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Creatinine and Creatininium Cation in Water Solution. Tautomerism and Quantitative Interpretation of the Solution Acidity Effect on ^{1}H, ^{13}C and ^{14}N NMR Chemical Shifts

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^{1}H, ^{13}C and ^{14}N NMR chemical shifts for creatinine in water solutions of various acidity have been measured. Analysis of these data enabled determination of the acidity constant of creatininium cation and the chemical shifts of the neutral and protonated forms of creatinine. Molecular energies and carbon and nitrogen magnetic shielding constants for various tautomeric structures of the investigated species have been calculated using the quantum chemistry method GIAO DFT B3LYP/6-311++G(2d,p). Compilation of the available experimental and theoretical results has provided additional information on the problem of tautomerism of this important biological molecule.

Key words: creatinine, protonation, tautomerism, ^{1}H, ^{13}C, ^{14}N-NMR, chemical shift calculations GIAO DFT

Creatinine (**1**) is excreted in urine as an end product of nitrogen metabolism in vertebrates. The excretion rate of creatinine mainly depends on the total mass of muscles and is remarkably constant for humans [1]. For healthy adults the excretion norm amounts to about 1.5–2 g per day. In medical analyses its level in urine is commonly used as an internal concentration reference and the concentration of other metabolites is expressed relative to creatinine rather than in absolute units. On the other hand, the abnormal excretion of creatinine is meaningful clinically as an indicator of a disease state [1,2]. Thus, creatinine is an important biological compound and it has attracted the interest of many investigators. Its structure and physicochemical properties including NMR spectra have been investigated many times [3–15]. The chemical shift differences between values measured for **1** in neutral and acidic water solutions were attributed to protonation of the neutral form of **1** below pH = 3 [3–6]. There was, however, no report in the chemical literature on the quantitative analysis of this solution acidity effect. The least squares analysis of the changes of ^{1}H, ^{13}C and ^{14}N chemical shifts of **1** in the function of the solution pH, performed within this work, yielded the acidity constant and chemical shift values of the protonated and neutral form of **1**. These chemical shifts were compared with theoretical ones calculated by the quan-

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tum mechanical method. The possibility of existence of creatinine and its salt in various tautomeric forms in water solutions was discussed in the light of available experimental data and DFT calculations.

EXPERIMENTAL

Throughout this work a commercial anhydrous creatinine (Sigma, C 4255) was used without further purification.

Measurements were performed for water solutions of selected concentrations of creatinine, NaCl and HCl (or NaOH), in order to ensure the appropriate ionic strength and pH of the sample. The last parameter was finally controlled directly in the NMR tube being thermostated at 30°C, using a pH electrode (Cole-Parmer NMR tube P-05990-30) and pH-meter (Cole-Parmer P-59002-00). This measuring system was standardized using pH = 1.68, pH = 4.01, pH = 7.00, and pH = 10.01 buffers. All the samples contained 5% of D_2O for providing a lock signal, and sodium 3-(trimethylsilyl)-1-propanesulphonate in concentration *ca.* 0.2 mole/1 mole of creatinine as ^1H and ^{13}C chemical shift reference. The precision of ^1H and ^{13}C chemical shift determination was better than 0.001 ppm and 0.01 ppm, respectively.

NMR spectra were recorded at the temperature 30°C controlled by the ethylene glycol sample, using Varian GEMINI 2000 spectrometer working at 4.7 T and Bruker Avance XL-500 spectrometer working at 11.7 T magnetic field. ^{14}N NMR chemical shifts were referenced *via* Bruker spectrometer frequency to the independently measured signal of CH_3NO_2 and were accurate to about ± 0.2 ppm.

Quantum mechanical calculations were performed using Gaussian98W program [16]. The applied level of theory DFT B3LYP/6-311++G(2d,p) was sufficient for reproducing satisfactorily the molecular geometry of creatinine known from crystallography [13] and it was believed that this theory could predict properly the gas phase structure as well as the reliable values of magnetic shielding parameters when using GIAO method [17].

