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Mixed-ligand complexes of copper(II) ions with L-glutamic acid in the systems with triamines and non-covalent interaction between bioligands in aqueous solution

Abstract: The interactions between L-glutamic acid (Glu) and 1,7-diamino-4-azaheptan (3,3-tri) and 1,8-diamino-4-azaoktan (Spd) in metal-free systems and in the appropriate Cu(II) ions complexes were studied by potentiometric and spectral methods. The composition and overall stability constants of complexes forming in the systems studied were determined and the reactions centres were identified. In the complex formation conditions, the ligands have positive and negative reaction centres, which are also the potential sites of metal coordination. Below pH 7 in metal-free systems, the terminal amine groups from both triamines and the oxygen atoms from −C(5)O− group as well as the amine group from Glu do not take part in the weak noncovalent interactions between the ligands, but at higher pH all available active centres of the ligands are involved in the interactions in the adducts formed and the inversion effect is observed. In the Cu(II)/Glu/triamine systems, in the species MLHL′, both 3,3-tri and Spd were found to coordinate Cu(II) ions in the same way, while only in the Cu(Glu)H(Spd) complex the oxygen atoms from −C(5)O− group of the amino acid do not take part in metallation. In the species MLL′ and MLL′OH with 3,3-tri, the chromophore formed was of different type than that formed in the corresponding species with Spd. This is related to the fact that the nitrogen atom from the amine group of Glu is inactive in the coordination. Thus, not only the length of the polyamine carbon chain but also the length of the amino acid carbon chain influence the interactions between the bioligands. The introduction of metal ions to Glu-Spd metal-free system excludes the interaction between the bioligands, and the ligands centres that do not take part in the weak interactions in the adduct (Glu)H(Spd) are involved in the metallation, but the centres that took part in the weak interactions are not involved in the coordination in a heteroligand complex.

Keywords: Copper(II), L-glutamic acid, triamines, adducts, mixed complexes

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1 Introduction

Copper(II) is an endogenous metal in the human brain which plays an integral role in neurotransmission [1] and together with amyloid β-peptide (Aβ) is found in the senile plaque formed in the Alzheimer’s disease (AD) [2-5]. Copper(II) ions are mainly found in the blood plasma in the form of mixed complexes with amino acids, peptides and other biomolecules [6-7]. The complexes with amino acids are particularly interesting as Cu(II) ions can split DNA in the presence of hydrogen peroxide and the various side groups of amino acids can bind specifically to DNA [8,9]. Glutamic acid (Glu), which is an endogenous amino acid, is the main stimulating neurotransmitter in the brains of mammals [10,11] this is related to its important role in the functions of learning and memorising and in the brain aging [12,13]. Glutamatic acid is a part of Aβ structure and is directly involved in neurological damage appearing as a consequence of cerebral stroke and degeneracy, including AIDS-dementia complex, Alzheimer’s, Parkinson’s diseases [14-16], sclerosis and epilepsy [17,18]. Glu is the most important oxidative substrate for the intestinal mucosa [19] and also plays a critical role in synaptic...
maintenance and plasticity, and formation and function of the cytoskeleton [20]. Along with amino acids, polyamines (PAs) [21,22], which are small organic cations indispensable for growth of eukaryotic cells, also play essential role in living organisms. PAs are involved in regulation of gene expression [23], stabilisation of chromat [24], prevention of endonuclease-mediated DNA fragmentation [25], inhibition of DNA damage [26,27] or in regulation of physical properties of biologic membranes [28]. At physiological pH, PAs interact with negative fragments of other biomolecules, i.e., amino acids, proteins and phospholipids. As studied earlier [29], the high basicity of donor PA atoms (positive centres of weak interactions) is the main factor determining the noncovalent interactions in amino acid/PA systems. These –NH₄⁺ donor PA groups can react with deprotonated carboxyl groups and/or –NH₂ group (negatively centres) of amino acids. In solution, when molecular complexes are forming, a shift of the acid-base equilibrium of the substrates is observed, along with a change in the concentration of hydrogen cations, which permits evaluation of the thermodynamic stability of the adducts formed [30-32]. As follows from the results obtained by the spectral and equilibrium methods, these interactions have ion-dipole or ion-ion character [30-35].

2 Experimental procedure

2.1 Materials

The compound 1,7-diamino-4-azaheptane (3,3-tri) – C₉H₁₀N₃ was purchased from Aldrich and 1,8-diamino-4-azaoctane, spermidine (Spd) – C₁₉H₂₁N₇ was purchased from Sigma. PA nitrates were prepared by dissolving a proper amount of free amine and addition of an equimolar amount of HNO₃. The white precipitate obtained was recrystallised, washed out with methanol, dried in desiccator over P₂O₅ or in the air. The ligands which were used as nitrate salts were subjected to elemental analysis whose results (%C, %N, %H) were in agreement with the theoretically calculated values (±0.5%). The elemental analysis was performed on an Elemental Analyzer CHN 2400, Perkin-Elmer. L-glutamic acid (Glu) C₅H₉NO₄ used without further purification, was bought from Sigma-Aldrich Co. Copper(II) nitrate(V) from POCh–Poland, was purified by recrystallisation from water. The concentration of Cu(II) ions was determined by the method of inductively coupled plasma optical mass spectroscopy (ICP MS) on Spectrometer Mass Varian.

