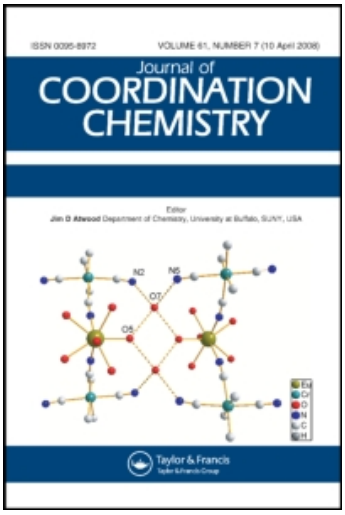

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Equilibrium models of Cr^{3+} and Cu^{2+} with glutamate

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Speciation diagrams and stability constants for glutamate (Glu) with (Cr^{3+}) and (Cu^{2+}) in aqueous solutions are presented. The current study covers a larger pH-range affording accurate results, and reveal a different set of species for Cu^{2+} and species not previously reported for Cr^{3+}. For the Cu^{2+} : Glu system, the most successful model that refined the potentiometric data contains the simple one-to-one complex, the *bis*-complex and the mono-hydroxo complex. The overall stability constants for Cu^{2+}–Glu complexes have respective values of $\log\beta_{110} = 7.6 \pm 0.2$, $\log\beta_{11\text{-}1} = 1.3 \pm 0.7$, $\log\beta_{120} = 13.6 \pm 0.2$. Attempts to refine the stability constant for the mono-protonated metal complex ($\log\beta_{111}$) that was reported in the literature indicated that this mono-protonated species did not form to an appreciable amount to be important for the model presented here. For the Cr^{3+} : Glu system, the overall stability constants for the complexes formed have the values of $\log\beta_{110} = 8.34 \pm 0.03$, $\log\beta_{11\text{-}1} = 1.9 \pm 0.1$ and $\log\beta_{11\text{-}2} = -4.6 \pm 0.1$. These results for Cr^{3+} system covers wider pH-range and have more accuracy than those reported previously. The NMR experiments for Glu revealed downfield shifts of all protons as pH values decrease from 11.21 to 2.85.

Keywords: Glutamate; Chromium; Copper; Equilibrium constants; Hydrolytic species; Speciation diagram; UV–Vis

1. Introduction

We initiated this study, in part, to better understand how trivalent chromium (Cr^{3+}) interacts with the glutamate residue in a peptide that forms what is known as Low Molecular Mass Chromium (LMMCr) complex [1, 2]. Although there are many studies regarding essential Cr^{3+} [1–18], the isolation and characterization of LMMCr has not been achieved, and thus its precise structure is still unknown [1, 2]. This is one of the reasons we have been interested in the interaction of Cr^{3+} with low-molecular-mass ligands [3, 4, 7]. The LMMCr has been suggested to contain four Cr^{3+} centers with an oligopeptide composed of glycine, cysteine, aspartic acid and glutamic acid (Glu), with

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the carboxylate side-chain amino acids comprising more than half of the total amino acid residues.

In contrast to chromium, copper exists in the +1 and +2 oxidation states with +2 being the most stable. Divalent copper (Cu^{2+}) is an essential cofactor in redox enzymes and hemocyanins [19]. In an adult human, there are about 280 milligrams copper and about 3 milligrams of chromium [20]. In mammals, Glu is a known neurotransmitter [21]. It has been established that the amino groups from many α-amino acids are converted in the liver to the amino group of glutamic acid [22, 23]. Stability constants of Cr^{3+} with Glu have been reported [24–26]. These studies disagree about models of the Cr^{3+}–Glu binary system in aqueous solutions.

Potentiometric titrations for Glu complexes of UO_2^{2+}, Cu^{2+}, Cr^{3+}, Fe^{3+}, La^{3+}, Nd^{3+}, Pr^{3+}, Zr^{4+} and Th^{4+} within the ~3.0 to ~9.0 pH-range (25°C, ionic strengths (I) of 0.1 M) examined reaction of Glu with Cu^{2+} and Glu with Cr^{3+} [24]. Their model for Cr^{3+} and Glu only included the Cr(Glu) one-to-one complex with a $\log\beta_1$ value of 9.7 [to be consistent with the literature, Cr(Glu) refers to the glutamic acid dianion with overall 2-charge. Similarly, HGlu refers to glutamic acid anion with overall 1-charge]. Their model for Cu^{2+} and Glu included the Cu(Glu) one-to-one complex and $Cu(Glu)_2$ with $\log\beta_1$ values of 8.23 and $\log\beta_2$ 14.28 [24].