RESULTS AND DISCUSSION

Chemical shift – solution acidity relationship. Theoretical calculations as well as all the available experimental data point out that in water solutions unprotonated creatinine exists predominantly as the amino tautomer **1a** (2-amino-1-methyl-2-imidazolin-4-one), which in acidic solution is protonated at N-3 (Fig. 1) [3,4,9,10]. The sensitivity of the proton, carbon and nitrogen chemical shifts of creatinine NMR signals on the solution acidity has been noticed by many authors [3–6]. The broad signal of amine protons, which occurs at $\delta = 8.5$ ppm in acidic solutions, at higher pH's, broadens further and finally disappears above approximately pH = 5.5. The obvious reason for such a behavior is the rapid exchange of $-NH_2$ and H_2O protons. The largest chemical shift change due to protonation is observed for the signal of N-3. This shift is as large as 57.7 ppm, which is, however, still only *ca.* 2/3 of the analogous shift observed for pyridine nitrogen. This finding was tentatively attributed to the delocalization of the positive charge in structure **2a** or to the presence of some amounts of another tautomer of creatininium cation in the equilibrium [3] (see below). Broad signals of two remaining nitrogens extensively overlap each other even at the magnetic field $B_0 = 11.7$ T and their common position changes only by about 4 ppm in the whole acidity range. Out of the four carbon signals those of sp^2 hybridization, C-2 and C-4, experience larger changes, which is understandable taking into account N-3 being the

protonation site. The remaining ^{1}H and ^{13}C NMR signals of creatinine change their positions much less, but the observed changes are perfectly systematic. All the chemical shift *vs.* pH relationships have the shapes of typical titration curves. The illustrative data are given in Table 1 and Fig. 2. According to our knowledge no attempt at quantitative description of this relationship has been published yet.

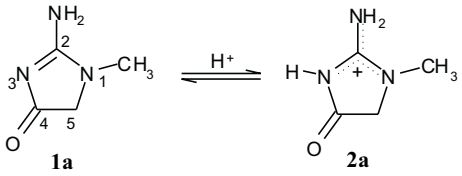


Figure 1. Tautomers **1a** and **2a** of creatinine and its cation being predominant in water solutions.

Table 1. The solution acidity dependence of ^{1}H, ^{13}C and ^{14}N chemical shifts of creatinine (0.3 mol/dm^3) in water solutions (ionic strength = 0.646; 303 K).

pH	C**H**$_3$	C**H**$_2$	C-1	C-5	C-2	C-4	N-3	N-1 + N-2
1.14	3.144	4.309	33.62	56.84	159.83	175.58	–235.4	–298.7
2.47	3.145	4.309	33.62	56.85	159.89	175.68	–235.7	–300.3
3.26	3.143	4.303	33.62	56.92	160.20	176.08	–234.8	–300.3
4.18	3.129	4.269	33.52	56.97	161.78	178.18	–227.0	–300.5
4.63	3.112	4.222	33.39	57.63	164.02	181.14	–216.0	–301.1
5.04	3.087	4.160	33.23	58.17	166.92	184.94	–202.5	–301.8
5.46	3.067	4.108	33.09	58.62	169.33	188.11	–190.0	–301.8
6.52	3.046	4.055	32.95	59.07	171.73	191.26	–179.6	–302.3
7.52	3.042	4.049	32.93	59.12	171.98	191.57	–178.3	–303.0
7.96	3.042	4.049	32.92	59.11	171.98	191.59	–178.2	–302.3
9.08	3.042	4.049	32.94	59.13	172.00	191.60	–177.8	–302.4

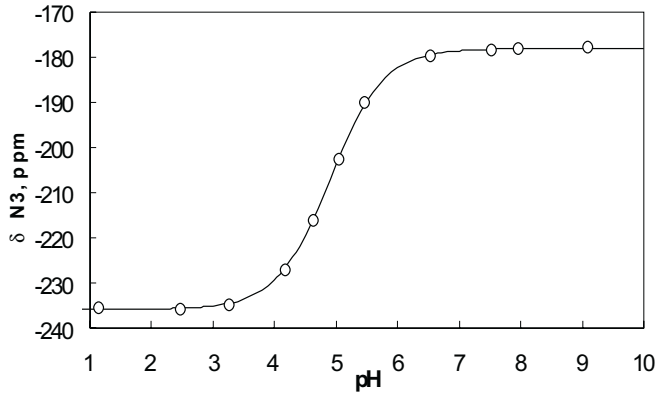


Figure 2. ^{14}N NMR chemical shift of N-3 signal of creatinine dependence on solution acidity (experimental points and the theoretical curve from least squares fitting).

