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ORIGINAL PAPER

Spectroscopic study of protonation of oligonucleotides containing adenine and cytosine‡

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Acidobasic properties of purine and pyrimidine bases (adenine, cytosine) and relevant nucleosides (adenosine, cytidine) were studied by means of glass-electrode potentiometry and the respective dissociation constants were determined under given experimental conditions (I = 0.1 M (NaCl), t = (25.0 ± 0.1) °C): adenine (pK_{HL} = 9.65 ± 0.04, pK_{H2L} = 4.18 ± 0.04), adenosine (pK_{H2L} = 3.59 ± 0.05), cytosine (pK_{H2L} = 4.56 ± 0.01), cytidine (pK_{H2L} = 4.16 ± 0.02). In addition, thermodynamic parameters for bases: adenine (ΔH^0 = (–17 ± 4) kJ mol^{-1}, ΔS^0 = (23 ± 13) J K^{-1} mol^{-1}), cytosine (ΔH^0 = (–22 ± 1) kJ mol^{-1}, ΔS^0 = (13 ± 5) J K^{-1} mol^{-1}) were calculated. Acidobasic behavior of oligonucleotides (5'CAC-CAC-CAC3' = $(CAC)_3$, 5'AAA-CCC-CCC3' = A_3C_6, 5'CCC-AAA-CCC3' = $C_3A_3C_3$) was studied under the same experimental conditions by molecular absorption spectroscopy. pH-dependent spectral datasets were analyzed by means of advanced chemometric techniques (EFA, MCR-ALS) and the presence of hemiprotonated species concerning (C^+–C) a non-canonical pair (*i*-motif) in titled oligonucleotides was proposed in order to explain experimental data obtained according to literature.

Keywords: oligonucleotides, adenine, cytosine, protonation, thermodynamics, chemometrics

Introduction

Adenine (A) and cytosine (C) are important building blocks of nucleic acids taking part in the process of genetic information transfer (Saenger, 1984; Burger, 1990; Sigel & Griesser, 2005; Gargallo et al., 1999). In addition, they are involved also in the transfer of energy, charge, and biological important compounds. Moreover, it was found that expansion of trinucleotide repetitive sequences in genomic DNA is related to inherited diseases, e.g. some kinds of cancer and many neurological diseases. One can find that the CCG repeating motif is accompanied with fragility of the X chromosom, CTC with Huntington chorea, and TTC with Friedrich ataxia (Mikelová et al., 2007; Trnková et al., 2003, 2006; Zemánek et al., 2005). On the contrary, the CCA repeating motif can be utilized as a very useful probe in human DNA analysis since it is the longest polymorphic microsatellite in the human genome (Mikelová et al., 2007; Trnková et al., 2003, 2006). The secondary structure and thus also its function in the process of genetic information transfer can be influenced by a pH change of the solution (Gargallo et al., 1996a, 1996b; Jaumot et al., 2004). Due to acidobasic properties of bioligands, the distribution of protonated species is dependent on solution pH and, therefore, it is important to know the bioligand dissociation constants. The thermodynamic parameters of bases were determined mostly by glass-electrode potentiometry (Burger, 1990; Martell et al., 2004). Large differences in the determined stability constant values can be found in literature. The protonation behavior of oligonucleotides was studied mostly by spectroscopic techniques (UV-VIS absorption, lu-

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minescence, and CD molecular spectroscopy) (Casassas et al., 1994, 1995; Gargallo et al., 1997a, 1997b; Vorlíčková et al., 2005). The aim of this work was to determine the dissociation constants of the titled bioligands and to study the influence of the base order on their acidobasic behavior since, in case of oligonucleotides, this behavior explains the stabilization and thus also the role of these sequences in gene (Jaumot et al., 2004; Vorlíčková et al., 2005).

Theoretical

Mathematical treatment of experimental data matrix obtained from acid-base spectrometric titrations results in an estimation of the matrix of species concentration profiles (C) dependent on the pK_a values, and in an estimation of the species of molar absorptivities (E) in order to obtain the best chemical model with a minimum residual error

$$A = CE + \text{Error} \tag{1}$$

Both OPIUM and Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) software packages estimate both matrices from the absorbance matrix (A) obtained experimentally by means of different mathematical approaches (Jaumot et al., 2004).