Cr^{3+} with Glu at 50°C ($I = 0.1$ M) in the pH-range of 2.0 to 4.3 showed different results from reference 24 [25]. The authors prepared a speciation diagram for the ternary Cr^{3+}–Cys–Glu system but not for the binary Cr^{3+}–Glu system [25]. Their model fit the titration data with the following species: Cr(Glu), Cr(H)(Glu), $Cr(Glu)_2$ and $Cr(H)(Glu)_2$ with the $\log\beta$ values of 11.39, 14.04, 18.96 and 23.91, respectively [25].

Another group [26] also studied Cr^{3+} with Glu, but at 25°C and ($I = 0.1$ M) and reported the same species Cr(Glu), Cr(H)(Glu), $Cr(Glu)_2$ and $Cr(H)(Glu)_2$ with $\log\beta$ values of 11.79, 14.58, 19.46, and 24.19, respectively; however the speciation model pH-value range was 2.5 to 4.5 [26].

We report careful potentiometric titrations and speciation diagrams for Glu individually with Cu^{2+} and Cr^{3+} over a broad pH-range. This has led to the discovery of new hydrolytic species; we report UV–Vis spectra and NMR studies.

2. Experimental

2.1. *Materials and equipments*

Analytical-reagent-grade L-(+)glutamic acid (Fisher Scientific 99% purity), Glu was received in its zwitterionic form, chromium nitrate nonahydrate, $Cr(NO_3)_3 \cdot 9H_2O$ (Fisher Scientific 99%), and copper sulfate pentahydrate $Cu(SO_4) \cdot 5H_2O$ (Fisher Scientific 98%), were used without purification. The pH-values of all solutions were adjusted using ~0.1 M sodium hydroxide solution. The pH values were measured using an Orion pH electrode model 720A+ connected to an Orion pH-meter. The ionic strength of all solutions was adjusted to 0.1 M by using 1.0 M $NaNO_3$ solution. D_2O ACROS grade, with 99.8% purity, was used in the preparation of the NMR-samples. The Dowex 50 × 8-100 ion exchange resin was nitrogen purged. Doubly de-ionized (D.I.) water was used to prepare all solutions.

2.2. *Standardization of the stock metal solutions*

All stock metal solutions were standardized by eluting a known volume (typically 1.0 mL) through the Dowex 50X8-100 resin packed in a 7×1 inch glass column and titrating the eluant with a standard NaOH solution. The stock metal ion concentrations were in the range of 0.05 M. Typically, seven to nine runs were averaged.

2.3. *Potentiometric titrations*

In metal-ligand potentiometric titrations, the NaOH solution was always the titrant. The methods used to prepare, standardize and prevent contamination of the titrant with atmospheric CO_2 have been described elsewhere [3, 4, 7, 27]. In a typical titration, 2.0 mL of 0.050 M Glu solution was added to a 100.0 mL volumetric flask, then 2.0 mL of stock Cu^{2+} or 2.0 mL Cr^{3+} solution and 10.0 mL of 1.0 M $NaNO_3$ solution were added before dilution to the mark with D. I. water. Before each titration, the metal-Glu mixtures were allowed to stir for 20 min to reach equilibrium. The NaOH solution was added in 100 μL increments using an Eppendorf micropipette with continuous stirring. The time intervals between additions of the NaOH solution were set to 3 min, which was sufficient to get each of the pH values stabilized and reach complete equilibrium. Typical titration experiment took about 5–6 h to be completed. Each potentiometric titration was performed in triplicate.

2.4. *Least-squares fitting*

The potentiometric data were analyzed by a nonlinear least-squares program using a customized version of the nonlinear least-squares program ORGLES from Oak Ridge National Laboratory [28]. In this program, each potentiometric data set was defined by one particular model, which is a set of proposed species that existed in solution under equilibrium. Each species was defined with the appropriate overall stability constant β. The overall stability constant β is expressed in its logarithm value. This program has been used successfully in several studies [27, 29, 30].

2.5. *^{1}H-NMR experiment*

All ^{1}H-NMR spectra were acquired at room temperature using 5 mm OD NMR sample tubes and deuterium oxide to lock and shim the sample on a JEOL 270 MHz NMR spectrometer. Since the Cr^{3+} and Cu^{2+} complexes are paramagnetic, their ^{1}H-NMR spectra could not be obtained. The solution pH was corrected by using equation (1) [31].

$$\mathrm{pD} = \mathrm{pH}_{(\text{meter reading})} + 0.40 \tag{1}$$

2.6. *UV–Vis spectrophotometer measurements*

All UV–Vis spectroscopy was conducted using the T60 high performance spectrophotometer in connection with UVWIN software version 5.0, both purchased from Advanced ChemTech., Louisville, KY. Samples were prepared in D.I. water at 25°C.

