The Complex Formation of Copper(II) with GHL* and HSA*

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The formation constants for complexes of copper(II) with GHL have been determined by means of pH titrations and ESR spectroscopy in aqueous solutions. GHL has an extremely high affinity for copper(II) and forms very stable 1:1 complexes and a comparatively weak 1:2 complex. The ϵ amino group of GHL seems not to be involved in complex formation as can be deduced from both equilibrium constants and ESR spectroscopy. The ternary system copper(II)–GHL–HSA was investigated by ESR spectroscopy and optical absorption spectroscopy in aqueous solution at physiological pH (7.4). At equimolar concentrations, copper(II), HSA and GHL form a ternary complex.

Introduction

GHL is coisolated with copper and iron from human plasma at an amount of about 200 ng/cm^3 [1, 2]. In combination with the transition metals copper, iron and zinc, GHL enhances the growth of cultured hepatoma cells, whereas the addition of these metals without GHL decreases the number of these cells [3]. GHL seems to bind the toxic transition metals in a utilizable form [3]. Pickart and Thaler [3] postulated a mechanism for the copper transport in plasma: GHL should act as a 'low weight' carrier and transfer copper to albumin.

Because of this assumed physiologial role and many other biological functions [4–13], more detailed equilibrium studies of such complexes seemed highly desirable. Another aim of this work was to test the utility of ESR spectroscopy in aqueous solutions of copper(II) [14–16] for the investigation of such systems.

Earlier investigations on copper(II)–GHL complexes have been performed using potentiometric titration [17], optical absorption spectroscopy [17, 18], crystallographic structure determination [19], high resolution NMR spectroscopy [20] and ESR spectroscopy in ethylene glycol–water glasses [18].

The ternary system copper(II)–GHL–HSA has been investigated so far only by means of equilibrium dialysis [17]. In addition to that, some biological investigations have been caried out on this system to reveal the biological importance and the mechanism of action of GHL (reviewed by Pickart [21]).

Experimental

Materials

$CuCl_2 \cdot 2H_2O$ (analytical grade) was dried at 130 °C until the weight was constant. GHL was obtained from Serva as acetate (analytical grade). HSA was obtained from Serva (pure, lyophil.). All solutions were prepared using CO_2 free distilled water. Acid and base were Titrisol products (Merck). All solutions contained 0.1 *M* $NaNO_3$.

Potentiometric Titrations

Titrations were carried out at various metal/ligand ratios ranging from 1:1 to 1:4. The copper chloride concentration was 0.001–0.0025 *M*. The systems were titrated with 0.5 *M* NaOH at 37 °C ± 0.3 °C using a 0.2 cm^3 burette (Gilmont) after adding an appropriate amount of HCl.

ESR Experiments

For the binary system copper(II)–GHL about 7 samples per titration were taken from the titration vessel by micropipette and frozen in liquid nitrogen immediately. The concentration of copper chloride

*Abbreviations: AH = L-histidine-N^2-L-alanyl; BSA = bovine serum albumin; ESR = electron spin resonance; GG = glycylglycine; GH = L-histidine-N^2-glycyl; GHL = L-lysine-N^2-(-N-glycyl-L-histidyl); HSA = human serum albumin; LH = L-histidine-N^2-L-leucyl; NMR = nuclear magnetic resonance; RG = receiver gain.

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was 0.0025 *M*. The titration volume was 3 cm^3. After defrosting the samples, the spectra were recorded at 37 °C.

For the ternary system the concentration of copper chloride was lowered to 0.00125 *M*. pH 7.4 was adjusted with 0.5 *M* NaOH. The ratios metal/HSA/GHL ranged from 1/1/0 up to 1/1/1. GHL was added as a solution using a 0.2 cm^3 burette. The samples were treated as described above.

Optical Absorption Spectra

The copper chloride concentration was 0.0005 or 0.00025 *M*. All measurements were carried out at pH 7.4 and T = 25 °C. The spectra were recorded at various ratios of copper(II)/HSA/GHL (from 1/1/0 to 1/1/1 and from 1/0/1 to 1/1/1), with a cell thickness of 1.0 cm.