OPIUM is a new computer program utilizing classical non-linear least-squares curve fitting method (Kývala et al., 1998). It necessitates the postulation of a chemical model where all species with defined stoichiometry and initial estimation of equilibrium constants are given. Then, this program is capable of solving the mass-balance equations simultaneously with absorbance data treatment according to Eq. (1) until the minimum least-square error function is achieved. The chemical model is changed if this procedure does not converge. In case of the study of biomacromolecules, where possible conformational and polyelectrolyte effects can cause failure of the initial hypothesis of a chemical model and the mass-action law is not fulfilled at the site level, application of this hard-modeling software is limited (Gargallo et al., 1996a, 1996b).

Applying a soft-modeling approach without any prior postulation of a chemical model, this problem can be overcome (de Juan et al., 2000; Jaumot et al., 2004). ALS as an example of the curve resolution method based on factor analysis of experimental data calculates the C and E matrices from the original absorbance data matrix and the procedure consists of the following steps (Gargallo et al., 1996a, 1996b, 1997a, 1997b):

1. The number of significant principal components (NC) present in all spectroscopic titration data is estimated. This value corresponds with the number of light-absorbing species in the system. Factor analysis is used for the decomposition of absorbance matrix (A)

$$A = UV^{\mathrm{T}} + E = A^* + E \tag{2}$$

where U and V^{T} are the score and the loading matrices of A matrix, respectively, for the pre-selected number of components, E is the residual error matrix including the variance not explained by U and V^{T}, and A^* is the reproduced data matrix based on the knowledge of U and V^{T}. The residual error matrix E is close to the experimental error or instrumental noise (e.g. 0.003 in UV-VIS molecular spectroscopy) when linearity is assumed.

2. The matrix form of generalized Bouger–Lambert –Beer law (BLBL) is solved by ALS procedure in order to obtain concentration (C) and molar absorptivity (E) profiles (see Eq. (1)). Starting point is the initial estimation of C data matrix obtained by the Evolving Factor Analysis (EFA) from the absorbance data matrix (A) considering the presence of NC light-absorbing species in solution. Also, this estimation is easier if initial estimation of molar absorptivities of certain species is known in case when only one species predominates in certain pH region. In case of known initial estimate of the concentration matrix (C), the ALS procedure starts using relationships derived from the generalized BLBL:

a) Firstly, the estimation of unknown species spectra is performed using the equation

$$E = C^+ D^* \tag{3}$$

where D^* is the reproduced data matrix for NC species and C^+ is the pseudo-inverse of the C matrix. The E matrix now consists of the current least-squares estimation of molar absorptivities.

b) Secondly, a new estimation of the concentration profile is calculated as

$$C = D^* E^+ \tag{4}$$

where E^+ is the pseudo-inverse of the E matrix.

Previous steps are repeated until the absorbance data matrix is well explained within the experimental error. The ALS process is usually run in a few iterations. Quality of the ALS results is evaluated by calculating the lack of fit, LOF, expressed as

$$\mathrm{LOF} = \sqrt{\frac{\sum_{ij} \left(A_{ij} - A^*_{ij}\right)^2}{\sum_{ij} A^2_{ij}}} \tag{5}$$

where A_{ij} are the experimental absorbance data and A^*_{ij} the reproduced absorbance data obtained by the ALS method, while the subscripts i and j refer to rows and columns of the original absorbance data matrix, respectively (de Juan et al., 1997).