Due to the rapid proton exchange, the observed position of a given signal in the spectrum, δ_{obs}, is the weighted average of the chemical shifts of the appropriate nuclei in the unprotonated (δ_1) and protonated (δ_2) forms, with populations of both species (p_1 and p_2) being the weighting factors:

$$\delta_{obs} = \delta_1 p_1 + \delta_2 p_2 \tag{1}$$

For a series of spectra recorded for solutions of the constant ionic strength the species populations can be expressed in terms of the acidity constant of protonated creatinine, K, and the solution pH:

$$p_1 = 1 - p_2 = K/(10^{-\mathrm{pH}} + K) \tag{2}$$

The least squares analysis of the data similar to those given in Table 1 and Fig. 2 yielded the values of the unknown parameters: K, δ_1 and δ_2. Such an analysis was performed for the data collected for various ionic strengths and creatinine concentrations. In every case the fit between experimental and calculated curves was excellent, usually of the order of the precision of chemical shift measurements. The pK values obtained for a given ionic strength, derived from dependences of various signals, diverged by less than 0.04 pK units, which was comparable with standard errors of these parameters. The acidity constant was ionic strength dependent (Table 2), but its value for low ionic strengths converged to the thermodynamic value ($pK_a = 4.76$ for 30°C), determined by other authors by spectrophotometric or potentiometric methods [11]. On the other hand, the limiting ^{1}H and ^{13}C chemical shifts seemed to be only marginally dependent on the applied measurement conditions.

Table 2. Acidity constants of creatininium cation, as determined from the chemical shift *vs*. pH relationships.

Creatinine concentration, mol/dm^3	Ionic Strength	NMR	Group	pK	δ_1	δ_2
0.3	0.646	^{1}H	C**H**$_3$	4.94± 0.01	3.042±0.001	3.145 ±0.001
			C**H**$_2$	4.92± 0.01	4.049±0.001	4.309 ±0.001
		^{13}C	**C**H$_3$	4.92± 0.01	32.93±0.01	33.63±0.01
			CH$_2$	4.93± 0.04	59.13±0.04	56.81±0.04
			C2	4.91± 0.01	172.01±0.02	159.86 ±0.02
			C4	4.90± 0.01	191.61±0.03	175.64 ±0.03
		^{14}N	**N**3	4.90± 0.01	–178.0± 0.2	–235.9±0.2
0.06	0.429	^{1}H	C**H**$_3$	4.94± 0.01	3.036±0.001	3.138 ±0.001
			C**H**$_2$	4.93± 0.01	4.049±0.001	4.303 ±0.001
0.06	0.10	^{13}C	**C**H$_3$	–	32.89±0.02	33.58±0.02
			CH$_2$	–	59.15±0.02	56.86±0.02
			C2	–	172.07±0.02	159.94±0.02
			C4	–	191.75±0.02	175.70±0.02
0.01	0.437	^{1}H	C**H**$_3$	4.94±0.01	3.032±0.001	3.135±0.001
			C**H**$_2$	4.92±0.01	4.045±0.001	4.301±0.001

Table 2 (continuation)

0.01	0.197	^{1}H	C**H**$_3$	4.90±0.03	3.032±0.001	3.133±0.001
			C**H**$_2$	4.90±0.02	4.046±0.002	4.296±0.002
0.01	0.080	^{1}H	C**H**$_3$	4.86±0.01	3.032±0.001	3.132±0.001
			C**H**$_2$	4.85±0.01	4.044±0.001	4.293±0.001
0.01	0.057	^{1}H	C**H**$_3$	4.84±0.02	3.030±0.001	3.129±0.001
			C**H**$_2$	4.85±0.01	4.042±0.001	4.290±0.001

Structure of creatinine and creatininium cation. Traditionally, in biochemical textbooks up to the eighties, creatinine was expressed as 2-imino-1-methylimidazolidin-4-one (**1b**). One can imagine, however, several tautomeric forms of creatinine molecule, two of which, **1a** and **1b**, appear to be energetically favourable (Fig. 3). Indeed, independently of the level of theory, calculations show that the imino form, **1b**, is the most energetically stable [4,10]. The numbers given in Fig. 3 denote relative energies calculated for structures optimized using DFT method, so one may expect that at least in the gas state **1b** form dominates.