The entire UV–Vis spectrum was scanned from 200 nm to 1100 nm using quartz cuvettes with optical path length of 1 cm. Reference cuvettes were used with all measurements and filled with an equal volume of D.I. water.

3. Results and discussion

3.1. *System standardization and Glu pKa values*

A standard phosphoric acid solution (H_3PO_4) was titrated to calibrate the whole potentiometric titration system before gathering the actual potentiometric titrations for either free Glu or the metal ion : Glu reaction system in different molar ratios. From figure 1, it is clear that when the free Glu was titrated without metal ion, it behaved as an H_2L ligand where two protons were titrated from the side-chain carboxylic acid and the ammonium groups. The pKa values of these groups are given in table 1. pKa values are in agreement with the corresponding literature pKa values, considering the many variables involved in the experimental set-up from lab to lab such as the sensitivity and accuracy of the pH-meter, the pH-electrode used, the calibration performed before each titration, the quality of the D.I. water, the operating temperature, and the ionic strength of the final solution [6, 24–26].

Table 1 also catalogues the stability constants measured for Cu^{2+} with Glu. Values for the overall stability constants are given in the form of $\log \beta$ for the equilibrium given in equation (2) and defined by equation (3). The stability constant for formation of the

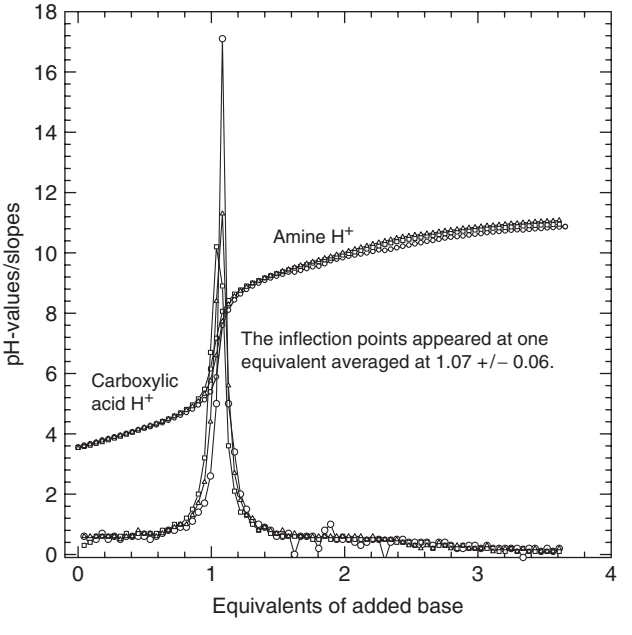

Figure 1. Titrations of free Glu with number of equivalents of added base along with slopes. The sharp inflection separates the carboxylic acid proton from the amine proton. Independent triplicate experiments are represented by circles, squares and triangles.

simple one-to-one complex is $\log_{110}$. The first index in the complex designation stands for the number of metal ions, the second stands for the number of Glu, and the third is that of protons. The hydroxide stoichiometry is designated as −1 in the nomenclature of the metal complexes. This mean that the 111 complex can be re-written as Cu(Glu)(H), the 110 complex can be re-written as Cu(Glu), and the 11-1 complex as Cu(Glu)(OH).

Figure 2 shows the speciation diagram of the free Glu derived from the potentiometric titrations shown in figure 1. This speciation diagram was generated by using the Hyss program [32] with pKw = 13.781 [33]. It is clear from figure 2 that the intersections of the individual plots of the free Glu species are the exact pKa values shown in table 1.

$$i(\mathrm{M}) + j(\mathrm{Glu}) + k(\mathrm{H}) \rightarrow [(\mathrm{M})_i(\mathrm{Glu})_j(\mathrm{H})_k] \tag{2}$$

$$\beta_{ijk} = \frac{[\mathrm{M}_i(\mathrm{Glu})_j\mathrm{H}_k]}{[\mathrm{M}]^i[\mathrm{Glu}]^j[\mathrm{H}]^k} \tag{3}$$

Table 1. pKa values of Glu and stability constants of the Cu^{2+} : Glu complexes.

Glu			Cu^{2+} : Glu			
pKa1	pKa2	pKa3	$\log_{110}$	$\log_{111}$	$\log_{11\text{-}1}$	$\log_{120}$
2.16	4.15[a]	9.58	8.32	2.89	b	14.92
	4.21 ± 0.08[c]	9.2 ± 0.1[c]	7.6 ± 0.2[c]	d	1.3 ± 0.7[c]	13.6 ± 0.2[c]
b	4.15[e]	9.20[e]	8.23[e]	b	b	14.28[e]
2.32[f]	4.22[f]	9.12[f]				
2.30[g]	4.53[g]	9.97[g]				

[a]Ref [6]; [b]not reported; [c]this study, 25°C, $I = 0.1$ M $NaNO_3$; [d]not observed; [e]ref [24]; [f]ref [25]; [g]ref [26].