Apparatus

For the pH measurements a Schott pH-meter CG 803 and an Ingold electrode calibrated with standard buffer solutions (Merck) were used.

The ESR spectra were recorded on a Varian E 104 spectrometer (calibrated microwave frequency = 9.097 GHz) in tubes with only 1 mm diamter (Wilmad Cat. Nr. 800) in order to reduce the dielectric losses caused by water as solvent. The optical absorption spectra were recorded on a Unicam 1800 Ultraviolet Spectrophotometer linked to a Unicam 1805 programme controller and a Unical AR 25 linear recorder.

Calculations

All calculations were carried out at the CDC Cyber 74 computer of the University of Innsbruck. 20–30 points per titration and 400 points per ESR spectrum (digitized with a Summagraphics ID 2000) were included.

Methods

The method to evaluate the formation constants of complexes from titration curves has already been described [22]. The method of evaluating formation constants from ESR spectra has not been applied very often [14–16].

The principle of the method is as follows (see also ref. [14]): The detected ESR spectra are a sum of spectra of species present in the solution. The basic assumption is that the amplitude of the ESR signal is proportional to the concentration of the species in solution. This assumption allows the evaluation of quantitative data. For this purpose the spectra are stored in their digitized form S_{ij} (i = number of digital points, 1–400; j = number of spectrum; S_{ij} = height of spectrum j at point i, corrected by the spectrum of the tube). S_{ij} can be expressed as the sum of different overlapping spectra:

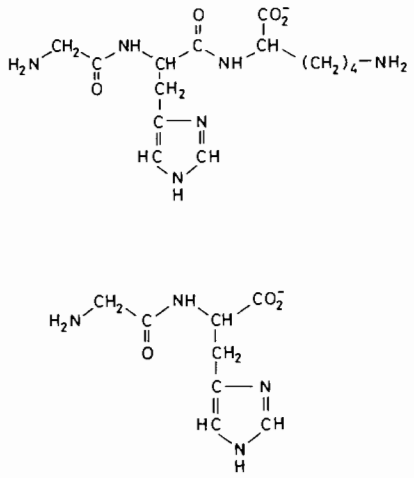

Fig. 1. If copper(II) binds to GHL to form $M_1H_0L_1$ at physiological pH no proton has to be removed (loss of the peptide proton but protonated ϵ amino group; the latter form is equal to the illustrated one in terms of macroscopic equilibrium).

$$S_{ij} = \sum_k R_{ik} \cdot X_{jk} \quad (1)$$

k = number of species, R_{ik} = height of spectra of pure species k at point i, X_{jk} = concentration of species k in spectrum j.

When K_k are the equilibrium constants, M_j the total metal concentration and L_j the total ligand concentration, one is able to evaluate X_{jk} for any pH value (titration point) if the ligand protonation constants are already known (*e.g.* from potentiometric titration of the pure ligand). The computation of the pure spectra R_{ik} of the species starts with an arbitrary set of K_k values. By solution of n·i equations (1) one can compute n pure spectra R_{ik}. After that S_{ij}^{calc} for all i and j are calculated and the difference F is a measure for the quality of the model

$$F = \sum_{j \neq n} \sum_i (\sum_k R_{ik} \cdot X_{jk} - S_{ij}^{exp})^2 \quad (2)$$

description within a given set of K_k. In the next step, the formation constants are varied systematically by iteration until the lowest F is obtained. It is of course possible to use different model descriptions, each with a 'best' set of K_k, and to compare F for the different models.