2.2 Potentiometric measurements

Potentiometric studies were performed on a Methrom 702 SM Titrino with an autoburette. A glass electrode Methrom 6.0233.100 was calibrated in terms of hydrogen ions concentration [36] with a preliminary use of borax (pH = 9.225) and phthalate (pH = 4.002) standard buffers. The concentration of ligands in the titrated systems was 1×10⁻² M in metal free-systems and 1×10⁻³ M in the ternary systems. The concentration ratio of L : L’ (where L = glutamic acid and L’ = triamine) in the samples studied was 1 : 1 and the ratio of Cu : L : L’ was 1 : 2.5 : 2.5. Potentiometric titrations were performed at the constant ionic strength μ = 0.1 M (KNO₃), at 20±1°C inert gas atmosphere (helium), using CO₂-free NaOH solution (about 0.2 M) as a titrant. Addition of NaOH solution did not change the ionic strength, because the measurements were performed starting from fully protonated polyamines, so –NH₄⁺ cations were replaced by equivalent amounts of Na⁺. The selection of the models and determination of the stability constants of the complexes were made using the SUPERQUAD [37] or HYPERQUAD [38] computer programs. Six titrations were performed for each particular system and 100-350 points for each titration curve were used for computer analyses. The SUPERQUAD and HYPERQUAD programs use the nonlinear method of least squares to minimize the sum (S) of squares of residuals between the observed quantities (fobs) and those calculated on the basis of the model (fcalc).

\[ S = \sum_{i} w_{i} (f_{i}^{\text{obs}} - f_{i}^{\text{calc}})^{2} \]

where \( n \) is the number of measurement and \( w_{i} \) is the statistical weight.

The testing usually begins with the simplest hypothesis and then in the next steps the models are expanded to include progressively more species, and the results are scrutinized to eliminate those species rejected in the refinement process. The criteria of correct choice of model are given in [39]. The above-described procedures were used for investigation of the binary systems amino acid-polyamine as well as coordination compounds forming in ternary systems with metals.

Determined \( pK_{a} \) was 13.89. Distribution of particular species was determined by the HALTAFALL computer program [40].
2.3 Spectral measurements

2.3.1 NMR spectroscopy

The samples for $^{13}$C NMR investigation were prepared by dissolving appropriate amounts of the species (Cu(II) ions, Glu, 3,3-tri and Spd) in D$_2$O. DNO and NaOD were used to adjust pD of solutions, correcting pH-readings (a pH meter N517 made by Mera-Tronik) according to the formula: 
\[ \text{pD} = \text{pH}_{\text{readings}} + 0.40 \] [41]. The concentration of ligands was 0.1 M, at the concentration rate of Cu(II) to those of the ligands of \( M : L : L' = 1 : 100 : 100 \). $^{13}$C NMR spectra were recorded on a NMR Gemini 300VT Varian spectrometer using dioxane as an internal standard. The positions of $^{13}$C NMR signals were converted to the TMS scale.

2.3.2 IR spectroscopy

IR spectra were taken on an ISS 66 v/S Bruker spectrometer in the range 4000-400 cm$^{-1}$; resolution of 2 cm$^{-1}$ in D$_2$O in the cells KRS-5 (pD of the solution was adjusted using NaOD or DCl, like in the preparation of samples for NMR). The concentration of the ligands was 0.15 M (ratio of \( L : L' = 1 : 1 \)).

2.3.3 Vis spectroscopy

Vis spectra were taken on a UV/Vis Thermo Fisher Scientific Evolution 300 Spectrophotometer. The samples were prepared in H$_2$O for the same ligand concentration as in samples for potentiometric titrations at the metal to ligand ratio of 1 : 2.5 : 2.5 using a Plastibrand PMMA cell with 1 cm path length. NaOH and HNO$_3$ were used to adjust pH of solutions.

2.3.4 EPR spectroscopy

The EPR spectra were recorded on an SE/X 2547 Radiopan Spectrophotometer at 77 K in glass capillary tubes of 130-μL capacity. Samples were made in a water:glycol mixture (3 : 1), and the metal to ligand ratio was 1 : 2.5 : 2.5. The concentration of the Cu(II) ions was 0.005 M. NaOH and HNO$_3$ were used to adjust pH of solutions.

3 Results and discussion

The ligands studied are presented in Scheme 1.

Potential centres of weak noncovalent interactions leading to formation of molecular complexes are the oxygen atoms of carboxyl groups and amine group in L-glutamic acid and amine groups in the triamines studied. These centres are also the potential sites of metal ions coordination by donor atoms. The conclusions on the mode of interactions in the adducts and heteroligand complexes formed in the systems studied were assumed as correct only when supported by potentiometric data being in full agreement with the data obtained by a few independent spectroscopic methods.

3.1 Non-covalent interactions in the Glu/3,3-tri and Glu/Spd systems

Except from our recent papers [42-44], no literature with information on the reactions taking place in amino acid-polyamine systems and in the analogues with Cu(II) ions has been found.

As described earlier [39,45], the release of proton in the reaction of H$_x$Glu + H$_y$(triamine) $\rightleftharpoons$ (Glu)H$_{(x+y)}$(triamine) + nH$^+$ permits the use of potentiometric method for the
determination of composition of the adducts forming in the metal-free systems studied and their stability constants (log $\beta$). The centres of interaction and mode of interaction were identified on the basis of the spectroscopic studies. The assignment of $^{13}$C NMR signals to individual carbon atoms from the triamines and glutamic acid studied has been made on the basis of literature data [46] and [47], respectively.