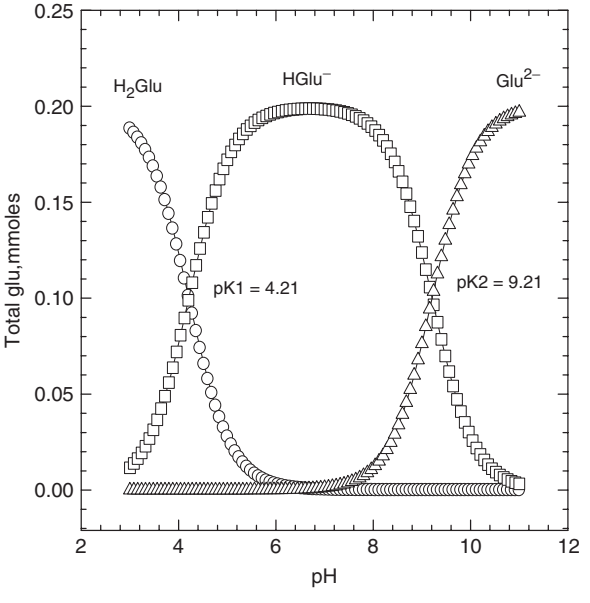

Figure 2. Speciation diagram of free Glu. The diagram was constructed using the Hyss program [32]. The cross points are the exact pKa-values for Glu.

3.2. Cu^{2+} : Glu system

Potentiometric titrations of the Cu^{2+} : Glu systems have been conducted in 1 : 1, 1 : 2 and 1 : 3 molar ratios. Figure 3 is a representative graph of the 1 : 3 Cu^{2+} : Glu titration in triplicate. In potentiometric titrations, the presence of a sharp inflection point indicates the formation of a single dominant species, since several species forming at different pH would result in a gradual change (like that observed before or after the inflection point). The position of the inflection point indicates the number of protons released via the formation of this dominant species [27, 30, 34, 35]. Table 2 shows the inflection point data for these titration curves. By using equation (4) for the simple one-to-one complex at 298 K, one can calculate the electrostatic binding energy of Cu^{2+} to Glu in aqueous solution at room temperature.

$$\Delta G^{\circ} = -RT \ln K_{\text{eq 1:1}} \tag{4}$$

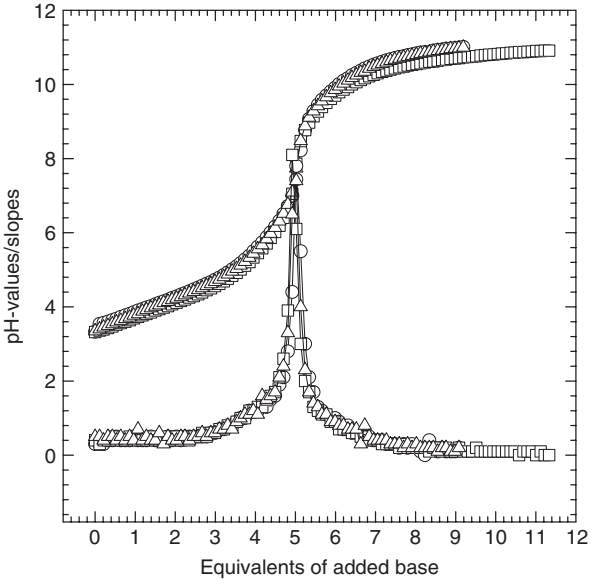

Figure 3. Potentiometric titration curves of Cu^{2+} : Glu in 1 : 3 molar ratio. Independent replicate experiments are represented by circles, squares and triangles.

Table 2. Inflection points for titrations of different Cu^{2+} : Glu molar ratios[a].

	Inflection point (eq NaOH)			
Molar ratios	0 : 1	1 : 1	1 : 2	1 : 3
Run #1	1.10	3.05	4.40	5.40
Run #2	1.00	3.05	4.30	5.30
Run #3	1.10	3.27	4.40	5.40
Average	1.07	3.12	4.37	5.37
St. dev. σ	0.06	0.13	0.06	0.06

[a]25°C, $I = 0.1$ M $NaNO_3$.

Substituting 7.64 from table 1 for $K_{eq\,1:1}$ of Cu(Glu) obtained ($8.315\,J\,K^{-1}\cdot mol$) (298 K) ($\ln 10^{7.64}$) $= -43.6\,kJ\,mol^{-1}$. This value lies in the expected range (20–70 $kJ\,mol^{-1}$) for most electrostatic interactions of low molecular weight ligands with divalent metal ions in aqueous solution under physiological conditions [21–23, 34–36].