Results and Discussion

Potentiometric Titration of GHL and GHL–Cu(II)

The general equilibrium involving metal ion M, proton H and ligand L can be written as:

TABLE 1. pK_{pqr} Values for GHL and Cu(II)–GHL.

p	q	r	pH titrations		ESR spectroscopy		Literature
			pK_{pqr}	dpK_{pqr}[a]	pK_{pqr}	dpK_{pqr}[b]	pK_{pqr}
0	4	1	−26.51	0.19			−27.81[17][d], −27.72[28][e]
0	3	1	−23.99	0.09			−24.90[17][d], −24.63[28][e], −25.37[29][f]
0	2	1	−17.66	0.10			−18.37[17][d], −18.18[28][e], −18.77[29][f]
0	1	1	−10.01	0.20			−10.44[17][d], −10.34[28][e], −10.71[29][f]
1	1	1	−19.00	0.68	n.d.[c]		
1	0	1	−16.12	0.10	−16.28	0.18	−16.44[17][d]
1	−1	1	−7.01	0.40	−6.94	0.25	−7.48[17][d]
1	−2	1	3.01	0.31	3.01	0.72	3.74[17][d]
1	1	2	−29.02	0.90	−29.02	0.69	−30.83[17][d]

[a]If pK_{pqr} is varied by $\pm dpK_{pqr}$ experimental error (Σ_i ($base^{calcul.}_{added}$ − $base^{exper.}_{added}$)^2 [22] is doubled. [b]If pK_{pqr} is varied by $\pm dpK_{pqr}$ experimental error (F; see eqn. 2) [14] is doubled. [c]n.d. not determined by ESR method. [d][17] Potentiometric titration, 25 °C, 0.15 *M* NaCl. [e][28] NMR, 37 °C, D_2O. [f][29] NMR, 25 °C, 0.08 *M* KCl.

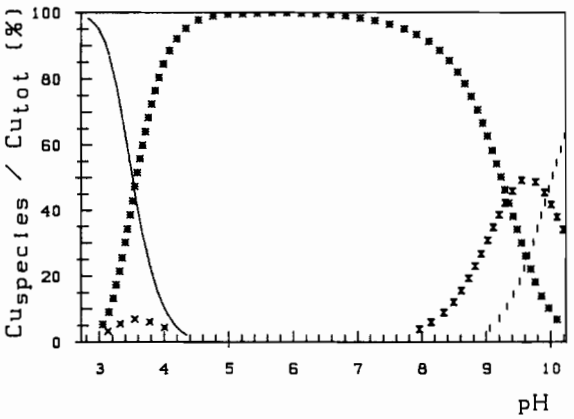

Fig. 2. Species distribution as a function of pH for Cu(II)–GHL. Concentration of copper chloride 0.0025 *M*. Concentration of GHL 0.0025 *M*. (Symbols as in Fig. 3).

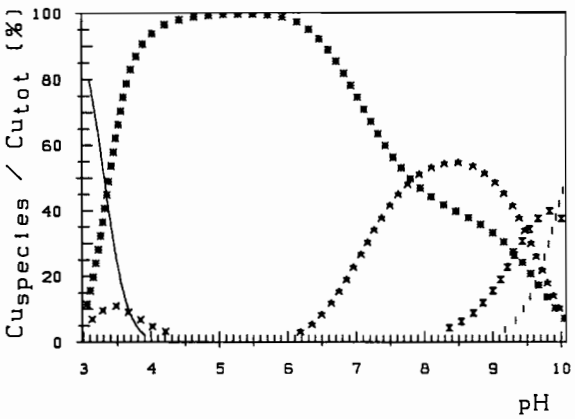

Fig. 3. Species distribution as a function of pH for Cu(II)–GHL. Concentration of copper chloride 0.0025 *M*. Concentration of GHL 0.0066 *M*. —— free copper(II), ×××× $M_1H_1L_1$, **** $M_1H_0L_1$, XXXX $M_1H_{-1}L_1$, |||| $M_1H_{-2}L_1$, ***** $M_1H_1L_2$.

