Noncovalent interactions in polyamine/nucleoside (or diaminocarboxylate) systems studied by potentiometric and NMR techniques

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Noncovalent interactions and formation of molecular complexes have been found to occur between a number of polyamines and adenosine or cytidine. Since these observed interactions affect the acid–base character of particular components of the systems the determination of overall stability constants of the complexes was possible on the basis of a computer analysis of potentiometric data and then equilibrium constants of the reactions were calculated. The comparison of the constants and the changes in the position of ^{13}C NMR signals provided grounds for a conclusion that mainly two (or three) $-NH_x^+$ groups of amine and electron-rich centres as well as the system of π electrons from purine and pyrimidine bases constitute sites for the interactions. The tendency of particular polyamines to form adducts depends on the number of nitrogen atoms as well as the length of the methylene chains in the molecules. Distributions of molecular complexes as a function of pH were calculated. The ranges of the occurrence of PA/Nuc molecular complexes overlap with those of nucleoside deprotonation and polyamine protonation, which confirms the validity of the assumed model of interactions. It was found that in the polyamine systems with diaminocarboxylates no adducts were formed.

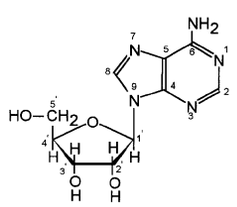
Introduction

Polyamines (PA, biogenic amines), mainly putrescine: $NH_2(CH_2)_4NH_2$, spermidine: $NH_2(CH_2)_3NH(CH_2)_4NH_2$ and spermine: $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, play an important role in many biological processes.[1–4] PAs occur in all types of living cells.[1,3,5,6] Moreover, increased concentration of amines is observed in young and cancer cells.[2,7–9] One pathway for the biosynthesis of polyamines and the characteristics of enzymes participating in the synthesis are already known.[9–12] PAs occur in physiological fluids as protonated species and in this form mainly they interact with other bioligands. Of special importance is the high affinity of polyamines for DNA and RNA and their influence on the biosynthesis of nucleic acids and proteins.[2,13,14] The presence of PAs is probably responsible for the structural changes in nucleic acids on several levels of organization and the character of the interactions depends on many factors.[15–24] Yet, the mechanism of interaction of polyamines in biological processes at the molecular level has not been fully recognized. The model, which accounts only for electrostatic interactions of PA with negative fragments of other molecules, does not provide an explanation for the high specificity of some of the reactions.[25]

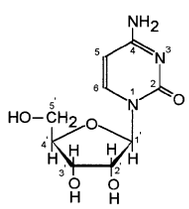
In our previous studies on ternary systems with metal ions, we proved the presence of molecular complexes of cytidine and adenosine with some polyamines.[26,27] Metal ions act as interfering agents because they affect the character of interactions: the centres participating in noncovalent interactions are at the same time sites of metal–donor atom bond formation. The results of our previous investigations enabled us to claim that only a careful (with the emphasis put on the fact that the interaction is weak and noncovalent) comparison of the interactions in the metal and metal-free systems may help us to understand the role of particular components in biological processes. The present paper describes the results of studies on the formation of molecular complexes of adenosine, cytidine and diaminocarboxylates with a number of polyamines.