No visible precipitates were observed for any of the Cu^{2+} : Glu titration experiments. The 1 : 3 titration curves showed well-defined and extended buffer regions between pH $\approx$ 3.5–6.5 (figure 3). The initial buffer regions were terminated with sharp and well-defined inflection points at 5.37 ± 0.06 equivalents. The titration continued to pH $\approx$ 11.50. Various models were taken into consideration to refine the potentiometric data. It appeared that the most successful model was the one that contained the stability constant values of the following species ($\log_{110}$, $\log_{11\text{-}1}$, and $\log_{120}$) based on the criteria of the goodness of fit that was described elsewhere [27, 30]. In this model, the pKa values of Glu were averaged from the least-squares refinement for the free Glu titrations. This model was used to refine the analysis of 1 : 1, 1 : 2 and 1 : 3 titration experiments.

A different model initially used $\log_{111} = 2.90$ for the mono-protonated complex stability constant along with the combination of $\log_{110}$, $\log_{11\text{-}1}$ and $\log_{120}$ mentioned above; the least-squares refinement did not converge rejecting the initial value of this species, indicating that it is a minor species. Other attempts to refine a different model with a reasonable stability constant value (such as 15–17) for a tri-nuclear species ended the least-square program in divergence and rejecting its presence.

To further confirm that the species present in the proposed model are the dominant species, figure 4 was constructed using the Hyss program [32]. It clearly shows that the $Cu(Glu)_2$ species is dominant in solution from pH $\sim$ 6 to 10. The monohydroxo

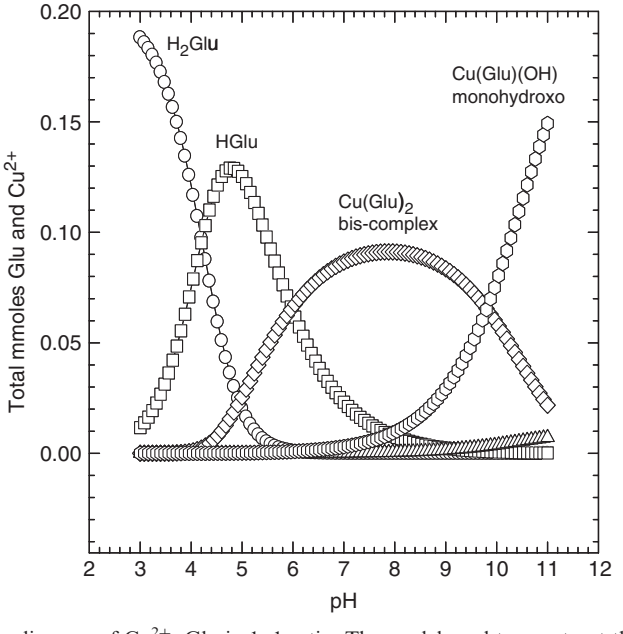

Figure 4. Speciation diagram of Cu^{2+} : Glu in 1 : 1 ratio. The model used to construct the diagram includes Cu(Glu), $Cu(Glu)_2$ and Cu(Glu)(OH). The mono-protonated CuH(Glu) complex was found to be less than 1% (Δ).

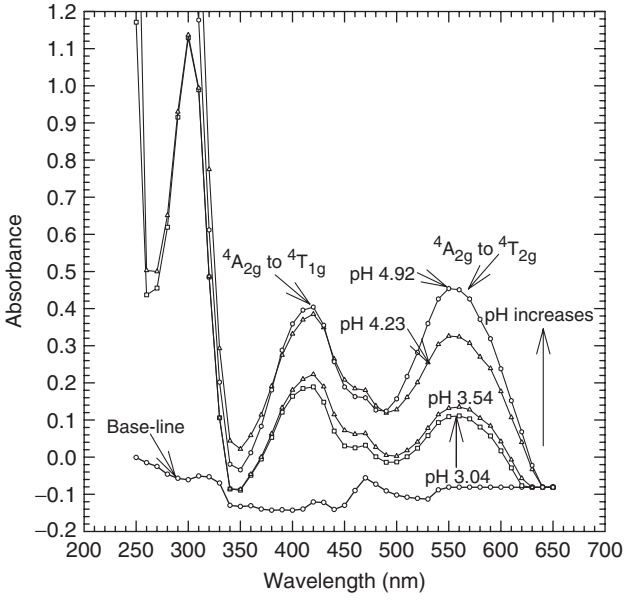

Figure 5. UV–Vis absorption spectra of Cr^{3+} : Glu in 1 : 2 ratio. $[Cu^{3+}] = 0.020$ M in 0.1 M ionic strength.

complex or Cu(Glu)(OH) species was formed to about 70%, while Cu(Glu) formed to about 2% between pH 4 and 5. The Cu(Glu)H was formed to less than 1%. The small contributions of the latter two species are not apparent in figure 4.