$$pM + qH + rL \rightleftharpoons M_pH_qL_r \quad (3)$$

where p, q and r are the stoichiometric numbers of M, H and L. The equilibrium constants are defined as:

$$pK_{pqr} = -\lg\left(\frac{M_pH_qL_r}{m^p h^q l^r}\right) \quad (4)$$

where m, h and l are the concentrations of free metal ion, H^+ and free ligand. L denotes the anionic form of GHL illustrated in Fig. 1. Table I shows the set of pK_{pqr} which fits the titration curves best. The values are in good agreement with those from Lau and Sarkar [17]. Figures 2 and 3 illustrate the species distribution as a function of pH for different metal/ligand ratios.

pK_{101} (M + L ⇌ ML) is very low indeed. Lau and Sarkar have compared the value of −16.44 [17] (see Table I) with that for GH–Cu(II) (−8.68 [23, 17]*), interpreting the difference of some orders of magnitude as being a consequence of the absence of the ϵ amino group in GH, which therefore should play an essential part in complex formation. Such a participation of the ϵ amino group of GHL is contradicted by some workers [18–20]. In our opinion, the two values are not comparable at all because different types of complexes would be compared; in the case of GHL L denotes the anion (Fig. 1); if copper(II)

*The pK_{pqr} values from Agarwal and Perrin for GH [23] are in good agreement with our work for AH and LH (unpublished results; the agreement is demonstrated in Fig. 4).

binds to the peptide nitrogen of histidine at this stage of protonation, no proton has to be removed (Fig. 1). In the case of GH, the fourth equivalent is not titrable in aqueous solutions of pure GH and one cannot formulate therefore, a corresponding equation for the complex formation of copper(II) with GH without the loss of a proton. If one wants to compare the formation constants of the two systems (Cu(II)–GHL and Cu(II)–GH) one would have to take into account the non-titrable pK_{0-11} of GH (Fig. 1; the loss of the proton from imidazole nitrogen N^1 would be the next deprotonation step).

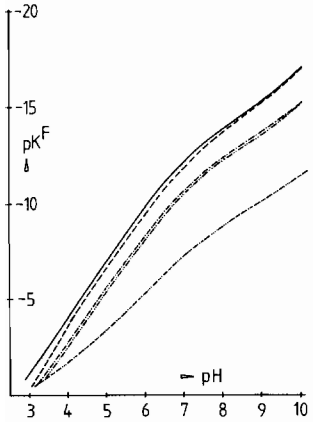

Fig. 4. pK^F as a function of pH for the complex formation of copper(II) with different ligands. Copper(II)/ligand = 1:1. ——— ligand = GHL (pK_{pqr} taken from Table I); - - - - - - - - ligand = GHL (pK_{pqr} taken from ref. [17]); —·—·— ligand = AH (pK_{pqr} taken from unpublished results); —··—··— ligand = GH (pK_{pqr} taken from ref. [23]); —···—···— ligand = GG (pK_{pqr} taken from ref. [14]).

In order to compare the competition of GHL and GH in the complex formation with copper(II), one can compare the pK^F of both ligands as a function of pH. pK^F is defined as the complex formation constant for a complex of M with a fictive ligand L^F ($L^F + M \rightleftharpoons ML^F$), which would bind M to the same extent as the real L in all complexes $M_pH_qL_r$ ($p \neq 0$) together. This diagram is shown in Fig. 4 for GHL, GH, AH and GG. GHL has the highest affinity to copper(II) over the whole pH range but it is not much higher than that for GH and AH, whereas the affinity of GG to copper(II) is considerably lower. One can deduce from Fig. 4 that the structure element GH in GH and GHL as well as the equivalent structure of AH is responsible for the high affinity to copper(II). Despite this the side chain of lysine in GHL seems to have a stabilisation effect of about 1.7 orders of magnitude, but it is not as great as that assumed by Lau and Sarkar [17].