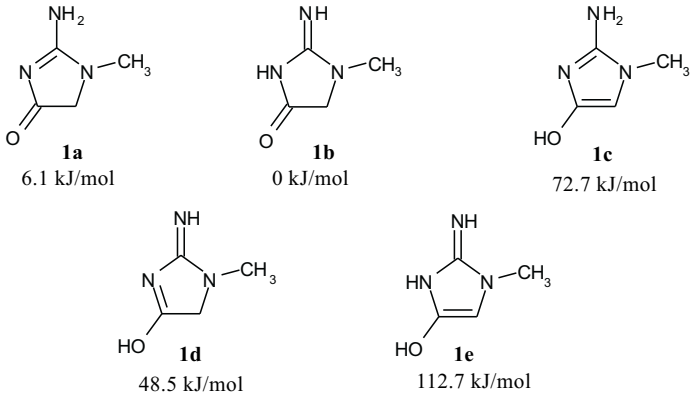


Figure 3. Creatinine tautomers and their relative energies [kJ/mol], as calculated by DFT B3LYP/6-311++G(2d,p) method.

On the other hand, after some initial misinterpretation, the crystallographic investigations have shown that in the solid state creatinine has the structure **1a** [12,13]. Simultaneously, more hints were pointing out that also in solutions the amino form was predominant. Those indications were mostly indirect, in the sense that they came from the studies in which physicochemical properties of creatinine and analogous compounds were compared. But, on the other hand, they originated from various independent sources and very different investigations, such as UV spectra, measurements of alkaline hydrolysis rates, interpretation of acidity constants [9,14] and also from NMR spectroscopy [4].

The results of quantum mechanical calculations do not contradict the above findings. It is understandable that theoretical calculations concerning isolated molecules can be insufficient for clarifying the situation in condensed phases, especially when the energy difference between possible structures is rather small. In such cases the interactions with the surrounding have to be taken into account. We calculated the

energies for monohydrates of two most probable tautomers of creatinine (structures shown in Fig. 4). The energy of the hydrate of **1b** is still lower than the energy of the hydrate of **1a**, but the difference is now really minimal. The calculation done by Craw, Hillier *et al*. [10], which additionally took into account the influence of a bulk solvent, has shown that, indeed, the tautomer **1a** of the higher dipole moment is the one which is more stable in water solutions.

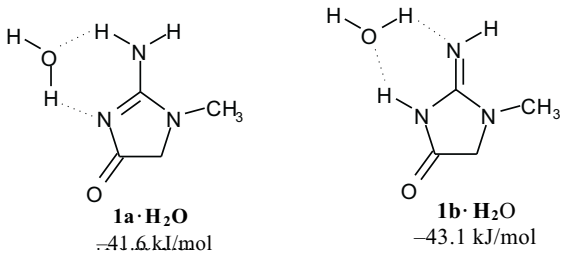


Figure 4. Structures and calculated energies of monohydrates of creatinine tautomers **1a** and **1b**. (Energies are given relative to the same level as those in Fig. 3 and were corrected by substraction from the total calculated energy the energy of a free water molecule).

Creatinine is a Brönsted base with four potential protonation sites: three nitrogen atoms and carbonyl oxygen (Fig. 5). A comparison of the calculated relative energies of the hypothetical structures of creatininium cation, **2a–2d**, allows excluding the protonation of nitrogens 1 and 2 from further considerations. Indeed, in the structures **2c** and **2d**, the positive charge would have to be localized exclusively on the appropriate nitrogen, while in the structures **2a** and **2b** it can be strongly delocalized. Apparently, protonation at N-3 is preferred, which is also confirmed by the chemical shift changes accompanying protonation.