### 3.1.1 Glu/3,3-tri system

Potentiometric studies in the system Glu-3,3-tri were performed in the pH range from 2.8 to 10.5. Table 1 presents the overall stability constants (log$\beta$) obtained on the basis of the computer analysis of potentiometric data for molecular complexes forming by L-glutamic acid and 3,3-tri together with the equilibrium constants of formation (log$K_e$) of these species.

In the system Glu-3,3-tri, the adducts (Glu)$H_\chi$(3,3-tri) are formed, where $\chi=2$-$5$. The molecular complex (Glu)$H_5$(3,3-tri) forms below pH 5 (Fig. 1a), that is in the pH range of deprotonation of the first carboxyl group of glutamic acid. In the pH range from 5 to 7 the dominant species is (Glu)$H_4$(3,3-tri), and above pH 7 the triprotonated complex is formed. The adduct (Glu)$H_2$(3,3-tri) occurs above pH 8.5.

The $^{13}$C NMR spectra of the adducts reveal changes in the signal positions with respect to those of analogous signals in the spectrum of free ligand. The changes appear as a consequence of changes in electronic density on the atoms localised near the centres of noncovalent interactions.

#### Table 1: Overall stability constants (log $\beta$) and equilibrium constants of adduct formation (log $K_e$) in Glu/3,3-tri and Glu/Spd systems.

| Species       | $\Sigma$ | $\chi^2$ | Equilibrium                                                                 | log $\beta$ | log $K_e$ |
|---------------|----------|----------|-----------------------------------------------------------------------------|-------------|-----------|
| (Glu)$H_5$(3,3-tri) |          |          | (Glu)$H_5$ + (3,3-tri) $\Leftrightarrow$ (Glu)$H_5$(3,3-tri)             | 43.92(1)    | 2.20      |
| (Glu)$H_4$(3,3-tri) | 18.64    | 7.26     | (Glu)$H_4$ + (3,3-tri) $\Leftrightarrow$ (Glu)$H_4$(3,3-tri)             | 39.78(1)    | 2.24      |
| (Glu)$H_3$(3,3-tri) |          |          | (Glu)$H_3$ + (3,3-tri) $\Leftrightarrow$ (Glu)$H_3$(3,3-tri)             | 31.86(1)    | 2.13      |
| (Glu)$H_2$(3,3-tri) |          |          | (Glu)$H_2$ + (3,3-tri) $\Leftrightarrow$ (Glu)$H_2$(3,3-tri)             | 22.33(2)    | 2.34      |
| (Glu)$H_4$(Spd)   | 27.29    | 17.30    | (Glu)$H_4$ + (Spd) $\Leftrightarrow$ (Glu)$H_4$(Spd)                     | 40.60(6)    | 1.47      |
| (Glu)H(Spd)       |          |          | (Glu) + (Spd) $\Leftrightarrow$ (Glu)H(Spd)                              | 32.74(4)    | 2.20      |
| (Glu)H(Spd)       |          |          | (Glu) + (Spd) $\Leftrightarrow$ (Glu)H(Spd)                              | 13.45(7)    | 2.48      |

*Overall protonation constants of the ligands: $H_3$(Glu), 15.96(1); $H_2$(Glu), 13.69(2); H(Glu), 9.51(1) – our laboratory; $H_5$(3,3-tri), 28.03(3); $H_4$(3,3-tri), 20.22(2); H(3,3-tri), 10.48(2) [49]; H(Spd), 29.62(2); H(Spd), 21.03(1); H(Spd), 10.97(1) [49];

![Figure 1](image_url)
The insignificant changes in positions of the signals assigned to C\(_{\text{C(2)}}\) and C\(_{\text{C(5)}}\) from Glu (Table 2) confirm that in the process of (Glu)H\(_5\) (3,3-tri) adduct formation, the –C\(_{\text{C(5)}}\)OOH and –NH\(_{\text{3}}^+\) groups from Glu, protonated at this pH, are not involved in the interaction with triamine (no negative reaction centres in the PA molecule), Scheme 2.

Above pH 3, the second carboxyl group from amino acid undergoes deprotonation and (Glu)H\(_4\) (3,3-tri) adduct is formed (Fig. 1a). At pH of 6, at which this species is dominant, the shifts of the signals assigned to C\(_{\text{C(1)}}\), C\(_{\text{C(2)}}\) and C\(_{\text{C(5)}}\) of Glu and C(1), C(2) and C(3) of triamine are 0.100, 0.032 and 0.007, 0.025, 0.051 ppm, respectively (Fig. 2a).

These changes point to the engagement of only –C\(_{\text{C(1)}}\)OO carboxyl group from Glu and –NH\(_{\text{3}}^+\) group from 3,3-tri in the interaction between the bioligands. So, in (Glu)H\(_5\) (3,3-tri) and (Glu)H\(_4\) (3,3-tri) adducts (Scheme 2), in contrast to the situation in the earlier studied Asp-3,3-tri system [43], the mode of interaction is similar, which is confirmed by comparable values of equilibrium constants of the relevant reactions logK\(_e\), equal to 2.20 and 2.24 for the two species, respectively. For the reason that different

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**Table 2**: Differences between \(^{13}\)C NMR chemical shifts for the ligands in the Glu/triamine systems in relation to the free isolated ligands [ppm].