Experimental

Adenosine (Ado) and cytidine (Cyd) were purchased from Sigma. Preparation of hydrochlorides of Ado and Cyd was described previously.[26,27] The results of elemental analyses (%C, %N and %H) were in agreement with calculated results (±0.5%). Ethylenediamine (en), 1,3-diaminopropane (tn), 1,4-diaminobutane (putrescine, Put), diethylenetriamine (2,2-tri,dien), 1,6-diamino-3-azahexane (2,3-tri), 1,7-diamino-4-azaheptane (3,3-tri), 1,8-diamino-4-azaoctane (spermidine, Spd), 1,12-diamino-4,9-diazadodecane (spermine, Spm) and their hydrochlorides were purchased from Sigma. DL-2,3-Diaminopropionic acid (dapa) was purchased from Aldrich, while DL-2,4-diamino butyric acid (daba) was obtained from Sigma. Potentiometric measurements were carried out using a Radiometer DTS 800 Multi-Tritration System with a GK-2401C electrode which had been calibrated for determination of hydrogen ion concentrations.[28] Concentrations of Ado·HCl, Cyd·HCl, tn·2HCl, Put·2HCl, dien·3HCl, 2,3-tri·3HCl, 3,3-tri·3HCl, Spd·3HCl, Spm·4HCl, dapa·HCl, daba·2HCl in the titrated samples ranged from 5×10^{-3} to 15×10^{-3} M. All titrations were performed under an argon atmosphere at an ionic strength $\mu = 0.1$ M (KNO_3), $T = 20 \pm 1$ °C, using as a titrant CO_2-free NaOH solution of concentration 0.5418 M. Addition of NaOH solution does not change the ionic strength because the measurements were performed starting from fully proto-



Ado



Cyd

NH_2-CH_2-CH_2-NH_2 **en**

NH_2-CH_2-CH_2-CH_2-NH_2 **tn**

NH_2-CH_2-CH_2-CH_2-CH_2-NH_2 **Put**

NH_2-CH_2-CH_2-NH-CH_2-CH_2-NH_2 **dien**

NH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-NH_2 **2,3-tri**

NH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-NH_2 **3,3-tri**

NH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-CH_2-NH_2 **Spd**

NH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-CH_2-NH-CH_2-CH_2-CH_2-NH_2 **Spm**

$CH_2(NH_2)CH(NH_2)COO^-$ **dapa**

$CH_2(NH_2)CH_2CH(NH_2)COO^-$ **daba**

Table 1 Overall stability constants of molecular complexes formed in Ado/PA and Cyd/PA systems[a]

	Cyd		Ado	
PA	Species	log β	Species	log β
en	—	—	—	—
tn	—	—	$AdoH_2tn$	20.44 (3)
Put	—	—	$AdoH_2Put$	22.04 (2)
dien	—	—	—	—
2,3-tri	—	—	$AdoH_3$(2,3-tri)	28.05 (4)
3,3-tri	—	—	$AdoH_3$(3,3-tri)	29.45 (3)
			$AdoH_2$(3,3-tri)	21.01 (2)
Spd	$CydH_3Spd$	31.42 (3)	$AdoH_3Spd$	30.38 (2)
			$AdoH_2Spd$	21.81 (2)
Spm	$CydH_4Spm$	40.46 (4)	$AdoH_4Spm$	40.61 (2)
	$CydH_3Spm$	32.10 (4)	$AdoH_3Spm$	32.27 (3)
	$CydH_2Spm$	23.12 (2)	$AdoH_2Spm$	23.08 (3)
			AdoHSpm	12.54 (3)

[a] There is no complex formation in all diaminocarboxylate/PA systems.

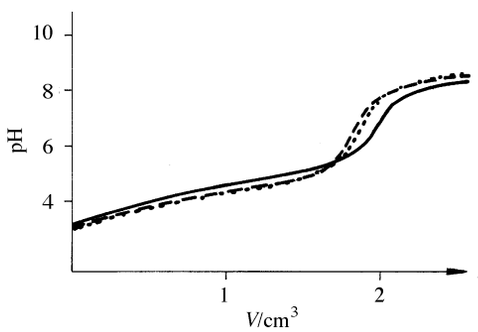


Fig. 1 Experimental (– – –) and simulated titration curves (—); adduct formation was not taken into account (· · ·); adduct formation was taken into account) for the Cyd/Spd system; c_{cyd} = 0.015, c_{Spd} = 0.015 M