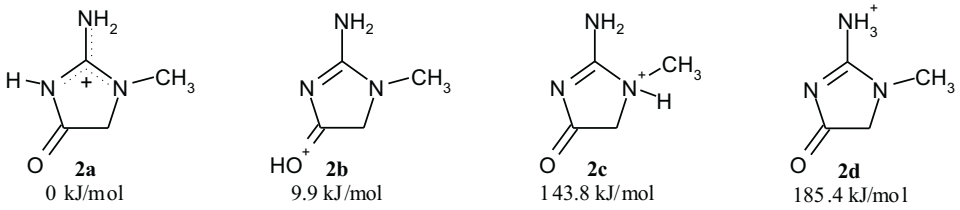


Figure 5. Hypothetical structures of creatininium cation tautomers and their relative energies [kJ/mol], as calculated by DFT B3LYP/6-311++G(2d,p) method.

Comparison of the experimental and theoretically calculated ^{13}C and ^{14}N chemical shifts. Having the set of the appropriate experimental data, we have calculated theoretically the carbon and nitrogen chemical shifts for the most probable tautomers of the neutral and protonated creatinine molecules. The obtained results are given in Table 3. The agreement between the experimental and the theoretical data is remarkably better for creatinine amino tautomer (**1a**) and creatininium cation possessing the proton at nitrogen-3 (**2a**), than for forms **1b** and **2b**, respectively. The comparison of

the absolute values of the nitrogen chemical shifts is perhaps not so appealing, but it is to be remembered that the calculations concern isolated molecules resting in the potential energy minimum. This is an idealized situation being very different from that actually occurring in measurements when molecules in a solution at ambient temperature undergo ro-vibrational motion and intermolecular interactions. Comparing the experimental and theoretical shielding data for nitrogen is always difficult, as the chemical shifts of this nucleus are very sensitive to the influence of the surrounding [18]. When, however, the protonation shifts are additionally taken into account, the conclusion is unambiguous: The agreement between experiment and theory exists only for forms **1a** and **2a** of the unprotonated and protonated creatinine, respectively.

Table 3. Comparison of the experimental and theoretical ^{13}C and ^{14}N NMR chemical shifts [ppm] for the neutral and protonated forms of creatinine ($\delta_{theor} = \sigma_{TMS} - \sigma_{theor}$, $\sigma_{TMS} = 183.06$; theoretical shielding constants, σ, were calculated using GIAO DFT 6-311++G(2d,p) method).

Atom	Neutral molecule			Protonated molecule		
	δ experimental	δ theoretical		δ experimental	δ theoretical	
		1a	**1b**		**2a**	**2b**
C2	172.01	179.30	161.37	159.86	160.89	172.89
C4	191.61	185.49	172.30	175.64	165.83	193.54
C5	59.13	60.30	57.96	56.81	57.39	61.03
C1	32.93	32.91	30.48	33.63	31.63	31.07
N3	–178.3	–166.2	–246.2	–235.7	–252.1	–149.4
N1	–302[a]	–316.1	–325.8	–299[a]	–301.8	–291.4
N2	–302[a]	–336.0	–238.2	–299[a]	–330.0	–314.9

[a] – average value.

Less abundant tautomers. It is to be stressed that the presence of small fractions of creatinine and creatininium cation tautomers other than **1a** and **2a** in solutions cannot be excluded on the basis of chemical shift or any other data. Furthermore, there are some hints suggesting that in solutions such species have to be present and play their important roles. Indeed, monitoring the width of NH_2 proton signal of creatinine in water solution one finds that even in acidic solutions the proton exchange process is base catalyzed. In the case of creatinine this problem is now under investigation, but on the basis of presently available data and the general knowledge concerning exchange of N-H protons [19,20] one may assume that such an exchange proceeds *via* the mechanism shown in Fig. 6. A Brönsted base abstracts a proton from the NH_2 group of protonated creatinine, most probably producing the tautomer **1b**. Assuming that the basicity of the imino nitrogen in **1b** is approximately the same as the appropriate nitrogen in 1,3-dimethyl-2-iminoimidazolidin-4-one ($pK_a = 8.07$ [9]) one can estimate the equilibrium population of **1b** form of creatinine:

