta_{mma}(t$=8 min)=0,65) is close to the initial fraction of monoaquated cisplatin in

stock solution ($\theta_{ma}(t=0)$, fig. 2A) and to the maximal total fraction of the monofunctional adducts θ_m(t=8 min) (fig. 2B):

$$\theta_{ma}(t=0) \approx \theta_{mma}(t=8\text{ min}) \approx \theta_m(t=8\text{ min}) \approx 0.65 \qquad (15)$$

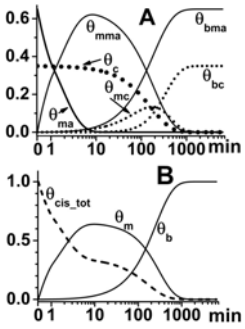

Fig. 2. A) Calculated time dependences of fractions of undissociated ($\theta_c(t)$) and monoaquated ($\theta_{ma}(t)$) cisplatin; θ_{mc}, θ_{mma}, θ_{bc}, θ_{bma} are the fractions of monofunctional (**m**) and bifunctional (**b**) adducts arisen from undissociated cisplatin (c) and monoaquated cisplatin (**ma**). B) Calculated time dependences of the total fractions of monofunctional ($\theta_m=\theta_{mc}+\theta_{mma}$), bifunctional ($\theta_b=\theta_{bc}+\theta_{bma}$) adducts, and the total fraction of unbound cisplatin ($\theta_{cis\_tot}=\theta_c+\theta_{ma}$). The following parameter values were used for calculation: $\theta_c(0)$=0.35, $\theta_{ma}(0)$=0.65, k_{mma}=9.7·10-3 s-1, k_{mc}=10.2·10^{-5} s-1 (1) and kb=9.2·10^{-5} s^{-1} (1).

Monofunctional adducts arisen from non-dissociated cisplatin cannot be accumulated, since the rates of their formation from non-dissociated cisplatin and transformation into bifunctional adducts are almost equal, and the maximal value of their fraction is much lower ($\theta_{mc}(t=3h)$=0,13, fig. 2A).

An increase in the fractions of bifunctional adducts arisen from monoaquated and non-dissociated cisplatin are characterized with S-shaped curves and half times about 2 and 6 hours, respectively (fig. 2A). For total fraction of bifunctional adducts ($\theta_{bma}+\theta_{bc}$), the half time is 3 hours (fig. 2B).

The time dependence of the relative thermal stability $\theta(t)=\delta Tm(t)/\delta T_{max}$ was calculated for parallel successive pseudo-first-order reactions (Equations (1) and (2)) with primary conditions $\theta_c(0)$=0,35, $\theta_{ma}(0)$=0,65 using Equations (13), (14). There is a good coincidence between calculated (1 calc) and experimental (1 exp) curves θ(t) (fig. 1). Both curves demonstrate a two-step shape. A decrease in the fraction of total cisplatin also demonstrates a similar two-step character (fig. 2B).

Besides experimental primary conditions ($\theta_c(0)$=0,35, $\theta_{ma}(0)$=0,65), additional modeling for two limiting cases was carried out, firstly, for cisplatin fully non-dissociated in stock solution ($\theta_c(0)$=1, $\theta_{ma}(0)$=0) and, secondly, for fully monoaquated cisplatin ($\theta_c(0)$=0, $\theta_{ma}(0)$=1). All three dependences $\theta(t)$ are shown in fig. 3. It is seen that the state of cisplatin in stock solution strongly influences temporal behavior of DNA stability. If all cisplatin in stock solution is monoaquated ($\theta_{ma}(0)$=1), then the first step is markedly higher relative to the present experiment, in which $\theta_{ma}(0)$=0,65. If cisplatin is fully non-dissociated ($\theta_{ma}(0)$=0), the "quick" first step disappears and the shape of the curve is mainly determined by accumulation of bifunctional adducts.