nated PA so $-NH_3^+$ cations were replaced by equivalent amounts of Na^+. At least 100 points were taken to produce each titration curve. Stability constants and stoichiometric compositions of the molecular complexes were determined with the SUPERQUAD computer program,[29] and distributions with the HALTAFALL program.[30] Selection and verification of models were carried out as described earlier.[31] Samples for ^{13}C NMR and IR studies were prepared by dissolving Ado, Cyd and hydrochlorides of the studied amines in D_2O. pH measurements were corrected according to the formula pD = pH + 0.40.[32] The concentration of ligands in samples for NMR studies was 0.05 M. ^{13}C NMR spectra were recorded on Jeol Fx 90Q and NMR Gemini-300 Varian spectrometers used dioxane as internal standard. The positions of ^{13}C NMR signals were converted relative to $SiMe_4$. The concentration of nucleosides and amines in samples for IR studies was 0.05 M (KRS-5 cuvette). IR measurements were taken using a Bruker IFS-113v spectrometer. Elemental analyses of the obtained hydrochlorides were performed on a CHN 2400 Perkin-Elmer elemental analyser.

Results and discussion

Potentiometric studies of the Ado/PA systems

By taking advantage of the fact that the observed noncovalent interactions bring about changes in the acid–base properties of the components of the systems, formation of molecular complexes was studied. Overall stability constants (log β) of the molecular complexes of polyamines (PA) with nucleosides (Nuc) were determined potentiometrically (Table 1).

It was observed that the experimentally obtained curves for the systems where adduct(s) formation occurred, and the curves obtained by computer simulation (HALTFALL program) calculated for a system free of the ligand–ligand interactions do not overlap (Fig. 1). This is related to the reversible process of molecular complex formation: eqn. (1). (For simplicity, ion charges except for those of the hydrogen cations are neglected in all formulae.) On the other hand, the overlapping of both curves (*i.e.* the experimental and simulated) indicate that both components of the mixture do not change their acid–base character, because molecular complexes of the studied type are not formed (example in Fig. 2). Characteristically, in some cases interactions of the components of the system cause an increase and in others a decrease in the pH of the system, which is a result of opposing effects. On the one hand, intermolecular interaction weakens the N–H bond in the amine (or Nuc) molecule, whereas on the other, the possibility of formation of hydrogen bonds leads to proton stabilization in the ligand. However, it should also be noted that adducts whose formation is accompanied by no changes in acid–base character of bioligands may occur in the system.

Table 2 Equilibrium constants of molecular complex formation

Reaction	log K
Ado + $H_2tn \rightleftharpoons AdoH_2tn$	0.80
Ado + $H_2Put \rightleftharpoons AdoH_2Put$	1.53
Ado + H_3(2,3-tri) $\rightleftharpoons AdoH_3$(2,3-tri)	1.89
Ado + H_3(3,3-tri) $\rightleftharpoons AdoH_3$(3,3-tri)	1.42
Ado + H_2(3,3-tri) $\rightleftharpoons AdoH_2$(3,3-tri)	0.79
Ado + $H_3Spd \rightleftharpoons AdoH_3Spd$	0.77
Ado + $H_2Spd \rightleftharpoons AdoH_2Spd$	0.78
Ado + $H_4Spm \rightleftharpoons AdoH_4Spm$	2.00
Ado + $H_3Spm \rightleftharpoons AdoH_3Spm$	1.88
Ado + $H_2Spm \rightleftharpoons AdoH_2Spm$	1.80
Ado + HSpm $\rightleftharpoons$ AdoHSpm	1.03
Cyd + $H_3Spm \rightleftharpoons CydH_3Spd$	1.81
Cyd + $H_4Spm \rightleftharpoons CydH_4Spm$	1.85
Cyd + $H_3Spm \rightleftharpoons CydH_3Spm$	1.71
Cyd + $H_2Spm \rightleftharpoons CydH_2Spm$	1.81