In order to reach only one physico-chemically meaningful solution of C and E matrices, some constraints are applied: (1) UV-VIS absorption spectra

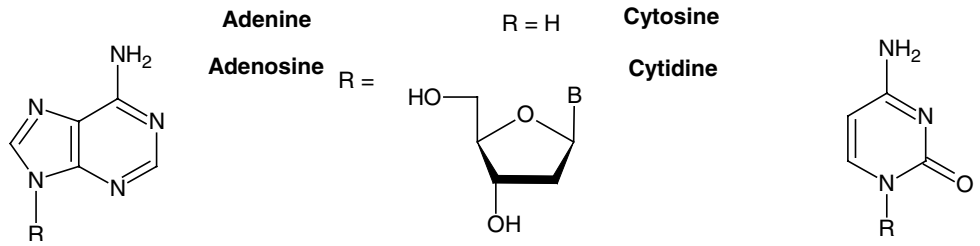

Fig. 1. Structural formulas of ligands (HL) discussed in the paper.

are positive, (2) species concentrations are positive and unimodal, i.e. there is only one maximum concentration for each species under the given experimental conditions, (3) the system is supposed to be closed, i.e. concentration of all absorbing species at each pH is constant, i.e. the ligand total concentration. A more detailed ALS procedure can be found elsewhere (Casassas et al., 1994, 1995; Gargallo et al., 1996a, 1996b; Jaumot et al., 2004). An estimation of the pK_a values related to different acid-base equilibria can be obtained from the concentration profiles determined by the ALS procedure. The pK_a value can be calculated at any point of the titration in order to follow its dependence on the protonation degree (Gargallo et al., 1996a, 1996b, 1997a). If pK_a has a constant value along the titration, no secondary effects (i.e. polyfunctional, conformational, or polyelectrolytic effects) are present. On the contrary, the observed dependence of pK_a on the protonation degree, reflected in higher standard deviation of the determined value in comparison with the previous case, means the presence of secondary effects when the mass-action law ruling the equilibria is valid only separately for each of the reaction sites of the biomacromolecule (Gargallo et al., 1996a, 1997a).

Experimental

Bases (adenine, cytosine), nucleosides (adenosine, cytidine) (Fig. 1), and other chemicals of the highest available purity were purchased from Sigma–Aldrich (St. Luis, USA). Oligonucleotides (ODNs, 5'CAC-CAC-CAC3' = $(CAC)_3$, 5'AAA-CCC-CCC3' = A_3C_6, 5'CCC-AAA-CCC3' = $C_3A_3C_3$) were synthesized by the company ThermoScientific (Ulm, Germany) (Mikelová et al., 2007). Samples were dissolved in triple redistilled water prepared in a Heraeus apparatus (Munich, Germany) and their solution concentration was calculated from the knowledge of molecular absorptivity of nucleosides at $\lambda = 260$ nm: $\varepsilon_{260\mathrm{nm}}(CAC)_3 = 8\ 956\ \mathrm{M^{-1}\ cm^{-1}}$, $\varepsilon_{260\mathrm{nm}}(A_3C_6) = 9\ 022\ \mathrm{M^{-1}\ cm^{-1}}$, $\varepsilon_{260\mathrm{nm}}C_3A_3C_3 = 8\ 867\ \mathrm{M^{-1}\ cm^{-1}}$ (Warshaw & Tinoco, 1966; Cantor et al., 1970).

Potentiometric determination of the dissociation constants of bases and the respective nucleosides was done by means of an automatic titrator Titrando 835 (Metrohm, Switzerland), where the combined glass LL electrode (Metrohm) was placed in a thermostated cell (JULABO, Germany) through which argon stream was passing with the aim to avoid carbon dioxide dissolution. The electrode employed for pH measurements during the acidobasic titration of ligand was calibrated prior to each titration using the following calibration function

$$E = E_0 + S\left(-\log[\mathrm{H^+}]\right) \tag{6}$$

where E_0 is the standard potential including mostly the contribution of the reference electrode and S corresponds to the value close to the Nernstian slope. Calibration parameters were obtained from HCl–NaOH acidobasic titration and were used for the calculation of free proton concentration. Precision represented by standard deviation of parameters did not exceed 0.3 mV for E_0 and $(0.1\,(-\log[\mathrm{H^+}]))\,/\mathrm{mV}$ for S.

Spectrophotometric data were measured by UV 4 equipment (Pye Unicam, UK) operating in the wavelength region of 220–850 nm (slit 0.2 nm). All experiments were carried out at the temperature $t = (25.0 \pm 0.1)$ °C or at other temperatures specified elsewhere, and the ionic strength $I = 0.1$ M (NaCl).