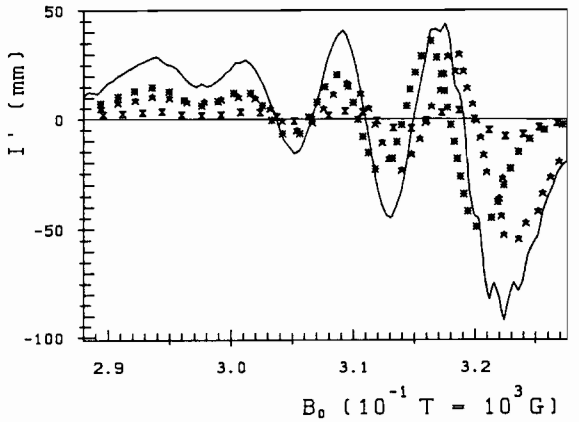

Fig. 5. Experimental (———) and calculated (- - - - - - -) spectra and the contribution of the spectra of the pure species (RG = 4.000). Concentration of copper chloride 0.0025 *M*. Concentration of GHL 0.0066 *M*. The dashed line may be covered by the one for the experimental spectrum. The symbols are the same as used in Fig. 3. pH = 8.79.

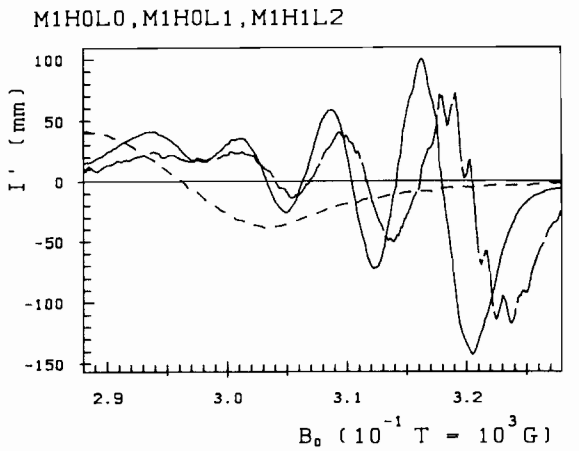

Fig. 6. Spectra of the pure species (0.0025 *M*; RG = 4.000). - - - - - - - copper chloride; ——— $M_1H_0L_1$; – – – $M_1H_1L_2$.

ESR Spectroscopy of GHL–Cu(II)

Quantitative ESR spectroscopy in aqueous solution is a complementary method to the pH titration method. By means of pH titration it is difficult, for example, to detect whether a complex is a monomer or dimer, whereas ESR spectroscopy in solution allows one to distinguish easily between them. The dimer shows a zero spectrum in most cases because of the copper–copper interaction under the given experimental conditions [14].

The formation constants for the complexes are listed in Table I. The complex $M_1H_1L_1$ could not be detected in reasonable amounts; the other formation constants agree with those obtained by means of pH titration within experimental error (Table I).

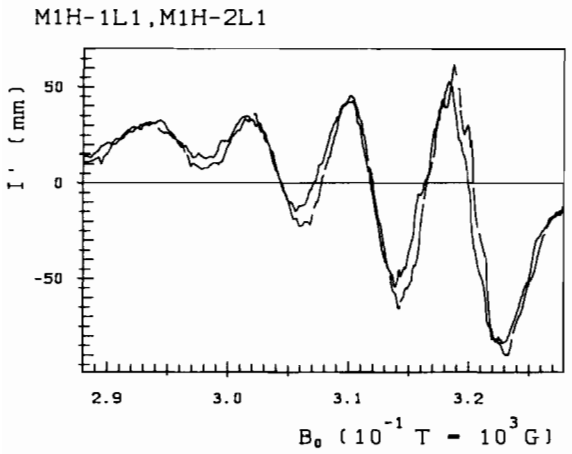

Fig. 7. Spectra of the pure species (0.0025 *M*; RG = 4.000). ——— $M_1H_{-1}L_1$; - - - $M_1H_{-2}L_1$.

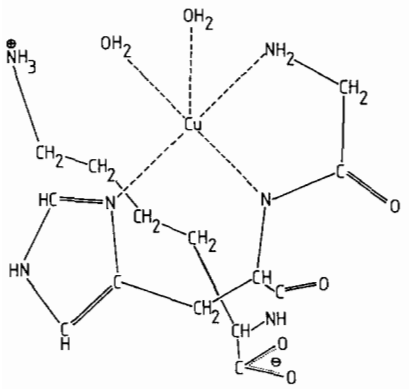

Fig. 8. Postulated structure for the complex $M_1H_0L_1$. This complex is deprotonated at one of the water molecules (first step; $M_1H_{-1}L_1$. The second deprotonation is the loss of a proton from the ϵ ammonium group ($M_1H_{-2}L_1$).