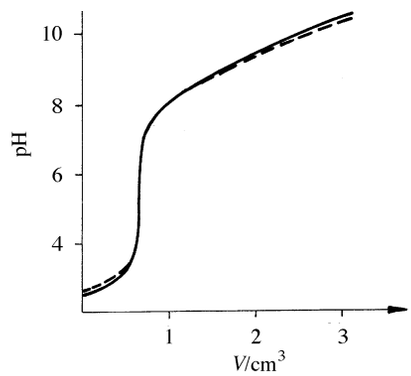


Fig. 2 Experimental (– – –) and simulated (—) curves for the daba/Spd system; c_{daba} = 0.015, c_{Spd} = 0.015 M

$$H_xPA + H_yNuc \rightleftharpoons (Nuc)H_{(x+y-n)}(PA) + nH^+ \quad (1)$$

In general, it was found that with increasing length of the methylene chain and with the growing amount of donor atoms in the PA molecule, the tendency to form molecular complexes also increases. A direct quantitative comparison of the determined log β is not possible due to the fact that ligands contain different numbers of protonated donor atoms. Thus, the equilibrium constants (log K) of the molecular complex formation were calculated: eqn. (2).

$$H_nPA + Nuc \rightleftharpoons (Nuc)H_n(PA)$$
$$\log K^{\mathbf{H}n\mathrm{PA}}_{\mathbf{(Nuc)H}n\mathrm{(PA)}} = \log \beta_{\mathbf{(Nuc)H}n\mathrm{(PA)}} - \log \beta_{\mathbf{Hn}\mathrm{PA}} \quad (2)$$

The values shown in Table 2 indicate an equilibrium shift and the tendency of the studied ligands to form adducts. Log K for a molecular complex in the Ado/Put system with a tetramethyl-

ene chain in a PA molecule is approximately one order of magnitude higher than in the system with tn (a trimethylene chain). Thus, differences in the length of the methylene chains in polyamines result in distinct changes in the affinity of the bioligands for Ado. Another factor influencing the interactions is the higher basicity of donor atoms of the PAs (successive values of protonation constants for Put: 10.83 and 9.68; for tn: 10.70 and 8.94).[33] Considerable differences in the values of formation constants of Put and tn adducts with Ado indicate that the ion–dipole interactions involve both $-NH_3^+$ groups of diamines (otherwise, the spatial factor would not be able to play such a significant role).

The character of interactions of a symmetric triamine—3,3-tri—in comparison with its asymmetric analogue—Spd—has proved to be different. The fact that the values of log *K* of the diprotonated molecular complex, $AdoH_2Spd$ and the triprotonated $AdoH_3Spd$ are so similar (Table 2) implies that mainly two amine groups are involved in the interaction. Taking into account the character of interactions (weak, noncovalent) it is difficult to firmly reject the possibility of a very slight participation of the third protonated amino group. Since polyamines with a tetramethylene chain have shown a greater tendency to form adducts (the comparison of Put with tn) it is reasonable to assume that the following Spd segment: NH_3^+–$(CH_2)_4$–NH_2^+– is the major part of PA that binds Ado. This is in agreement with the observed results of the interaction of spermidine with 5′-AMP.[34] These authors suggested that the trimethylene fragment of PA binds the phosphate moiety of the nucleotide, while the tetramethylene segment reacts with N(7) of the nucleotide. Recently, it was found that interactions of polyamines with pyrophosphate groups intensify with decreasing number of methylene groups between the nitrogen atoms of the PA.[35] It can be concluded that the trimethylene fragment of amine shows a higher affinity to the phosphate moiety, while the tetramethylene part has more affinity with the purine base. The NMR studies presented below verify the above conclusions.