Potentiometric experimental data (adenine, adenosine, cytosine, cytidine) obtained by several repetitive measurements (usually 2–3 times) were treated by the OPIUM program (Kývala et al., 1998) using the hard-model approach while the spectrophotometric data were analyzed by the MCR-ALS soft-modeling software package (Jaumot et al., 2004; de Juan et al., 2000) written in MATLAB (v. 7, The Mathwork Inc., Natick, USA). In addition, the SPECFIT program (Gampp et al., 1985) was utilized to verify the soft-model approach combined with the EFA (de Juan et al., 2000).

Results and discussion

Our study can be divided into two steps. Firstly, the comparison of our results concerning the protonation of ODNs with the dissociation constants of parent bases and nucleosides was performed. Since there are some discrepancies on the dissociation constants in literature (Martell et al., 2004), probably due to incorrect calibration of the glass electrode, the values were determined by potentiometric acid-base titrations and

Table 1. Dissociation constants and thermodynamic parameters of studied ODNs

Ligand	Dissociation constants (pK_{H2L})				Thermodynamic parameters	
	Temperature/ °C				ΔH^0	ΔS^0
	15	25	37	45	kJ mol^{-1}	J K^{-1} mol^{-1}
Adenine	–	4.18(4)*	4.03(1)	4.00(1)	–17(4)	23(13)
Adenosine	–	3.59(1)	–	–	–	
Cytosine	4.73(1)	4.56(1)	4.42(1)	4.35(1)	–22(1)	13(1)
Cytidine	–	4.16(2)	–	–	–	
Hydroxide	14.24(1)	13.78(1)	13.48(1)	–	–59(9)	69(30)

*pK_{HL} (N9) = 9.65(4). Values in brackets correspond to the respective standard deviations.

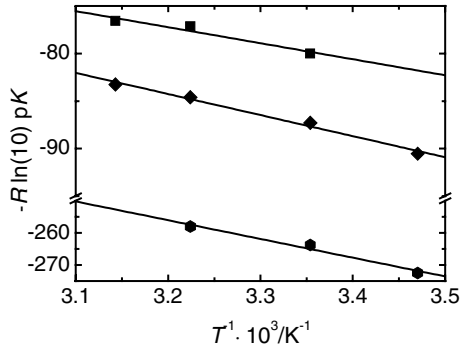

Fig. 2. van't Hoff plot of equilibrium constants as function of temperature for adenine (squares), cytosine (diamonds), and water (circles).

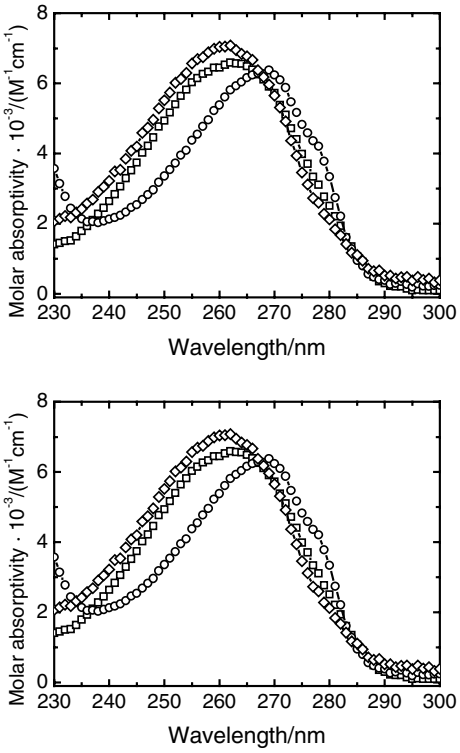

Fig. 3. Calculated absorption profiles for various protonated adenine (a) and adenosine (b) species: H_2L^+ (squares), HL (diamonds), and L^- (circles).

they are given for various temperatures in Table 1. Dissociation constants of the bases were determined at several temperatures and the thermodynamic parameters were determined from the temperature dependence (Fig. 2). In order to verify the reliability of the methodology used in this study, the ionic product of water was also determined for various temperatures and the obtained values are in agreement with values given in literature (pK_w = 13.78(1), ΔH^0 = –56.48 kJ mol^{-1}, ΔS^0 = 73.6 J K^{-1} mol^{-1}) (Martell et al., 2004).

