A New Contribution to the Determination of the Complex Equilibria of Oxidised Glutathione with Proton and Copper(II) in Aqueous Solution†

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The stability constants of the complexes formed between copper(II) and oxidised glutathione have previously been determined. In the present work, using identical experimental conditions, we have essentially confirmed the model previously proposed, above pH 5. However, for the range pH 2—5 we support a later model proposed by other workers which questioned the correctness of the earlier one.

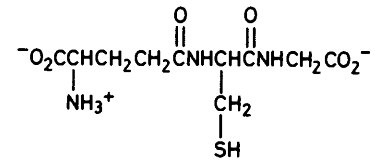
Glutathione (Hglut) is a naturally occurring tripeptide consisting of the sequence γ-L-glutamyl-L-cysteinylglycine. Due to its large number of potential binding sites, its co-ordination chemistry is characterized by the formation of protonated and polynuclear complexes.[1] From the biological point of view, glutathione is considered to be a normal and essential constituent of all living cells [2] and is generally the most abundant intracellular non-protein thiol. Accordingly, it is involved in numerous cellular processes, one of the most important being the protection of cellular membranes and constituents from oxidation by hydroperoxides.[3] In erythrocytes for instance, Hglut is oxidised into glut-glut as it consumes hydroperoxides, and both the reduced and oxidised forms coexist.[1]

These redox properties have recently been mentioned as playing a part in the regulation of the penicillamine : oxidised penicillamine ratio in erythrocytes of rheumatoid arthritis patients on penicillamine therapy.[4] Attention has also been drawn to copper–glutathione interactions in blood plasma, in relation with the use of this metal against the same disease. On this occasion, the protonation constants of glut-glut, as well as the equilibrium constants of its copper(II) complexes, were determined by Micheloni *et al.*[5] but the correctness of the model proposed for the complexation equilibria was questioned by another group of authors [6] in a more recent investigation.

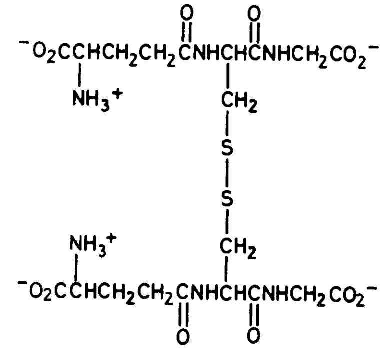
In view of the unfortunate implications of such a discrepancy on the use of these constants for various biological applications, we decided to attempt to resolve this matter. The present paper thus reports the experimental determinations of the equilibrium constants of the complexes formed by glut-glut with proton and copper(II) ions. For the sake of a better comparison, these determinations were carried out under the same conditions of temperature and ionic medium as in the previous study mentioned above.[5]

Experimental

Reagents.—All our experiments were carried out in sodium perchlorate (0.15 mol dm^{-3}), which has already been used by Micheloni *et al.*[5] as a background electrolyte. This salt was purchased from Merck (analytical grade) and was purified as described in a previous study.[7]



Hglut



glut-glut

The copper perchlorate stock solution was prepared by dissolving crystals supplied by the G. Frederick Smith Chemical Co. and was made slightly acid by adding Normatom grade Prolabo perchloric acid, so as to prevent hydrolysis and absorption of carbon dioxide. The metal content of the solution was determined by complexometric titration using ethylenediaminetetra-acetate (edta),[8] the acid concentration being deduced from direct potentiometric readings. Potassium hydroxide solutions were prepared by diluting the contents of BDH concentrated volumetric solution vials with freshly boiled deionised water, which was saturated with nitrogen before use. The titre of these solutions was standardized against Prolabo potassium hydrogenphthalate (analytical grade), and they were proved to be carbonate-free from the features of the Gran titration plots.[9]

Specific difficulties have previously been reported in the purification of oxidised glutathione.[5,6] In particular, the discrepancy concerning the composition of the model proposed for the copper(II)–glut-glut complexation equilibria had been interpreted by Abello *et al.*[6] in terms of an imperfect purification of the oxidised glutathione used by Micheloni *et al.*,[5] this being reflected in the corresponding microanalytical results. The present approach was thus different.

We purchased glut-glut from the Sigma Chemical Co., as Grade III crystalline (substantially ethanol-free) free acid. The purity of the product was expected to be better than 99%. Nevertheless, the microanalysis of this sample revealed the presence of water, as can be seen from Table 1, where the results of the two earlier studies are also reported. It is clear from the examination of the element to carbon ratios that only H and O were affected to a significant extent. Due to the

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† *Supplementary data available* (No. SUP 23321; 12 pp.): full titration data and formation plots.