| Systems       | pH | \(\text{Glu}\) | \(\text{C(1)}\) | \(\text{C(2)}\) | \(\text{C(3)}\) | \(\text{C(4)}\) | \(\text{C(5)}\) | \(\text{PA}\) | \(\text{C(1)}\) | \(\text{C(2)}\) | \(\text{C(3)}\) | \(\text{C(4)}\) | \(\text{C(5)}\) | \(\text{C(6)}\) | \(\text{C(7)}\) |
|---------------|----|--------------|----------------|----------------|----------------|----------------|----------------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Glu/3,3-tri   | 3.0| 0.160        | 0.033          | 0.047          | 0.060          | 0.043          | 0.005          | 0.020        | 0.059          |                |                |                |                |                |                |
|               | 6.0| 0.100        | 0.004          | 0.050          | 0.011          | 0.032          | 0.007          | 0.025        | 0.051          |                |                |                |                |                |                |
|               | 8.5| 0.548        | 0.074          | 0.054          | 0.020          | 0.320          | 0.385          | 0.866        | 0.237          |                |                |                |                |                |                |
|               | 10.0| 0.820        | 0.110          | 0.046          | 0.497          | 0.139          | 0.833          | 0.987        | 0.827          |                |                |                |                |                |                |
| Glu/Spd       | 6.0| 0.055        | 0.037          | 0.035          | 0.004          | 0.008          | 0.037          | 0.033        | 0.058          | 1.259          | 0.359          | 0.693          | 0.247          | 0.083          | 0.634          | 0.758          |
|               | 9.0| 1.180        | 0.177          | 0.059          | 0.597          | 0.292          | 0.034          | 0.047        | 0.075          | 0.034          | 0.059          | 0.041          |                |                |                |
|               | 10.0| 0.758        | 0.024          | 0.020          | 0.018          | 0.154          |                |                |                |                |                |                |                |                |                |

**Scheme 2**: Tentative mode of interaction in adducts formation in Glu/3,3-tri system (||||||| intermolecular interaction).
stoichiometric compositions of the species formed, the values of \( \log \beta \) cannot be directly applied in analysis of the character of these interactions.

The (Glu)\( H(3,3\text{-tri}) \) complex forms in the pH range in which the first amine group from 3,3-tri undergoes deprotonation (Fig. 1a). The \(^{13}C \) NMR spectrum of (Glu)\( H(3,3\text{-tri}) \) adduct at pH = 8.5 shows changes in the chemical shifts assigned to C(1), C(2) and C(3) from triamine by 0.385, 0.866 and 0.237 ppm, respectively. These changes imply that the deprotonated terminal –NH\(_2\) group (a negative reaction centre) and the protonated –NH\(_2\)+ and –NH\(_3\)+ groups from 3,3-tri (positive reaction centres) are involved in the weak noncovalent interactions with the amino acid. The changes in positions of the signals assigned to C(1), C(2) and C(5) from Glu of 0.548, 0.074 and 0.320 ppm, respectively, point to the participation of all reaction centres from Glu in the interactions. Therefore, in (Glu)\( H(3,3\text{-tri}) \) adduct, the interactions take place according to the inversion effect (similarly as in the species (Asp)\( H(3,3\text{-tri}) \) [43]), which means that depending on pH the amine groups from triamine can act as a positive or negative reaction centre, in (Glu)\( H(3,3\text{-tri}) \) the amine group is a positive reaction centre, while in (Glu)\( H(3,3\text{-tri}) \) it is a negative reaction centre. The character of interaction depends on the degree of protonation. The mode of interaction proposed for (Glu)\( H(3,3\text{-tri}) \) adduct, (Scheme 2), is confirmed by IR results. At pH 6.0, the band corresponding to the stretching vibrations of N-H in 3,3-tri in the IR spectrum of (Glu)\( H(3,3\text{-tri}) \) appears at 3395 cm\(^{-1}\), while in the IR spectrum of (Glu)\( H(3,3\text{-tri}) \) at pH of 8.5 this band is shifted to 3406 cm\(^{-1}\), which confirms the changes in the mode of interaction of triamine in the triprotonated adduct. GAUSSIAN 03 program (Ground State method/ DFT /B3LYP/LAND2DZ level, solvation model) [48] was used to calculate partial charges. For –NH\(_2\), –NH\(_3\)+ and –NH\(_3\)+ groups from H(3,3-tri) the partial charges are -0.021, -0.340 and +0.104, respectively, while for H(Glu) –C(1)OO, –C(5)OO and –NH\(_3\)+ they are -0.784, -0.830 and +0.523, respectively. Although these values alone cannot be treated as direct evidence of the mode of interactions, when interpreted together with the results obtained by the other methods they show that the carboxyl groups from Glu (negative partial charge) can interact only with the protonated amine groups from triamine (positive partial charge), while the –NH\(_2\) group from this ligand (negative partial charge) can interact only with –NH\(_3\)+ group from Glu (positive partial charge), which confirms the inversion in the mode of interaction in (Glu)\( H(3,3\text{-tri}) \) adduct (Scheme 2).

At pH 10, at which the dominant adduct is (Glu)\( H(3,3\text{-tri}) \), the \(^{13}C \) NMR spectrum (Table 2) proves that all functional groups from the molecules of both ligands take part in the interactions. In the IR spectrum of this species the band at 3420 cm\(^{-1}\) corresponding to the valence stretching vibrations of N-H from 3,3-tri is shifted by 14 cm\(^{-1}\) with respect to the position of the corresponding band in the IR spectrum of (Glu)\( H(3,3\text{-tri}) \), which means that a proton has been dissociated off from triamine (log\( K = 9.74 \) [49]) and the interaction between the ligands takes place according to the reaction H(Glu) + H(3,3-tri) \( \rightarrow \) (Glu)\( H(3,3\text{-tri}) \) and the inversion model, Scheme 2. The partial charges for H(3,3-tri) on –NH\(_2\), –NH\(_3\)+ and –NH\(_3\)+ groups are -0.042 and +0.301, while for H(Glu) they are -0.784, -0.830 and +0.523 on the groups -C(1)OO, -C(5)OO and –NH\(_3\)+, respectively.