Some interesting phenomena can be deduced from these results. Generally, purine compounds possess less basicity than pyrimidines. A substitution on the nitrogen atom of the base molecule (adenine, cytosine) leads in both cases to a basicity decrease of the nucleoside molecule. The same trend can be expected for the short ODNs containing both bases while the resolution of the site where the protonation takes place is difficult. In addition, the dissociation constants and thermodynamic parameters determined for the studied bases under the same experimental conditions (t = 25 °C, I = 0.1 M (NaCl)) are in agreement with the data given in literature (Martell et al., 2004): adenine (pK_{H2L} = 4.22(6), ΔH^0 = –17 kJ mol^{-1}, ΔS^0 = 23 J K^{-1} mol^{-1}, pK_{HL} = 9.67(9)), cytosine (pK_{H2L} = 4.57(7), ΔH^0 = –18 kJ mol^{-1}, ΔS^0 = 24 J K^{-1} mol^{-1}).

The next step was the study of protonation behavior of short ODNs containing three adenine and six cytosine molecules in order to understand the phenomena observed during electrochemical (Mikelová et al., 2007) and chromatographic studies (Trnková et al., 2008). Due to the limited amount of ODNs, molecular spectroscopy seems to be the most suitable experimental technique for their analysis. Although spectral profiles of protonated species are not very different (Fig. 3), it can be expected that the whole evaluation of the spectral dataset using advanced chemometric techniques (EFA, MCR-ALS) will help to resolve this problem as demonstrated by the study of poly(C) by means of UV-VIS molecular spectroscopy (Gargallo et al., 1996a, 1996b, 1997a, 1997b).

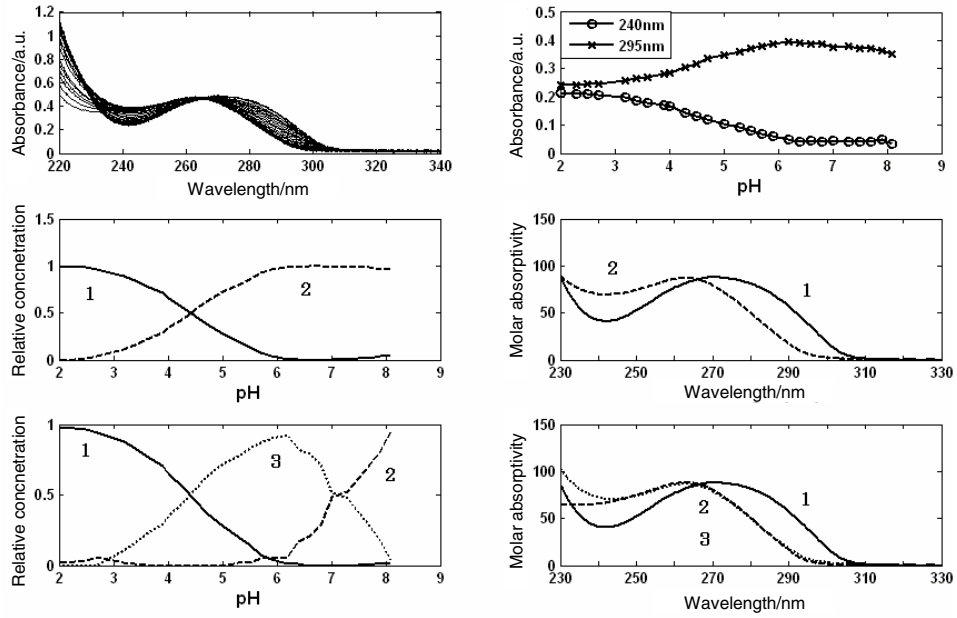

Fig. 4. MCR-ALS analysis of experimental spectroscopic data for $(CAC)_3$ oligonucleotide ($c = 5.35 \times 10^{-5}$ M, $t = 25\,°C$, $I = 0.1$ M (NaCl)). Molar absorptivities were divided by factor 100. 1: protonated species, 2: deprotonated species, 3: hemiprotonated (C^+-C) species.