Table 1. Oxidised glutathione microanalyses

	Analysis (%)					Ratio of percentages			
	C	H	N	S	O	H/C	N/C	S/C	O/C
Calculated for $C_{20}H_{32}O_{12}S_2$ (M = 612.64)	39.2	5.25	13.7	10.45	31.35	13.4	35.0	26.7	79.95
glut-glut Koch-Light (ref. 5)	39.3	5.95	11.85			15.15	30.15		
glut-glut Fluka (ref. 6)	39.45	5.45	13.55	10.25	31.2	13.85	34.35	26.15	79.05
glut-glut Sigma (this work)	37.55	5.45	12.9	10.0	34.1	14.45	34.4	26.65	90.9
Calculated for $C_{20}H_{32}N_6O_{12}S_2 \cdot 1.527H_2O$ (M = 640.15)	37.55	5.55	13.15	10.0	33.8	14.8	35.0	26.6	90.1

Table 2. Summary of the titration data used for calculating stability constants *

System	C_M	C_L	C_H	$-\log$ [H] range
	mmol dm^{-3}			
Proton-glut-glut		3.04	10.46	2.2—10.6
		5.93	16.40	2.0—10.4
		8.83	41.45	1.6—10.3
		11.70	33.10	1.9—10.4
Copper-glut-glut	4.97	3.04	10.86	2.1—4.0
	6.95	3.04	11.07	2.1—3.7
	4.97	5.93	16.84	2.0—9.0
	9.93	5.93	17.27	2.0—3.5
	4.97	8.83	41.89	1.9—9.4
	2.98	8.83	41.71	1.9—9.5
	4.97	11.70	33.54	1.9—9.4

* Initial total concentrations: copper (C_M), ligand (C_L), strong acid (C_H).

purification problems discussed above [5,6] it was decided to determine instead the water content of the sample by titration. Accordingly, a solution made by dissolving a weighed amount of glut-glut in aqueous perchloric acid (Merck; previously standardized) was Gran titrated. The sample was found to contain 1.527 mol of H_2O per mol of peptide. Taking this proportion of water into account in the molecular weight of the product, new theoretical percentages were calculated for each element, as well as for each element : carbon ratio. The comparison of these new theoretical values with the experimental ones resulted in quite a satisfactory agreement (see Table 1). It was thus decided to use the sample of glut-glut without further purification, the water content of any fresh solution being systematically checked in the way above.

Experimental Conditions.—The investigation of the protonation and metal complex equilibria was carried out by measuring the e.m.f.s of an electrochemical cell opposing a Corning glass electrode (g.e.) to a saturated NaCl Ingold calomel electrode (s.c.e.), the arrangement of which was as in expression (1).

$$\text{g.e./glut-glut, } Cu^{2+}, \text{ NaClO}_4 \text{ (0.15 mol dm}^{-3}\text{)/ NaCl (s.c.e.)/}Hg_2Cl_2\text{–Hg} \quad (1)$$

The potentiometric equipment was identical to that previously described.[10] The temperature in the reaction cell was maintained at 37 ± 0.02 °C by circulating thermostatted water through the Ingold cell system. All the work was performed under an atmosphere of purified nitrogen. Every titration carried out in the presence of copper was stopped whenever a steady drift occurred in the mV-meter readings, this denoting the formation of a precipitate in the solution. Table 2 reports the overall initial concentrations of the reactants, along with the pH range pertaining to each titration. The pH symbol actually corresponds to $-\log$[H] throughout this study, the electrode system being calibrated in terms of concentrations.

Calculation Procedures.—The selection of the most appropriate set of constants to account for the complexation equilibria was done through our usual two-step approach. The first step consists of the refinement of the approximate equilibrium constants of the complexes which appear likely to exist from the features of the formation curves. As far as the ligand protonation is concerned, the case is simple, since all the formation curves which express the average number of protons

$$\bar{r} = (C_H + C_L N_{dp} - C_{OH} + [OH^-] - [H^+])/C_L \quad (2)$$

bound to a mol of ligand ($\bar{r}$) as a function of pH for various ligand and strong acid concentrations are superimposed. This indeed means that all the species formed correspond to the LH_r stoicheiometry. In equation (2), N_{dp} stands for the number of dissociable protons of the ligand, C_H, C_L, and C_{OH} representing the total concentrations of strong acid, ligand, and alkali, respectively.