### 3.1.2: Glu/Spd system

In contrast to the system with 3,3-tri, in the system Glu-Spd the process of complexation starts above pH 3
(Fig. 1b) with formation of (Glu)H$_2$(Spd) adduct. In the pH range from 6.5 to 10.5, the formation of (Glu)H$_2$(Spd) is observed, while above pH 9.5, the (Glu)H(Spd) species is formed. The overall stability constants of these species (log$\beta$) and the equilibrium constants of formation (log$K_e$) are presented in Table 1.

The $^{13}$C NMR spectrum of the tetraprotonated species forming according to the reaction H(Glu) + H$_2$(Spd)$\rightarrow$ (Glu)H$_2$(Spd), at pH 6, shows changes in the chemical shifts of C(3) and C(4) from Spd relative to those in the spectrum of free ligand by 0.058 and 0.120 ppm, respectively, while the change in the signal assigned to the carbon atom from -C$_3$O$^-$ group in Glu is 0.055 ppm. These changes mean that only the –NH$_2$ amine group from Spd and -C$_3$O$^-$ carboxyl group from Glu are involved in the interaction between the bioligands. Much smaller chemical shift values assigned to the other carbon atoms from the molecules of both ligands (Table 2) suggest that there is no ligand1 - ligand2 type interaction between –NH$_2$ and –C$_3$O$^-$ groups from Glu and terminal protonated amine groups from Spd.

Above pH 7, the first proton is dissociated off from the Spd molecule and the formation of (Glu)H$_3$(Spd) adduct starts. At pH 9, the $^{13}$C NMR spectrum (Table 2, Fig. 2b) shows changes in the positions of signals assigned to carbon atoms localised near the reaction centres with respect to their positions in the spectrum of the free ligand. As follows from the changes, the deprotonated amine group from Spd (a negative reaction centre, partial charge of -0.003) is involved in the interactions between the bioligands and this group can only interact with the still protonated –NH$_2$ group from the amino acid (a positive reaction centre, partial charge +0.523). Depending on the degree of protonation, triamine can interact through positive or negative reaction centres. In the species (Glu)H$_3$(Spd) the subsequent reaction centres from both ligands are involved in the interaction, which is indicated by the equilibrium constant of formation log$K_e$ = 2.20 (log$K_e$(Glu(H$_3$(Spd)) = log$\beta_a$(Glu)$_4$(Spd) - log$\beta_a$(Glu)$ = 32.74 \cdot 9.51 \cdot 21.03 = 2.20$), higher than that obtained for the (Glu)H$_2$(Spd) adduct, log$K_e$ = 1.47. Thus the interaction in (Glu)H$_2$(Spd) takes place according to the effect of inversion.

No formation of a diprotonated species is observed (in detectable amounts), which is known to form in the analogous system with Asp [43]. In the pH range of (Glu)H(Spd) formation, the amino acid is already fully deprotonated (all reaction centres are negative) and can interact with the still partly protonated triamine. Analysis of the $^{13}$C NMR spectra (Table 2) reveals that only oxygen atoms from both Glu carboxyl groups and the protonated (a positive reaction centre) secondary amine group from Spd are involved in the interaction between the bioligands.

The deprotonated terminal amine groups from triamine (negative reaction centres) do not take part in the weak noncovalent interactions (no positive reaction centres in the molecule of Glu). In the IR spectrum of (Glu)H$_3$(Spd), the band assigned to the valence stretching vibrations N-H in the Spd molecule at pH = 9.0 appears at 3405 cm$^{-1}$ and is shifted to 3422 cm$^{-1}$ at pH 10.5 in the spectrum of (Glu)H(Spd) (Fig. 2). This result confirms a different mode of interaction between triamine and Glu in the monoprotonated adduct and triprotonated species.

### 3.2 Ternary Cu(II)/Glu/triamine systems

Computer analysis of the potentiometric titration data obtained for ternary systems were made using the literature values of the protonation constants of triamines [49] and Glu and the overall stability constants (log$\beta$) of the complexes formed in binary systems of Cu(II) ions with Glu, Cu(II) ions with 3,3-tri and Spd [49]. The hydrolysis constants of Cu(II) ions were also taken from literature [50].

#### 3.2.1 Cu(II)/Glu/3,3-tri system

The overall stability constants (log$\beta$) and equilibrium constants of formation (log$K_e$) of complexes in ternary systems Cu(II)-Glu-3,3-tri are given in Table 3.

Computer analysis of potentiometric titration data (program SUPERQUAD, HYPERQUAD) for Cu(II)/Glu/3,3-tri system, indicated the presence of the monoprotonated complex Cu(Glu)H(3,3-tri), Cu(Glu)(3,3-tri) species and the hydroxo complex Cu(Glu)(3,3-tri)(OH). In contrast to the data for the analogous system with Asp [43], no formation of molecular complexes (ML$^{-}$L$^{-}$) with a fully protonated polyamine in the outer coordination sphere involvement in noncovalent interactions with the anchoring binary connection of Cu(II) ions with amino acid. As illustrated in Fig. 3, the process of complexation in the Cu(II)/Glu/3,3-tri system starts at pH close to 5.5, at which the second amine group of 3,3-tri undergoes deprotonation and formation of Cu(Glu)(3,3-tri) species starts, binding about 60% Cu(II) ions at physiological pH.