3.3. *Cr^{3+} : Glu system*

Figure 5 shows the UV–Vis absorption spectra of the Cr^{3+} : Glu system in a 1 : 2 molar ratio with 0.1 M $NaNO_3$ to match the conditions of the potentiometric experiments. The characteristic peaks of the Cr^{3+} ion at 420 nm and 555 nm were assigned to the $^4A_{2g}$ to $^4T_{1g}$ and $^4A_{2g}$ to $^4T_{2g}$ electronic transitions, respectively. The intense peak at 300 nm was assigned to the nitrate ion. These assignments are in excellent agreement with Tanabe–Sugano diagrams, and they have been confirmed elsewhere [7, 35].

Before titrating the Cr^{3+} : Glu systems, we titrated a 6.97×10^{-4} M hexa-aqua Cr^{3+} solution. Figure 6 is a representative potentiometric titration graph of the free Cr^{3+} ion. It appears that there are two inflection points, one with a minor value of one equivalent and one with a major slope that appeared at three equivalents. For the hexa-aqua Cr^{3+} titration, the inflections were always at 1.0 and 3.0 equivalents for the minor and the major slopes, respectively. The molar ratios reported in table 3 were based on the titrations of 2.0 mL 0.03485 M Cr^{3+} mixed with 2.0, 3.0, 4.0 and 5.0 mL of Glu of 0.050 M.

Figure 7 is a representative graph of potentiometric titrations in triplicate for the Cr^{3+} : Glu in 1 : 3.59 molar ratio. Superimposing the graphs proves that the data are reproducible. Table 3 is the summary of all Cr^{3+} : Glu titration runs. It is obvious from figure 7 and table 3 that there are more equivalents of protons released when Glu is mixed with Cr^{3+} compared to that from free Cr^{3+} ion. The obvious source of the extra

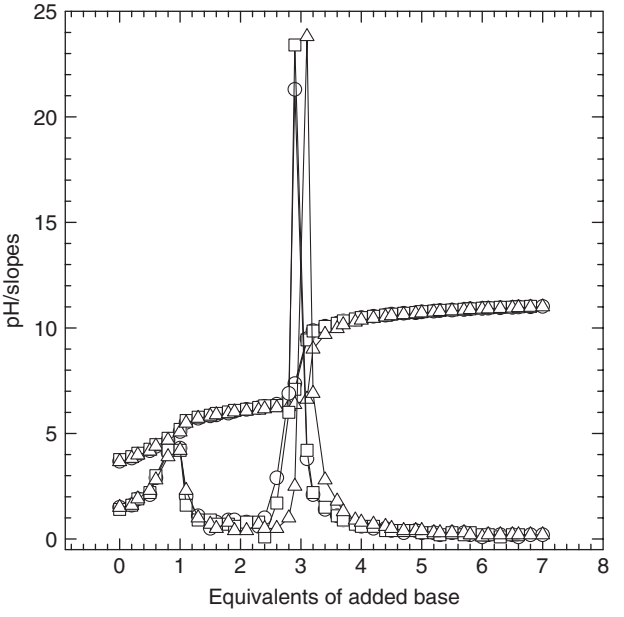

Figure 6. Potentiometric titration curves of 6.97×10^{-4} M hexa aqua Cr^{3+} in 0.1 M $NaNO_3$. The major inflection appeared at 3 equivalents indicating the hydrolysis of the metal ion via loss of three protons (see figure 7 for comparison).

Table 3. Inflection points for Cr^{3+} : Glu titrations in different ratios[a].

	Minor inflection point (Eq NaOH)		Major inflection point (Eq NaOH)	
Cr^{3+} : Glu	Average	σ	Average	σ
1 : 0	1.00	0.06	3.00	0.06
1 : 1.43	2.30	0.42	4.35	0.07
1 : 2.15	3.20	0.00	5.07	0.12
1 : 2.87	4.27	0.23	6.03	0.31
1 : 3.59	5.10	0.11	6.70	0.12

[a]All measurements are in triplicate at 25°C and 0.1 M $NaNO_3$.