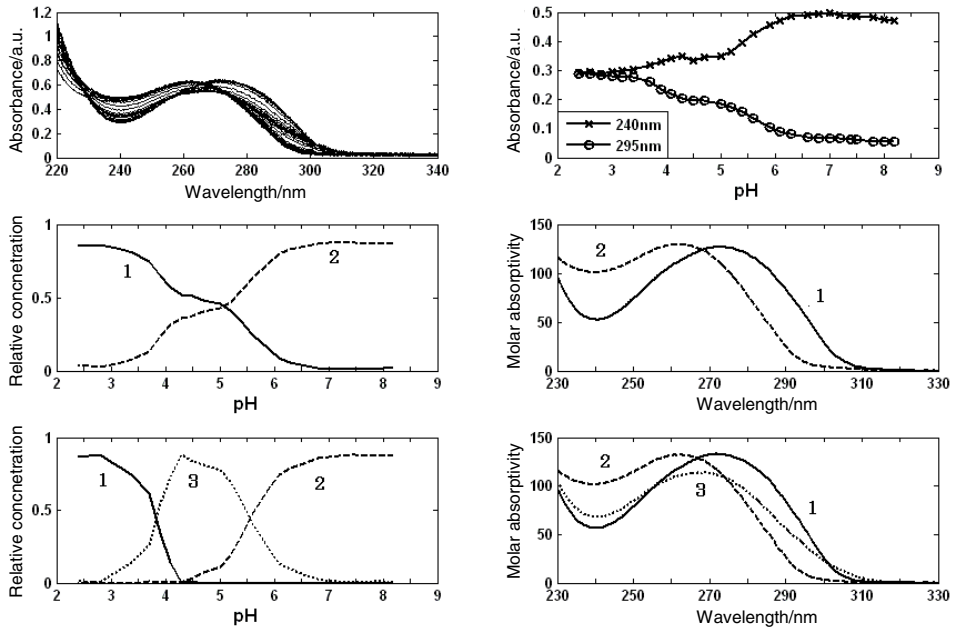

Fig. 5. MCR-ALS analysis of experimental spectroscopic data for A_3C_6 oligonucleotide [$c = 5.70 \times 10^{-5}$ M, $t = 25\,°C$, $I = 0.1$ M (NaCl)]. Molar absorptivities were divided by factor 100. 1: protonated species, 2: deprotonated species, 3: hemiprotonated (C^+-C) species.

Examples of ODNs with different repetitive structural motifs were used in the protonation behavior study. Examples of spectral data for $(CAC)_3$ and A_3C_6 are given in Figs. 4 and 5. While no crossing points are visible for $(CAC)_3$, one isosbestic point and two jumps related to the two-step deprotonation mechanism can be distinguished for A_3C_6. The last observation means that more than two species having different spectral profiles are present in the solution in this case and, therefore, this hypothesis was tested for all three ODNs investigated. Parameters such as standard deviation of fit, $s(A)$, lack of fit (LOF), and contribution to variance (VAR) are generally used to determine the number of light-absorbing species in solution (Lubal & Havel, 1997a, 1997b; Jaumot et al., 2004). While there are no doubts about the rank of the absorbance matrix being equal to 3 and 2 for the A_3C_6 and $(CAC)_3$ biomolecules, respectively, (see Ta-

Table 2. Results of MCR-ALS procedure for studied ODNs

Number of species	$(CAC)_3$			$C_3A_3C_3$			A_3C_6		
	$s(A) \cdot 10^3$	LOF / %	VAR / %	$s(A) \cdot 10^3$	LOF / %	VAR / %	$s(A) \cdot 10^3$	LOF / %	VAR / %
2	8.48	1.36	99.90	5.84	1.67	99.96	14.18	3.63	99.83
3	2.90	0.91	99.99	3.92	1.26	99.98	5.57	1.37	99.97
4	1.88	0.56	100.00	3.82	1.24	99.98	3.43	0.89	99.99
pK_a(2 species)	4.4(2)			4.4(2)			4.7(4)		
pK_a(3 species)	4.3(2); 7.2(2)			3.8(2); 5.0(2)			4.0(2); 5.7(2)		