Concerning metal complex equilibria, the difficulty arises from the fact that any stoicheiometry allowed by the coordination chemistry rules is possible among all the $M_qL_pH_r$ species. The stoicheiometric numbers of the actually existing complexes can be estimated from specific graphical integrations, which lead to the calculation of their possible range of existence.[11,12] Such considerations were used by Abello *et al.*[6] in their search for the species formed in the copper(II)-glut-glut system. However, the formation curves which express the average number of ligands bound to a mol of metal ion ($\bar{p}$) as

$$\bar{p} = (C_L - \sum_0^r [LH_r])/C_M \quad (3)$$

a function of the free ligand concentration for the different metal to ligand ratios investigated [equation (3)] can also be used to advantage. As already emphasized,[7] the calculation of *experimental* values of $\bar{p}$ is possible without any theoretical restriction, and is applicable to any complex stoicheiometry. If the complexes formed are of the ML_p type, all the formation curves are superimposed and the experimental definition of $\bar{p}$ strictly corresponds to its theoretical definition.[13] If not, different shapes can be seen: a ' tail ' is characteristic of hydroxy-species,[14] whereas a ' hump ' does denote the formation of protonated species.[15] In the latter cases, advantage is taken from

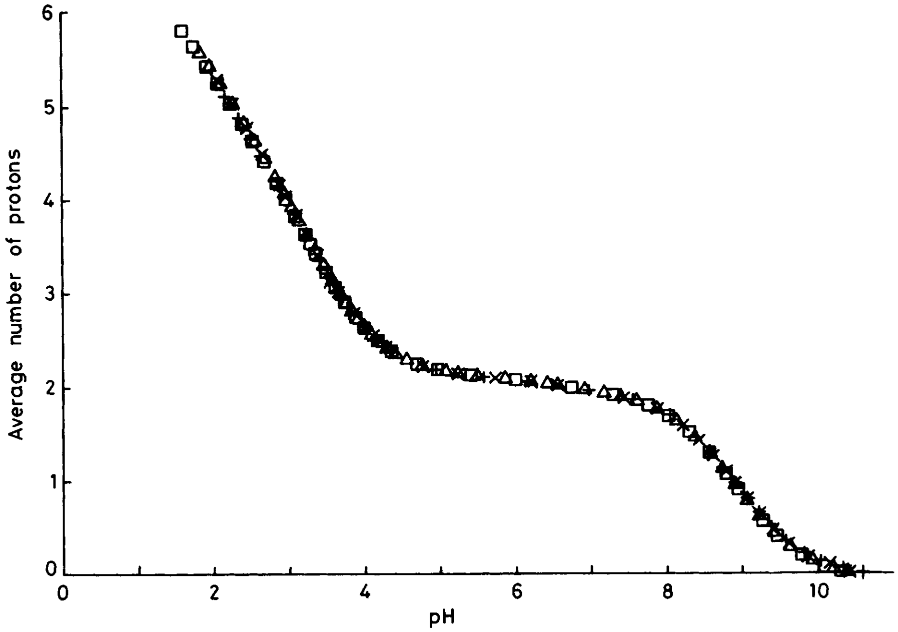

Figure 1. Experimental protonation curves for oxidised glutathione; for the sake of clarity, about every fourth experimental point only has been plotted. C_L,C_H = 3.04, 10.46(+); 5.93, 16.40 (×); 8.83, 41.45 (□); and 11.70, 33.10 mmol dm^{-3} (△)

the peculiar features of the curves in the assessment of the type of complexes formed. The protonation curves obtained in the presence of different concentrations of the metal can also be informative.[16] Once more, we used this kind of approach to estimate the stoicheiometries of the species existing in the copper(II)–glut-glut system.

The refinement of the preliminary constants of every model built up on these considerations was carried out by means of the MINIQUAD program.[17] The comparison of the sum of squares and R factors relative to the various sets of constants examined can throw light on the choice of the ' best ' model, but it frequently occurs that some of these sets appear to be as likely to exist. The use of graphical simulations of the experimental formation curves is then highly desirable to help in the ultimate discrimination.