At pH 9.3 the dominant species is Cu(Glu)(3,3-tri), while the hydroxo complex Cu(Glu)(3,3-tri)(OH) binds about 70% of copper ions above pH 10.5. As has been observed, there is a clear relation between the d-d transition energy, EPR parameters and the number of donor atoms in the inner coordination sphere of Cu(II) ion complexes [32,45,51-53].
Table 3: Overall stability constants (log β) and equilibrium constants (log Kₚ) of complexes formation in Cu(II)/Glu/3,3-tri or Spd systems.

| Species                  | Equilibrium of formation *                                                                 | log β  | log Kₚ |
|--------------------------|---------------------------------------------------------------------------------------------|--------|--------|
| Cu(Glu)(H)(3,3-tri)      | Cu⁺⁺ + H⁺ + Glu + 3,3-tri \( \rightleftharpoons \) Cu(Glu)(H)(3,3-tri)⁺⁺                  | 26.92(5)| 7.92   |
| Cu(Glu)(3,3-tri)         | Cu⁺⁺ + Glu + 3,3-tri \( \rightleftharpoons \) Cu(Glu)(3,3-tri)⁺⁺                      | 18.28(6)| 9.76   |
| Cu(Glu)(3,3-tri)(OH)     | Cu⁺⁺ + 3,3-tri + H⁺ \( \rightleftharpoons \) Cu(Glu)(3,3-tri)⁺⁺ + H⁺                     | 8.40(8)| -      |
| Cu(Glu)(H)(Spd)          | Cu⁺⁺ + H⁺ + Glu + Spd \( \rightleftharpoons \) Cu(Glu)(H)(Spd)⁺⁺                   | 26.90(9)| 7.41   |
| Cu(Glu)(Spd)             | Cu⁺⁺ + Glu + Spd \( \rightleftharpoons \) Cu(Glu)(Spd)⁺⁺                           | 18.00(9)| 9.48   |
| Cu(Glu)(Spd)(OH)         | Cu⁺⁺ + Glu + Spd + H₂O \( \rightleftharpoons \) Cu(Glu)(Spd)⁺⁺ + H⁺                 | 8.02(8)| -      |

*Overall stability constants (log β) of complexes formation in binary systems: CuH(Glu), 13.03(5); Cu(Glu), 8.52(5); Cu(Glu)₂, 15.01(7); Cu(Glu)(OH), 1.85(6) – our laboratory; CuH(3,3-tri), 18.87(18); Cu(3,3-tri), 13.71(3); Cu(3,3-tri)₂, 18.48(24); Cu(3,3-tri)(OH), 3.14(5) [49]; Cu(Spd), 18.91(7); Cu(Spd), 11.70(6); Cu(Spd)₂, 17.13(12); Cu(Spd)(OH), 2.90(11); Cu(Spd)₂(OH), 6.72(12) [49].

Table 4: Vis and EPR spectral data for Cu(II)/Glu/triamine systems.

| Species        | pH  | λₑ max (nm) | ε [M⁻¹ cm⁻¹] | EPR   | g₁   | A₁ (10⁶ cm⁻¹) |
|----------------|-----|-------------|--------------|-------|------|--------------|
| Cu(Glu)(H)(3,3-tri) | 8.0 | 624         | 77.2         | 2.232 | 2.035 | 179          |
| Cu(Glu)(3,3-tri)   | 9.5 | 645         | 93.1         | 2.280 | 2.035 | 182          |
| Cu(Glu)(3,3-tri)(OH) | 10.5 | 642 | 99.0         | 2.278 | 2.035 | 185          |
| Cu(Glu)(H)(Spd)    | 8.6 | 605         | 92.8         | 2.234 | 2.040 | 184          |
| Cu(Glu)(Spd)       | 9.5 | 598         | 117.2        | 2.228 | 2.040 | 184          |
| Cu(Glu)(Spd)(OH)   | 10.5 | 599 | 127.2        | 2.231 | 2.038 | 193          |

Figure 3: Distribution diagram for the Cu(II)/Glu/3,3-tri system; percentage of the species refers to total metal; Cₜₜₜ=5.4x10⁻⁸M; Cₜₜₜ=1x10⁻³M; Cₜₜₜ=1x10⁻³M, at 20°C, μ=0.1 mol L⁻¹(KNO₃).

The energy of d-d transitions (λₑ max = 624 nm) and EPR parameters (g₁ = 2.232 and A₁ = 179), Table 4, at pH 8.0 point to the copper ion coordination in Cu(Glu)(H)(3,3-tri) species through three nitrogen atoms from the molecules of both ligands and oxygen atoms from the deprotonated carboxyl groups of Glu, i.e., the {3N,O} chromophore. (Results of EPR studies are discussed in 3.2.2. paragraph to a greater extent).

At pH 8.0, in the ¹³C NMR spectrum of this complex the changes in positions of signals assigned to C₁, C₆, and C₉ from Glu, with respect to their positions in the spectra of free ligands are 0.061, 0.060 and 0.099 ppm, while the signals assigned to C(1) and C(3) from 3,3-tri are shifted by 0.269 and 0.093 ppm, respectively, (Table 5). These shifts indicate that the nitrogen atom from –NH₂ as well as the oxygen atoms from both carboxyl groups of Glu and the nitrogen atoms from both deprotonated terminal amine groups of triamine are involved in the metallation. This conclusion is confirmed by the type of chromophore forming in Cu(Glu)(H)(3,3-tri) species identified on the basis of Vis and EPR spectra.