A different behaviour has been observed for the symmetrical amine 3,3-tri, which has trimethylene chains. Differences in the values of log *K* between the di- and tri-protonated adduct suggest a different type of interaction, most probably with participation of two and three amino groups, respectively, in $AdoH_2$ (3,3-tri) and $AdoH_3$ (3,3-tri). The proposed mechanism of diprotonation of symmetrical amines[36,37] implies that charges are distributed on the primary nitrogen atoms, which clearly indicates that the terminal atoms are mainly involved in the interactions with Nuc. In the Ado adduct with triprotonated 3,3-tri, a secondary amino group probably also takes part in the interaction. As follows from the values of log *K* of the nucleoside adduct with 2,3-tri, the interaction mechanism is likely to be similar to that for 3,3-tri, including all the donor atoms of amine (contrary to the case for the $AdoH_3Spd$ adduct). Because of the complex character of these interactions, special care should be taken in any attempt to compare the values of log *K* for adducts of different bioligands (particularly those with differing numbers of nitrogen atoms). We assume, however, that in general any increase in the number of protonated nitrogen atoms on the centres of interactions leads to an increase in adduct stability. This is confirmed by the values of *K* for complexes formed in the Ado/spm system (Table 2). Together with an increasing number of $-NH_x^+$ groups, the equilibrium constants get bigger. Occurrence of a long side chain and only one active group in PA can account for the clearly lower value of log *K* for the complex AdoHSpm than that for the other adducts of this amine. Fig. 3 presents an example of the distribution of molecular complexes in Ado/PA systems. The pH ranges in which adducts occur overlap with the ranges in which the nucleosides are deprotonated, while the polyamine is protonated, which provides additional proof for the proposed interaction model. Adducts occur in the pH range above *ca.* 4 (log $K^{H}_{HAdo} = 3.92$)[38] and they decompose under polyamine dissociation conditions. Relative to the total concentration of the components of the system, 10–40% of the total amount of bioligands occurs in the form of a molecular complex (the above value depends on the PA used).

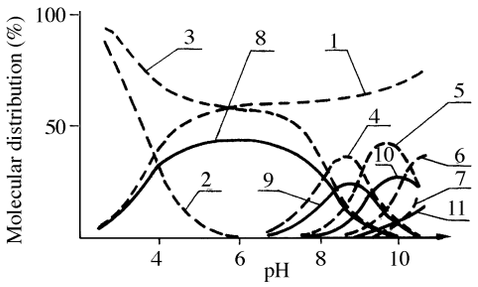


Fig. 3 Distribution diagram for the Ado/Spm system: 1, Ado; 2, HAdo; 3, H_4Spm; 4, H_3Spm; 5, H_2Spm; 6, HSpm; 7, Spm; 8, $AdoH_4Spm$; 9, $AdoH_3Spm$; 10, $AdoH_2Spm$; 11, AdoHSpm; $c_{Ado} = 0.015$, $c_{Spm} = 0.015$ M

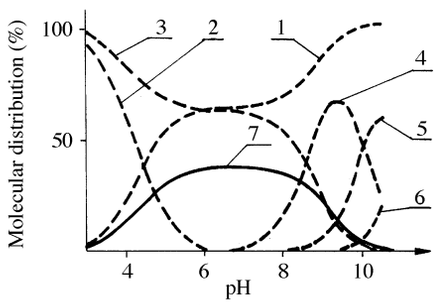


Fig. 4 Distribution diagram for the Cyd/Spd system: 1, Cyd; 2, HCyd; 3, H_3Spd; 4, H_2Spd; 5, HSpd; 6, Spd; 7, $CydH_3Spd$; $c_{Ado} = 0.15$, $c_{Spm} = 0.015$ M

Potentiometric studies of the Cyd/PA system

Unlike Ado, another nucleoside, cytidine forms molecular complexes with a number of the studied polyamines only when the PA molecule has long methylene chains and at least three amine groups. There is no doubt that this condition is related to the mutual structural matching of both components of the adduct. Similarly, as in the case of the systems with Ado, the longest of the studied polyamines, Spm, forms a series of complexes with Cyd, though no detectable monoprotonated species is formed, which corresponds to a relatively low stability of the AdoHSpm complex (Table 2). This provides some evidence for our conclusion that at least two $-NH_x^+$ groups should be present in a PA molecule for an effective interaction. Fig. 4 shows an example of complex distribution as a function of pH. Molecular complexes are formed at pH *ca.* 4 (log $K^{H}_{HCyd} = 4.49$)[38] and undergo decomposition at high pH. At most, *ca.* 10–40% of the total amount of the components occurs in the form of molecular complexes.

In contrast to nucleoside, molecular complexes were not found in any of the studied systems with 2,3-diaminopropionate and 2,4-diaminobutyrate ions (at least in the studied concentration ranges of substrates).