Values in brackets correspond to the respective standard deviations.

ble 2), it is not clear how to resolve this problem for $C_3A_3C_3$. The solution can be found also in the calculated distribution diagrams and physico-chemical parameters for two and three protonated species for the ODNs displayed in Figs. 4 and 5, while the presence of two species for $(CAC)_3$ and three species for A_3C_6 is very probable. The vicinity of crossing points of species abundance in the distribution diagram was used for the estimation of dissociation constants of ODNs (Table 2). The same procedure was applied for $C_3A_3C_3$ (experimental data and their evaluation are not shown) and the presence of three species is also assumed according to the available statistical and physico-chemical parameters.

On the basis of concentration profiles (examples in Figs. 4 a 5), the dissociation constants of ODNs were estimated (Table 2). Their standard deviations are higher than the values obtained for parent bases probably due to secondary structure effects. The value of the dissociation constant of $(CAC)_3$ is between those of adenine and cytosine while the two dissociation constants values found for $C_3A_3C_3$ and A_3C_6 nucleotides are either lower or higher than those of the parent bases. Similar behavior was observed for poly(A) and poly(C) nucleotides (Casassas et al., 1995; Gargallo et al., 1996a, 1996b; Izquierdo-Ridorsa et al., 1996) and the obtained dissociation constants are roughly in agreement with the values found in literature (Gargallo et al., 1997a). The authors observed also the formation of hemiprotonated species concerning a (C^+-C) non-canonical pair (Fig. 6) in poly(C) from the three-way absorption, fluorescence, and CD spectral datasets in aqueous (Casassas et al., 1995; Gargallo et al., 1996a, 1996b) or mixed water–organic solvent solutions (de Juan et al., 1997a). In addition, the melting-point analysis of UV-VIS spectra (Gargallo et al., 1997a) shows the importance of this species for stabilization of the ODN secondary structure

$$[\text{poly(C)-poly}(C^+)]_{\text{ds}} \rightarrow [\text{poly(C)-poly}(C^+)]_{\text{rc}} \quad t_{\text{m}} = 68\,^{\circ}\text{C} \tag{7}$$

In our case, the differences $(5.7 - 4.0) = 1.7$ and $(5.0 - 3.8) = 1.2$ in the dissociation constants for both ODNs (see Table 2) were caused by secondary-structure effects. The presence of this (C^+-C) hemiprotonated species in solution of $C_3A_3C_3$ and A_3C_6 ODN's is very probable since similar patterns were observed in calculated concentration and spectral profiles (Figs. 4 and 5) as in case of poly(C) (Gargallo et al., 1996a, 1996b). This is also supported by the fact that the studied ODN's show different retention times on the reverse-phase chromatographic column (11.0 min for $(CAC)_3$ vs. 21.6 min for A_3C_6 (Trnková et al., 2009)).

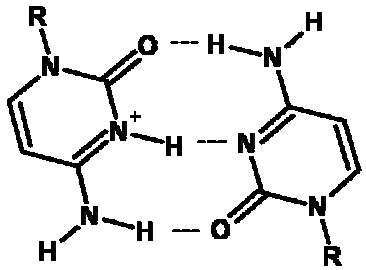

Fig. 6. Structure of hemiprotonated cytosine species, (C^+-C) pair.

Conclusions

This study demonstrates that the protonation of adenine and cytosine is an exothermic reaction and the binding of a sugar part on the base moiety leads to a decrease of the basicity in both cases (adenine vs. adenosine, cytosine vs. cytidine). The order of bases on an oligonucleotide molecule is also a significant aspect of its stabilization. The stabilization of ODNs secondary structure is observed in case of the presence of a repetitive motif concerning cytosine (in our case at least a CCC motif for A_3C_6 and $C_3A_3C_3$ ODNs is required). On the contrary, this stabilization cannot be achieved if the order of bases has random character, e.g. $(CAC)_3$. The facts presented in this paper can be relevant for analytical determination of respective ODNs in mixture by HPLC (Trnková et al., 2009) or voltammetric methods (Mikelová et al., 2007).

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