This kind of simulation constitutes the second step of our approach (see supplementary data). It can be achieved by means of the PSEUDOPLOT program,[18] on the basis of the analytical overall concentrations in equations (2) and (3) together with the equilibrium constants of the model being tested. It is noteworthy that this program has been used by Micheloni *et al.*[5] in their study of the present system.

The graphical comparisons of the complex formation curves are preferable to those of the protonation curves in the presence of the metal, as was done by Abello *et al.*[6] for example. Clearly, the complex formation curve is much more sensitive to the species composition than the protonation curves (see supplementary data). Nevertheless, better to illustrate the differences between the models considered in the present study, both types of curves were used.

Results and Discussion

The protonation curves that we have determined for different ligand and strong acid concentrations (Figure 1) appear to be more superimposable and reproducible than those given by Micheloni *et al.*[5] Clearly this is due to every fresh solution in this work being Gran titrated for strong acid and ligand content.

If we compare our protonation constants to those previously determined under the same experimental conditions,[5] both of these sets have similar orders of magnitude, but much better standard deviations have been obtained for our data (Table 3). In addition to the considerations developed above, this improved accuracy could also originate from the larger pH range investigated (Figure 1).

As far as the copper(II)–glut-glut system is concerned, a number of observations can be made when examining the experimental formation curve (Figure 2). The parallel drawings obtained for the different metal to ligand ratios may be characteristic of the existence of polynuclear species. In addition to this peculiarity, the fact that the bottom of the curves are slightly lifted from the abscissa may indicate the formation of protonated species. Similarly, evidence of a ' tail ' can be seen at the upper part of the curves, the shape of which directly depends on the metal to ligand ratio investigated; the existence of a hydroxy-species of 1 : 1 stoicheiometry should thus also be suspected.

All these observations can be helpful in designing the set of constants to be the most likely to account for the experimental data. However, we began our search of this ' best ' set by considering the models already proposed by the two former groups of authors.[5,6] All the species mentioned by Micheloni *et al.*[5] were refined by MINIQUAD, but, among those given by Abello *et al.*,[6] the constant pertaining to ML_2H_2 was rejected. It is worth noting at this stage that the sum of squares and R factor was found to be slightly better for the model proposed by the latter authors, but regarding the graphical comparisons, neither of the corresponding PSEUDOPLOT curves satisfactorily fitted the experimental one (see supplementary data).

We thus grouped together all the constants composing both of the previous sets to form the next model to be tested. In that case also, the constant of the ML_2H_2 complex turned out to be negative, confirming the non-existence of this species. The addition of ML_2 to the model proposed by Abello *et al.*[6] resulted in significantly better sum of squares and R factor (see supplementaty data), but the ' tail ' of the experimental formation curves was not correctly simulated, in that the drawings

Table 3. Formation constants for the proton- and copper(II)–oxidised glutathione systems *

System	p	q	r	log β	S	R	n	Notes
Proton–glut-glut	1	0	1	9.146 ± 0.006	0.448×10^{-5}	0.004 76	462	Present work
	1	0	2	17.601 ± 0.005				
	1	0	3	21.645 ± 0.011				
	1	0	4	24.834 ± 0.016				
	1	0	5	27.514 ± 0.020				
	1	0	6	29.499 ± 0.020				
Copper–glut-glut	1	1	0	13.369 ± 0.008	0.125×10^{-5}	0.002 45	483	Present work
	1	1	1	17.730 ± 0.007				
	1	1	2	20.664 ± 0.043				
	1	1	3	24.512 ± 0.012				
	2	1	0	15.440 ± 0.066				
	1	2	0	16.009 ± 0.058				
	1	2	1	20.197 ± 0.040				
	1	1	−1	3.368 ± 0.029				
	1	1	0	13.723 ± 0.022			142	Ref. 5
	1	1	1	17.693 ± 0.029				(37 °C, 0.15 mol dm^{-3} NaClO$_4$)
	1	1	2	21.494 ± 0.026				
	2	1	0	17.482 ± 0.086				
	1	2	0	16.063 ± 0.248				
	1	1	0	13.74 ± 0.02			174	Ref. 6
	1	1	1	18.20 ± 0.04				(25 °C, 0.15 mol dm^{-3} KNO$_3$)
	1	1	2	21.55 ± 0.02				
	1	1	3	24.51 ± 0.04				
	2	1	2	34.19 ± 0.08				
	1	2	0	16.26 ± 0.08				
	1	2	1	20.09 ± 0.12				

* General complex formula is Cu$_q$(glut-glut)$_p$H$_r$; n = number of experimental observations, S = sum of squares residuals, R = crystallographic factor (see ref. 17).