^{13}C NMR studies of Nuc/PA systems

Tables 3 and 4 present results of ^{13}C NMR studies of a few chosen Nuc/PA systems. The positions of signals for particular carbon atoms in the bioligands were assigned on the basis of literature data.[34,39,40] In spite of the complex character of the NMR signal changes resulting from noncovalent reactions, a thorough analysis of the changes provided much information about the mode of intermolecular interactions. The adduct $CydH_3Spd$ occurs in the pH limit from *ca.* 3–10 (Fig. 4). In the range of the maximum concentration of the molecular complex (pH of *ca.* 8), the signals from the C(2) and C(4) carbon atoms of cytidine are shifted by 0.098 and 0.085 ppm, respectively (see

Table 3 ^{13}C NMR signals (ppm) for the Cyd/Spd system and their changes in relation to single ligands (in parentheses)

Cyd					Spd						
pH	C(2)	C(4)	C(5)	C(6)	C(4)	C(3)	C(7)	C(1)	C(2)	C(6)	C(5)
2	149.030 (0.000)	159.830 (0.003)	95.785 (0.003)	144.965 (0.003)	47.947 (0.001)	45.420 (0.000)	39.716 (0.000)	37.490 (0.000)	24.825 (0.002)	24.655 (0.000)	23.663 (0.002)
4	152.738 (3.180)	162.588 (2.462)	96.279 (0.430)	143.892 (0.928)	47.939 (0.049)	45.418 (0.056)	39.707 (0.046)	37.481 (0.048)	24.824 (0.026)	26.657 (0.035)	23.666 (0.046)
6	158.186 (0.109)	166.747 (0.122)	97.020 (0.092)	142.222 (0.057)	47.950 (0.032)	45.431 (0.037)	39.717 (0.039)	37.491 (0.032)	24.841 (0.040)	24.677 (0.012)	23.679 (0.017)
8	158.275 (0.098)	166.799 (0.085)	97.899 (0.042)	142.322 (0.003)	48.058 (0.258)	45.650 (0.239)	39.804 (0.712)	37.759 (0.330)	25.386 (0.130)	25.028 (0.220)	24.092 (0.160)
10	158.376 (0.003)	166.785 (0.002)	97.008 (0.007)	142.380 (0.005)	48.862 (0.024)	46.758 (0.027)	40.843 (0.037)	39.090 (0.013)	30.666 (0.017)	28.764 (0.020)	26.102 (0.012)

Table 4 ^{13}C NMR signals (ppm) for the Ado/Spd system and their changes in relation to single ligands (in parentheses)

Ado						Spd						
pH	C(6)	C(2)	C(4)	C(8)	C(5)	C(4)	C(3)	C(7)	C(1)	C(2)	C(6)	C(5)
2	153.724 (0.003)	150.757 (0.002)	148.331 (0.005)	143.581 (0.007)	119.757 (0.004)	47.919 (0.001)	45.436 (0.000)	39.895 (0.002)	37.999 (0.003)	24.719 (0.001)	24.613 (0.002)	23.614 (0.002)
4	155.397 (0.514)	152.074 (0.787)	148.986 (0.035)	141.518 (0.307)	119.688 (0.180)	47.940 (0.027)	45.418 (0.026)	39.708 (0.027)	37.483 (0.026)	24.827 (0.029)	24.656 (0.034)	23.656 (0.036)
6	156.193 (0.342)	152.776 (0.081)	149.077 (0.081)	141.398 (0.070)	119.703 (0.239)	47.929 (0.011)	45.407 (0.013)	39.700 (0.017)	37.474 (0.015)	24.816 (0.015)	24.644 (0.019)	23.644 (0.019)
8	156.115 (0.509)	153.113 (0.733)	148.979 (0.011)	141.197 (0.272)	119.687 (0.064)	48.402 (0.387)	46.364 (0.480)	40.203 (0.280)	38.694 (0.600)	28.006 (1.800)	26.120 (0.870)	25.386 (0.084)
10	156.201 (0.498)	153.097 (0.668)	149.151 (0.076)	141.334 (0.234)	117.777 (2.020)	49.304 (0.620)	47.053 (0.420)	41.331 (0.690)	39.587 (0.310)	32.499 (2.620)	30.511 (2.530)	26.885 (1.040)