Computer modeling of a change in $\theta(t)= \delta T_m(t)/\delta T_{max}$ without thermal impact of monofunctional adducts formed from non-dissociated cisplatin demonstrates their weak influence on $\theta(t)$ (fig. 3). It means that the second step in the curve $\theta(t)= \delta T_m(t)/\delta T_{max}$ mainly reflects accumulation of final bifunctional adducts.

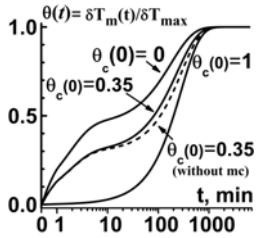

Fig. 3. Calculated time dependences of a relative change in melting temperature at various starting conditions: $\theta_c(0)=0$, $\theta_{ma}(0)=1$; $\theta_c(0)=1$, $\theta_{ma}(0)=0$; $\theta_c(0)=0.35$, $\theta_{ma}(0)=0.65$ (curve **1 calc** from fig. 1); and the same dependence calculated without thermal impact of monofunctional adducts formed from undissociated cisplatin. The values of $\theta_c(0)$ are shown for curve identification.

The first step of $\theta(t)$ that corresponds to accumulation of monofunctional adducts from monoaquated cisplatin is well resolved because $t_{1/2a}=t_{1/2mma}$=1,2 min is a hundred times lower than the time of transformation of monofunctional adducts into bifunctional ones ($t_{1/2b}\approx t_{1/2mb}\approx$ 2h) and the time of formation of monofunctional adducts from non-dissociated cisplatin ($t_{1/2mc}\sim$2h). Therefore monofunctional adducts arisen from monoaquated cisplatin can be accumulated before their further transformation and give a resolved step.

Monofunctional adducts arisen from non-dissociated cisplatin cannot be accumulated before their further transformation and give a resolved step, since the rates of their formation from non-dissociated cisplatin and transformation into bifunctional adducts are almost equal. The maximal contribution of monofunctional adducts from non-dissociated cisplatin in $\theta(t)$ is only 0.06 (fig. 3) and the maximal value of their fraction $\theta_{mc}(t$=3h)=0,13, i.e., both the impact and fraction of monofunctional adducts originated from monoaquated cisplatin is five times higher: their maximal contribution in $\theta(t)$ is 0.29 (fig. 1, curve 1a) and $\theta_{mma}(t$=8 min) is close to 0,65 (fig. 2A).

Thus the results of modeling demonstrate that, during the time interval $0<t<8$ min, only monofunctional adducts arisen from monoaquated cisplatin from stock solution determine the shift in melting temperature $\delta T_m(t)$. The maximal fraction of monofunctional adducts and their maximal impact on DNA melting temperature occur at t=8 min. That maximal fraction is equal to the initial fraction of monoaquated cisplatin in incubation medium (and in stock cisplatin solution). At $t>10$ hours, only bifunctional adducts determine the value of thermal stability and their fraction is close to unity. The separate determination and comparison of the impacts of monofunctional adducts at t=8 min and bifunctional adducts at $t>10$ h, and further calculation of the per adduct thermal effect demonstrate a two-fold stronger thermal destabilization of the double helix by a bifunctional adduct relative to a monofunctional one.

A kinetic curve of a change in DNA thermal stability under incubation with cisplatin is characterized with a two-step shape. The first "quick" step is caused by formation and accumulation of monofunctional adducts arisen from monoaquated cisplatin from stock solution. The second step is caused by all other processes that are much slower, but it predominately reflects the accumulation of bifunctional adducts, although there is some influence of intermediate monofunctional adducts arisen from initially non-dissociated cisplatin. The state of cisplatin in stock solution strongly influences the shape of the kinetic curve of a change DNA in thermal stability. If all cisplatin is initially monoaquated, then the first step is the highest. If cisplatin is fully non-dissociated, the "quick" first step is absent and a change in thermal stability mainly reflects the accumulation of bifunctional adducts.

In this case, the fraction and impact of monofunctional adducts is small, because of they cannot be accumulated in a large amount, since the rates of their formation from non-dissociated cisplatin and transformation into bifunctional adducts are almost equal.

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