The problematical question of credibility of NMR measurements of compounds with paramagnetic ions had been discussed earlier [33,35,42,54,55]. In order to minimise the effect of the NMR signal broadening, the spectra were recorded at low concentrations of Cu(II) ions in the pH ranges of the complex dominance that are similar to the pH ranges at higher concentrations of metal ions and ligands. Therefore, it was found possible to identify the mode of coordination on the basis of changes in the signal positions, because significant changes in the chemical shifts of some atoms were observed only in the pH ranges in which the formation of complexes took place. Moreover, the equilibrium constant of formation of Cu(Glu)(H)(3,3-tri)⁺⁺ \( \rightleftharpoons \) Cu(Glu)⁺⁺ + H(3,3-tri)⁺⁺, log Kₑ = logβCu(Glu)(H)(3,3-tri)⁻⁻ - logβCu(Glu)⁺⁺ - logβH(3,3-tri)⁺⁺ = 26.92 - 8.52 - 10.48 = 792 (Table 3) is similar to log Kₑ = 8.62 of the binary species Cu(Put) (Put: abbr. of
putrescine) and $\log K_e = 8.39$ determined for CuH(3,3-tri) species, in which two nitrogen atoms from PA take part in the coordination [49]. It suggests the mode of triamine coordination with Cu(II) ions in a heteroligand complex with formation of a six-membered ring similar to that in a binary complex.

Another complex Cu(Glu)(3,3-tri) is formed according to the reaction: Cu(Glu)$^{2+}$ + (3,3-tri) $\rightarrow$ Cu(Glu)(3,3-tri)$^{2+}$. In the $^{13}$C NMR spectrum recorded at pH 9.5, the changes in positions of the signals assigned to $C_{(1)}$, $C_{(2)}$ and $C_{(3)}$ of Glu are 0.075, 0.024 and 0.118 ppm, respectively, which points to the participation of only oxygen atoms from the two carboxyl groups of Glu in the coordination. The changes in positions of the signals assigned to C(1) and C(3) from PA are 0.065 and 0.065 ppm, respectively, which prove also the oxygen atoms from the carboxyl groups of amino acid and two nitrogen atoms from the PA amine groups are involved in the metallation. Because of the symmetric structure of 3,3-tri, it is difficult to identify which exact amine groups from PA are engaged in the coordination.

### 3.2.2. Cu(II)/Glu/Spd system

Similarly as in the system with 3,3-tri, also in Cu(II)/Glu/Spd, the process of complexation starts with deprotonation of the second amine group from Spd (Fig. 4).

In the pH range from 6.5 to 10.0, the dominant species is Cu(Glu)(H(Spd)). The equilibrium constant of its formation is $\log K_e = 7.41$ (Table 3), which is close to that of CuH(Spd) complex with [2N] type chromophore, $\log K_e = 7.94$ [49], which indicates that in the heteroligand complex H(Spd) is attached to the anchoring Cu(Glu). The positions of d-d band and EPR parameters at pH 8 (Table 4), $\lambda_{\text{max}} = 605$ nm, $g_\text{g} = 2.234$ and $A_e = 184$ for the heteroligand system correspond to the formation of $\{3\text{N}_2\text{O}_2\}$ chromophore. As follows from the analysis of changes in the NMR signals assigned to the carbon atoms localised near the reaction centres, in the $^{13}$C NMR spectrum, the coordination sites in complex Cu(Glu)H(Spd) are the oxygen atoms from the carboxyl group at $C_{(1)}$ and the nitrogen atom from the amine group of Glu (the shifts of signals of $C_{(1)}$ and $C_{(2)}$ Glu are 0.151 and 0.060 ppm, respectively) as well as the terminal amine groups from Spd (the shifts of signals of C(1) and C(7) are 0.100 and 0.430 ppm), which is in agreement with the Vis and EPR results. The mode of interaction in complex Cu(Glu)H(Spd), Scheme 3, shows that the introduction of metal ions to the Glu-Spd metal-free system excludes the interaction between the bioligands. Moreover, the ligand centres that do not take part in the weak noncovalent interactions in (Glu)H(Spd) adduct are involved in the metallation, while those that are involved in ligand1 · ligand2 interactions do not take part in the coordination in the heteroligand complex.

### Table 5: Differences between $^{13}$C NMR chemical shifts for the ligands in the Cu(II)/Glu/3,3-tri and Cu(II)/Glu/Spd systems in relation to the free ligands [ppm].