Table 3). This indicates the involvement of the N(3) atom in the interaction as the shift of the signal from C(6) atom which is not a neighbour of N(3) is only 0.003 ppm. Changes in the positions of NMR signals from the carbon atoms in spermidine, expressed in ppm, are: C(1), 0.330; C(3), 0.239; C(4), 0.258; C(7), 0.712. These values testify to the involvement of $-NH_3^+$ and $-NH_2^+$ groups in the interaction with the nucleoside. At pH 10 at which the $CydH_3Spd$ adduct concentration is close to zero, the shift of ^{13}C NMR signals is considerably smaller. For example the shifts (ppm) of the signals from the Cyd atoms are: C(2), 0.003; C(4), 0.002; C(6), 0.005; while those for the Spd molecular are: C(1), 0.013; C(3), 0.027; C(4), 0.024; C(7), 0.037.

Similar dependences have been observed for the system Ado/Spd for which the chemical shift values are given in Table 4. At pH 10 at which the $AdoH_2Spd$ adduct is present (potentiometric studies) the chemical shift values (ppm) are: for Ado, C(6), 0.498; C(2), 0.668; C(8), 0.234; C(5), 2.020; for Spd, C(1), 0.310; C(3), 0.420; C(4), 0.620; C(7), 0.690. The formation of molecular complexes of the type studied was excluded in the pH ranges within which no changes in NMR signals were observed, see for example, Tables 3 and 4, pH 2.

Similar analyses have been performed for all investigated systems. The results obtained corroborate the conclusions of potentiometric studies, indicating that the most effective centres of interactions are two or three $-NH_x^+$ groups of PA and they enter into ion–dipole interactions with electron-rich nitrogen atoms of nucleosides—mainly with the N(7) and N(1) atoms of Ado, and the N(3) atom of Cyd, and probably also with π electrons of base rings from nucleoside molecules. Participation of the last type of centres, as suggested previously,[34] is in agreement with our observations concerning the comparison of the interactions of nucleosides, 2,3-diaminopropionate and 2,4-diaminobutyrate ions with polyamines. Despite the presence of a negative $-COO^-$ group in both amino acids, a lack of ring π electrons distinctly reduces the tendency of adducts to format (this also confirms that a bioligand forming a molecular complex should have two active centres). Moreover, amino groups from diaminocarboxylates undergo deprotonation at significantly higher pH than in the case of nucleosides (successive protonation constants for dapa and daba: 9.23, 6.82 and 9.89, 8.37, respectively)[41] and in the competitive reactions in the systems, protons can block the active centres.

Recently, the process of formation of molecular complexes in the dien systems with polycarboxylic acids has been described.[42] On the basis of our findings we assume that the character of interactions is far more complex than in the model with only electrostatic interactions, as suggested in that paper. Moreover, such a model does not account for the bioactivity of biogenic amines (Put, Spd, Spm, *i.e.* those with relatively long methylene chains) in comparison with other PAs.

It was found that oxygen atoms of the cytidine carbonyl group do not take part in the interaction. To supplement the conclusions obtained from the magnetic resonance studies, the effect of interactions on IR spectra was monitored. Though it seems that even in the case of the signal due to the carbonyl group in Cyd (1654 cm^{-1}), *i.e.* the group being relatively sensitive to the influence of its environment, the changes of IR band positions cannot be too profound. Therefore, this type of result should not be considered significant, yet this complementary test indicates that involvement of the −C=O group in reactions is hardly probable in spite of the presence of a large net charge on the oxygen atom. On the other hand (though no unambiguous experimental evidence is available), participation of the exocyclic $-NH_2$ groups (in Ado and Cyd) should be excluded due to the relatively low electron density on the nitrogen atom.

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