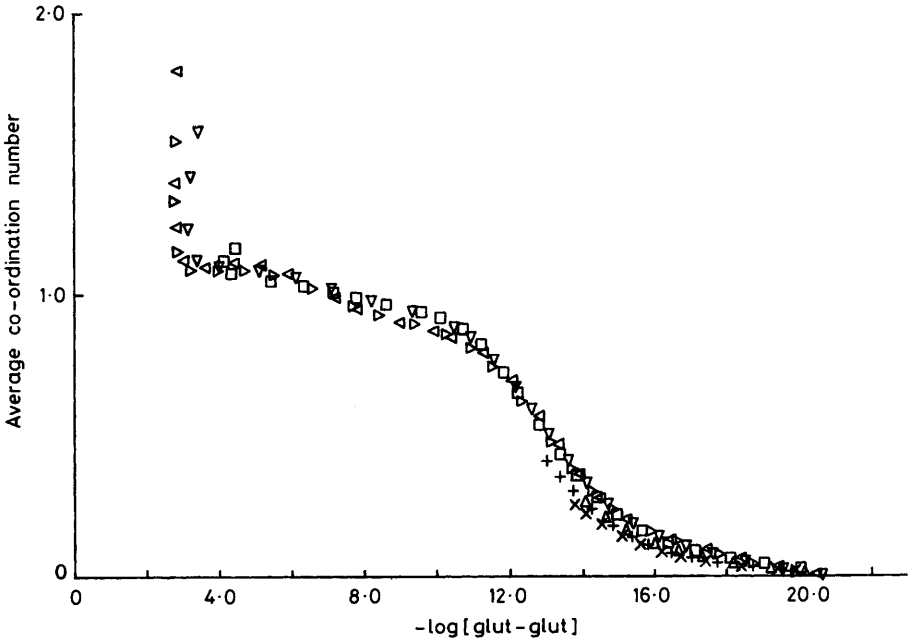

Figure 2. Experimental formation curves of the copper(II)–oxidised glutathione system; every fourth experimental point has been plotted. C_M,C_L = 4.97, 3.04 (+); 6.95, 3.04 (×); 4.97, 5.93 (□); 9.93, 5.93 (△); 4.97, 8.83 (▽); 2.98, 8.83 (◁); 4.97, 11.70 mmol dm^{-3} (▷)

were not split in relation to the different metal to ligand ratios. The species ML(OH) was thus proposed instead. This improved to a significant extent both sum of squares and R factor, as well as the graphical simulations (see supplementaty data). The reintegration of ML$_2$ into this last model still slightly improved the numerical and graphical fits. For this reason, this ultimate model was selected as the ' best ' one, the corresponding results being shown in Table 3 and Figure 3. Finally, it is to be noted that, by analogy with cystine,[10] the possible polymerization of the ML complex was envisaged. But, although the matrix solving tended to favour the highest degree of polymerization offered, the resulting sums of