equivalents of protons is the carboxylic acid and the amine groups of the Glu ligand. In contrast to the Cu^{2+} : Glu titration system, the most successful model that refined the potentiometric titration data of the Cr^{3+} : Glu system contains Cr(Glu), Cr(Glu)OH, and $Cr(Glu)(OH)_2$. The overall stability constants for these complexes have values $\log \beta_{110} = 8.34 \pm 0.03$, $\log \beta_{11\text{-}1} = 1.9 \pm 0.1$ and $\beta_{11\text{-}2} = -4.6 \pm 0.1$. Table 4 is the summary for these data. To the best of our knowledge, these values of the hydrolyzed species have not been reported previously. Figure 8 is the speciation diagram of the Cr^{3+}–Glu titration system in 1 : 1 ratio. The model used to fit the titration data contained Cr(Glu) formed ~23% between pH 4 and 6, Cr(Glu)(OH) formed ~25% between pH 6 and 8 and $Cr(Glu)(OH)_2$ formed ~99% above pH 8. By using equation (4), one may calculate the electrostatic binding energy for Cr(Glu) = $(8.315\,J\,K^{-1}\,mol)(298\,K)(\ln 10^{8.34}) = -47.6\,kJ\,mol^{-1}$.

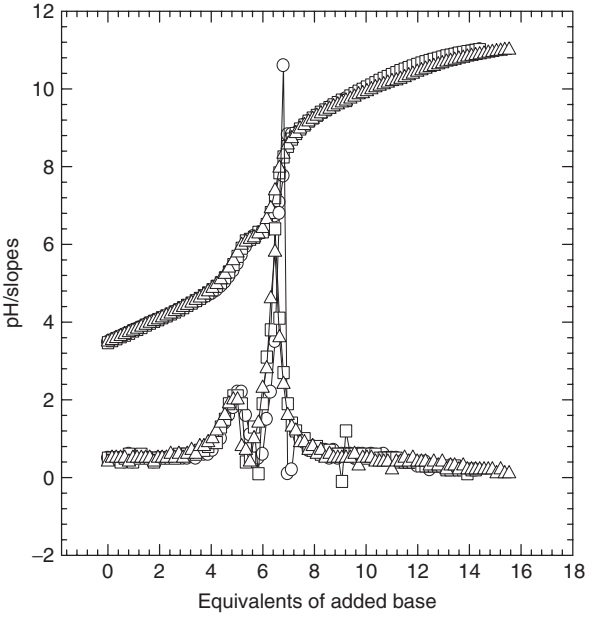

Figure 7. Potentiometric titrations of Cr^{3+}:Glu in 1:3.59 molar ratios in 0.1 M $NaNO_3$. The major inflections were averaged at 6.70 +/− 0.12 (see figure 6 for comparison).

Table 4. Stability constants of the Cr^{3+}:Glu complexes[a].

Species/Ref.	$\log_{111}$	$\log_{110}$	$\log_{11\text{-}1}$	$\log_{11\text{-}2}$	$\log_{121}$	$\log_{120}$	Remarks
This study[a]	b	8.34 ± 0.03	1.9 ± 0.1	−4.6 ± 0.1	b	b	pH range 3 to 11
Ref. 24[a]	c	9.70	c	c	c	c	pH range 2 to 9
Ref. 25[d]	14.04	11.39	c	c	23.91	18.96	pH range 2 to 4.3
Ref. 26[a]	14.58	11.79	c	c	24.19	19.46	pH range 2 to 4.3

[a]25°C $I = 0.1$ $NaNO_3$; [b]not observed; [c]not reported; [d]50°C $I = 0.1$.

3.4. *^{1}H-NMR spectra*

The ^{1}H-NMR spectra of Glu in D_2O were recorded at pH 2.85, 4.44, 7.90 and 11.21. A representative ^{1}H-NMR spectrum of 0.050 M Glu in D_2O at pH 4.44 is included in the supplementary figures. All CH proton signals shifted downfield as the pH decreased. For example in figure 9, the triplet of the β methylene group shifted from about 2.20 to 2.55 ppm when the pH decreased from 11.2 to 2.9. The shift in the peaks is consistent with addition of protons and loss of electron density near the methylene protons.

4. Conclusion

Previous studies of stability constants for the Cr^{3+}–Glu were only between pH 2.0 and 4.5 [25, 26] and did not report the presence of any of the hydrolytic species [24–26].