| Systems       | pH | Glu C(1) | Glu C(2) | Glu C(3) | Glu C(7) | PA C(1) | PA C(2) | PA C(3) | PA C(6) | PA C(7) |
|---------------|----|----------|----------|----------|----------|---------|---------|---------|---------|---------|
| Cu(II)/Glu/3,3-tri | 8.0 | 0.061  | 0.060  | 0.057  | 0.269  | 0.105  | 0.105  | 0.059  | 0.105  | 0.105  |
|               | 9.5 | 0.075  | 0.024  | 0.017  | 0.065  | 0.026  | 0.026  | 0.065  | 0.026  | 0.026  |
|               | 10.5 | 0.598 | 0.078  | 0.166  | 0.178  | 0.427  | 0.458  | 2.790  | 0.178  | 2.790  |
| Cu(II)/Glu/Spd | 8.5 | 0.151  | 0.060  | 0.057  | 0.008  | 0.040  | 0.090  | 0.430  | 0.100  | 0.430  |
|               | 9.5 | -      | 0.756  | 0.057  | 0.081  | 0.102  | 0.085  | 0.007  | 0.082  | 0.007  |
|               | 10.5 | -     | 0.308  | 0.196  | 0.127  | 0.222  | 0.227  | 0.124  | 0.178  | 0.131  |
The complex Cu(Glu)(Spd) begins to form above pH 7.5 and at pH 9.5 it binds about 60% of Cu$^{2+}$ ions. The Vis and EPR parameters (Table 4) obtained for the complex, indicate formation of $\{3N,O\}$ chromophore. An example of a EPR spectrum of the studied sample is shown in the Fig. 5 together with simulated spectrum (dashed line).

The spectrum is characteristic of Cu(II) ion in axially elongated octahedral or square planar geometry. The low field lines are quartet of hyperfine lines from Cu$^{2+}$ ($I=3/2$) with hyperfine splitting $A_{\perp}$. In high field region two strong lines are present. The EPR parameters depend of course on the geometry of the complex. However, according to our experience [32,52], a clear relation between their values and the number of donor atoms involved in coordination is also observed. Moreover, the $g_{\perp}$ values are typical for Cu(II) binding via nitrogen and oxygen atoms and are lower than these for Cu(II) - oxygen coordination [56].

The signals assigned to C(1), C(3), C(4) and C(7) from Spd are shifted with respect to their positions in the free ligand spectrum by 0.085, 0.050, 0.082 and 0.023 ppm (Table 5). It means that only the terminal –NH$_2$ group at the shorter chain and the secondary -NH group of Spd are involved in the coordination forming a six-membered ring. This conclusion is in full agreement with the equilibrium analysis. The equilibrium constant of formation of this species, characterising the attachment of Spd to the anchoring complex Cu(Glu), is $\log K_e = 9.48$ and is lower than $\log K_e = 11.70$ of the complex Cu(Spd) ($\{3N\}$ chromophore [49]) and similar to $\log K_e = 9.68$ of the binary form Cu(tn) [49], in which two nitrogen atoms from 1,3-diaminopropane take part in the coordination with Cu(II) ions and a six-membered ring is also formed. Moreover, the changes in positions of the signals assigned to C$_{\text{11}}$, C$_{\text{22}}$ and C$_{\text{45}}$ from Glu in the $^{13}$C NMR spectra (Table 5) prove that in the complex Cu(Glu)(Spd), oxygen atoms
from both carboxyl groups and the nitrogen atom from the amine group of this ligand take part in metallation.

At pH 10.5, the Vis and EPR parameters (Table 4) obtained for Cu(Glu)(Spd)(OH), indicate the formation of \{3N,O\} chromophore, in contrast to the result for the corresponding system with 3,3-tri. Moreover, analysis of the \(^{13}\)C NMR spectrum of this species reveals changes in positions of the signals assigned to C\(_{13}\), C\(_{12}\) and C\(_{10}\) of Glu and C(1), C(3) and C(4) of Spd (Table 5) with respect to their positions in the spectrum of the free ligand. These changes suggest that besides OH group, also the oxygen atoms from both carboxyl groups and the nitrogen atom from –NH\(_2\) group of Glu and two nitrogen atoms from the amine groups at the shorter Spd chain take part in the metallation.

4 Conclusions

The main centres of interactions in the adducts forming in the systems of Glu-triamine are the oxygen atoms from carboxyl groups and the nitrogen atom from the amine group of Glu and the nitrogen atoms from the amine groups of triamine. Moreover, these centres are also potential sites of metal ions coordination. At lower pH in the metal-free systems, the terminal amine groups of the two triamines studied, the oxygen atoms from –C\(_{15}\)OO and the amine group from Glu are not engaged in the weak noncovalent interactions between ligands, in contrast to the situation in the system Asp-triamine [43]). Thus, not only the length of the polyamine carbon chain [42-44] but also the length of the amino acid carbon chain influences the interactions between the bioligands. In the adducts forming above pH 7, all available active centres of the ligands are involved in the interaction and the inversion effect is observed, similarly as in the system of Asp-triamine [43]). The amine groups of the polyamine could act either as positive or negative centres of interaction, depending on pH (the character of interaction depends on the degree of protonation). In the system Cu(II)-triamine the following species are formed: MLHL', MLL' and MLL'OH (where L = Glu, L' = triamine). In contrast to the situation in the systems with a shorter chain Asp [42,43], no formation of ML-L' type molecular complexes was observed, with polyamine in the outer coordination sphere engaged in noncovalent interactions with the anchoring binary complex ML. In the monoprotonated species, the coordination of 3,3-tri and Spd to copper(II) ions is of the same character, but in disparity to the 3,3-tri species, in the complex Cu(Glu)H(Spd) the oxygen atoms from –C\(_{15}\)OO of the amino acid are not engaged in the metallation.

In the species MLL' and MLL'OH, forming in the system with 3,3-tri, the type of chromophore is different than in the analogous species with spermidine, because of no participation of the amine group from the amino acid in the coordination. It was observed, the introduction of metal ions to the Glu-Spd metal-free system excludes the interaction between bioligands and only these centres which do not take part in weak interactions in (Glu)H(Spd) adduct are involved in the metallation, while those which take part in the above interactions are not engaged in the metal bonding.

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