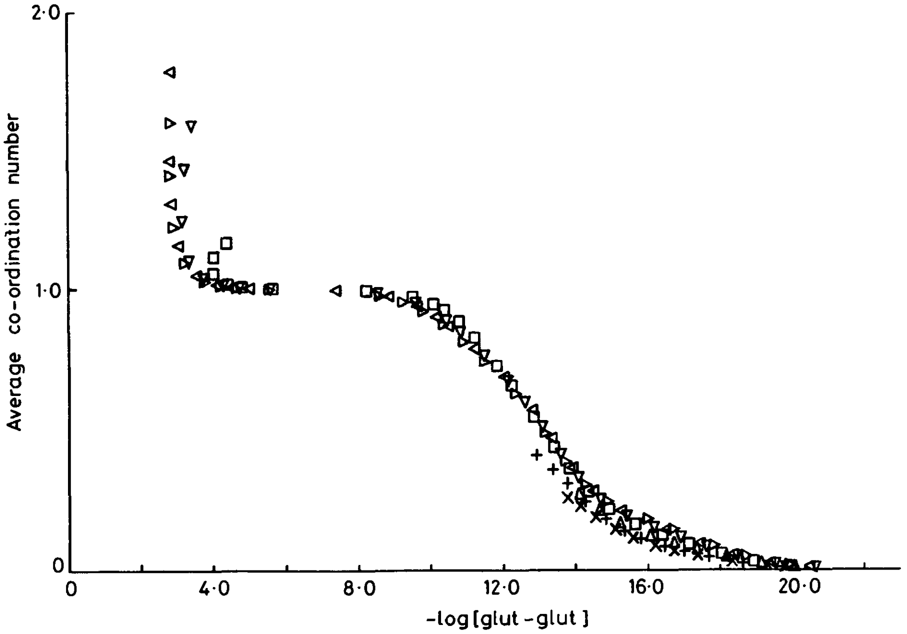

Figure 3. Copper(II)–oxidised glutathione formation curves as obtained from PSEUDOPLOT simulation based on the data in Table 3; key to symbols as in Figure 2

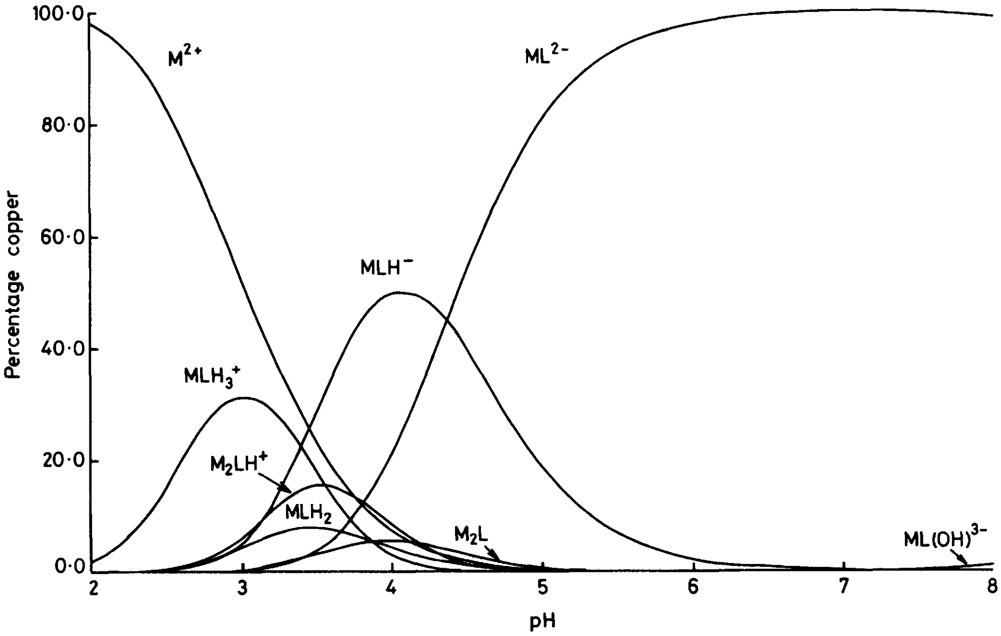

Figure 4. COMICS distribution of the complexes in the copper(II)–oxidised glutathione system for C_{Cu} = 4.00 mmol dm^{-3} and $C_{glut-glut}$ = 4.60 mmol dm^{-3}

squares and *R* factors were worse, as were the graphical simulations (see supplementary data).

In order to visualize the differences between our results and those given by Micheloni *et al.*[5] we have plotted the distribution of the various species in the copper(II)–glut-glut system as a function of pH, using the same overall concentrations of the reactants as these authors. The calculations were run with the help of the COMICS program.[19] The resulting drawings are shown in Figure 4.

Examination of Figure 4 shows that our results confirm those obtained by Micheloni *et al.*[5] only as far as the formation range of the main complex ML is concerned. In fact, the existence of minor species has been established in the present study, which might be of some importance under specific biological conditions. In particular, our characterization of MLH_3 brings about an important change in the complex distribution between pH 2 and 4. The substitution of the latter species for MLH_2 does significantly lower the concentration of electrically neutral complexes, which tends now to become negligible. This result would have to be taken into account for the biological applications envisaged by the above authors.[5]

As for the comparison of our results with those obtained by Abello *et al.*,[6] the only notable difference between the two models lies in the fact that we did not confirm the existence of the ML_2H_2 complex. Their characterization of this species stems presumably from the low selectivity of the SCOGS program,[20] which is known to be less rigorous than MINIQUAD.[7]

The present work did not aim to discuss the possible structures of the complexes in the copper(II)–glut-glut system. The few studies previously devoted to this matter were mentioned in the work of Abello *et al.*,[6] in which the related references can be found.

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