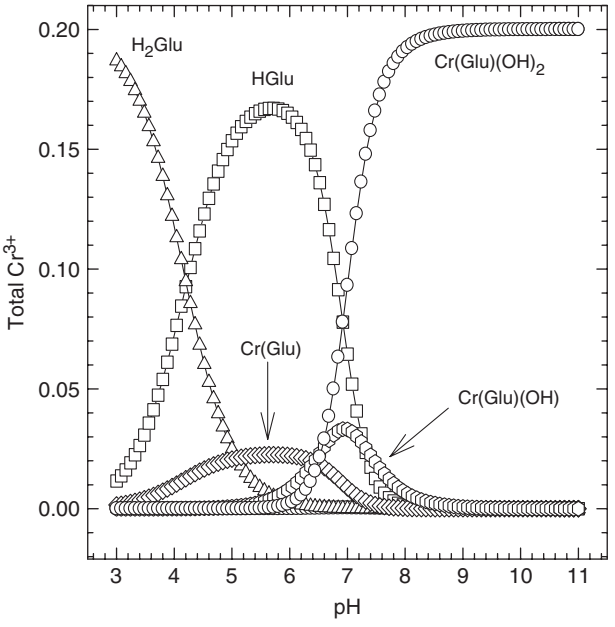

Figure 8. Speciation of Cr^{3+} : Glu in 1 : 1 ratio. The model includes CrGlu, Cr(Glu)(OH) and $Cr(Glu)(OH)_2$.

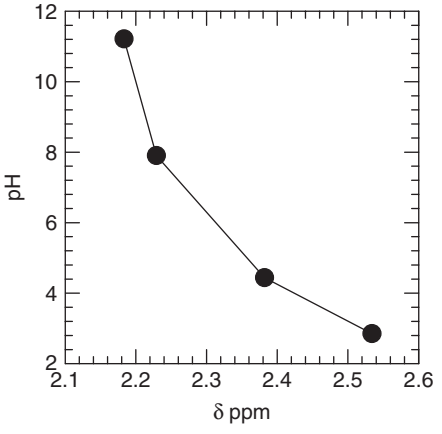

Figure 9. pH dependence of proton-NMR chemical shifts for glutamic acid β-methylene protons.

In contrast, our results reveal two new hydrolytic Cr^{3+} species: Cr(Glu)OH and $Cr(Glu)(OH)_2$. Furthermore, from the potentiometric titration data, we were able to measure an internally consistent set of stability constants for both the Cu^{2+} : Glu and the Cr^{3+} : Glu reaction systems. The most successful model for the Cu^{2+} : Glu reaction system contained Cu(Glu), $Cu(Glu)_2$ and Cu(Glu)OH. A model that included values Cu(Glu)H ($\log \beta_{111}$) or $Cu_3(Glu)_3H$ ($\log \beta_{331}$) was not successful. The most successful model for the Cr^{3+} : Glu system contained Cr(Glu), Cr(Glu)OH and $Cr(Glu)(OH)_2$. Similarly, a model that included a value for the bis-complex $Cr(Glu)_2$ was not successful.

A literature survey indicates that many studies investigated the Cr^{3+} : Glu system without reporting stability constants in aqueous solutions [37–40]. Stability constants are crucial estimates to understanding the stabilities of metal species [6]. The findings in this study are in good agreement with those in the literature [6, 37–41]. A report about Cr^{3+} showed that the hydrolysis of Cr^{3+} derivatives of insulin are rich in glutamic acid [37]. Glutamate is reported to be tri-dentate with Cr^{3+} and coordinated facially via the NH_2 group and the (Oα) and the (Oγ) [39]. We propose a similar coordination of the formed species in this study. The appearance of both the mono- and the di-hydroxo complexes is good evidence that the Cr^{3+} : Glu system undergoes hydrolysis, due to the tendency of hard metal ion such as Cr^{3+} to hydrolyze [11–13, 35, 42–45].

By closely examining the number of protons released from both the Cu^{2+} : Glu and the Cr^{3+} : Glu systems (figure 3, figure 7, table 2, and table 3), one might expect formation of hydrolytic species because more than the two protons of the free Glu were titrated. Based on the values of the stability constants, the increased intensity of the λ_{max} of the UV–Vis absorption spectra with pH, and the change in the chemical shifts of the NMR experiments for free Glu, we can show explicitly the species present in solution in equations (5–9) in which M^{n+} stands for either Cr^{3+} or Cu^{2+}. Equation (9) is only for the Cr^{3+} : Glu system.

$$H_2Glu + OH^- \rightarrow HGlu^- + H_2O \tag{5}$$

$$HGlu^- + OH^- \rightarrow Glu^{2-} + H_2O \tag{6}$$

$$M^{n+} + H_2Glu \rightarrow M(Glu)^{+n-2} + 2H^+ \tag{7}$$

$$M(Glu)^{+n-2} + OH^- \rightarrow M^{n+}(Glu^{2-})(OH^-)^{+n-3} \tag{8}$$

$$M^{n+}(Glu^{2-})(OH^-)^{+n-3} + OH^- \rightarrow M^{n+}(Glu^{2-})(OH^-)_2^{+n-4} \tag{9}$$

Others [46] also suggested the presence of the Cu^{2+}-hydroxo species shown in equation (8), further confirming our proposed speciation diagrams.

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