$$K_a = [\mathbf{1a}][H^+]/[\mathbf{2}] = 10^{-4.8} \quad (3)$$

$$K_{a'} = [\mathbf{1b}][H^+]/[\mathbf{2}] \approx 10^{-8.1} \quad (4)$$

$$[\mathbf{1b}]/[\mathbf{1a}] = K_{a'}/K_a \approx 10^{-3.3} \quad (5)$$

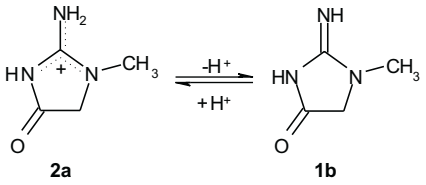
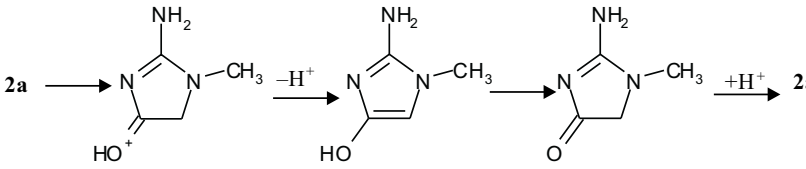


Figure 6. Hypothetical mechanism of -NH_2 proton exchange in creatininium cation leading to formation of the tautomer **1b**.

Moreover, Srinivasan and Stewart [15] have shown that in D_2O solutions CH_2 protons of creatinine undergo exchange on deuterons and that this process can be catalyzed by bases and acids. In basic solutions the exchange probably proceeds through the anion **3** (Fig. 7) possessing delocalized charge, which in the next step can be deuterated on carbon or oxygen. If the latter possibility occurs, the enolic form **1c** (2-amino-4-hydroxy-1-methylimidazol**)** is formed. Finally, one may suppose that in moderately acidic media, where the proton-deuteron exchange also occurs [15], the protonation on oxygen should facilitate deprotonation of C-5 more effectively than the protonation on N-3 and that this process probably proceeds through forms **2b** and **1c**:



(6)

It is to be stressed that the results of our calculations of energies of creatininium cation do not actually contradict the above hypothesis, as cations **2a** and **2b** are energetically not very far from each other and solvation (not included in the calculation) may substantially modify the situation.

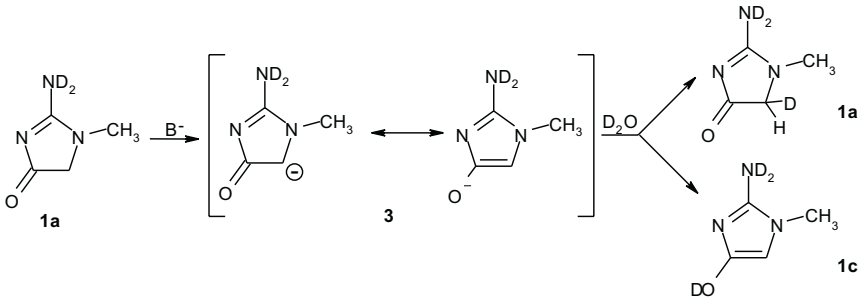


Figure 7. The base catalyzed proton/deuteron exchange at C-5 of creatinine, which may lead to formation of the tautomer **1c**.

CONCLUSIONS

Taking into account the fact that NMR spectroscopy has been proved to be a method of choice for urine assays [21,22] and that creatinine is a reference metabolite in such analyses, we believe that reported above precise ^{1}H and ^{13}C NMR chemical shift data for this compound in water solutions of various acidity are of potential usefulness. The analysis of these data as well as of the ^{14}N NMR chemical shifts has provided the acidity constant of creatininium cation (pK = 4.8). Comparison of the experimental ^{13}C and ^{14}N chemical shifts of the neutral and protonated forms of creatinine with the magnetic shielding constants calculated using the quantum chemistry method GIAO DFT B3LYP/6-311++G(2d,p) for various tautomeric structures of the investigated species supports the view that **1a** and **2a** forms of creatinine predominate in neutral and acidic water solutions, respectively. Compilation of the available experimental and theoretical results has allowed us to suppose that small amounts of **1b**, **1c** and **2b** creatinine forms are also present in water solutions, which extends the discussion on the problem of tautomerism of this important biological molecule.

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