Different models were applied to the system, but the one presented here fits the experimental data best. Figure 5 shows one example of experimental and calculated spectra. Figures 6 and 7 show the spectra of the pure species. The comparison of the spectra $M_1H_{-1}L_1$ and $M_1H_{-2}L_1$ leads to the conclusion that the proton removed from the complex $M_1H_{-1}L_1$ resides at a large distance from copper(II), as the spectrum is influenced only to a minor extent (Fig. 7). This observation is consistent with the hypothesis that the ϵ amino group is not involved in complex formation; in addition, the complex $M_1H_{-1}L_1$ loses its proton at almost the same pH as the free ligand $M_0H_1L_1$ ($pK_{1-11} - pK_{1-21} \cong pK_{011}$).

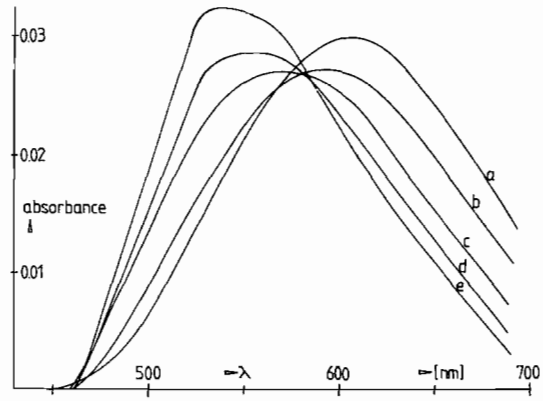

Fig. 9. Optical absorbance of different mixtures Cu(II)/GHL/HSA from 1/1/0 to 1/1/1. Concentration of copper chloride 0.0005 *M*. Concentration of GHL 0.0005 *M*. a, 1/1/0.00; b, 1/1/0.13; c, 1/1/0.40; d, 1/1/0.60; e, 1/1/1.00.

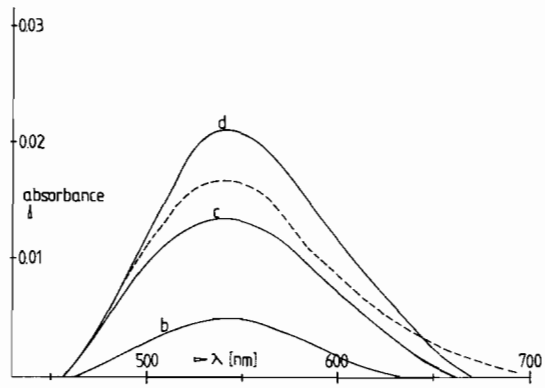

Fig. 10. Resulting spectra after subtraction multiple of Cu(II)–GHL spectrum from experimental spectra (Fig. 9; for further details see text). b, c, d, see Fig. 9. ------- (dashed line), spectrum for Cu(II)–HSA 1:1, 0.00025 *M*.

Postulated Structures of the Complexes $M_pH_qL_r$

The species $M_1H_0L_1$ dominates over a wide pH range. Our postulated structure is illustrated in Fig. 8. The ϵ amino group is not involved in complex formation. The latter assumption agrees well with the polymeric crystal structure [19].

Optical Absorption Spectroscopy of Cu(II)–GHL, Cu(II)–HSA and Cu(II)–GHL–HSA at pH 7.4

Addition of GHL up to equimolar ratios of copper(II)–HSA in copper(II)–HSA (1:1) containing solutions hardly alters the absorption spectra (reference being the same solution of copper(II)–HSA (1:1), 0.00025 *M* as was used as sample before adding GHL). Therefore zero spectra are obtained by addition of GHL.

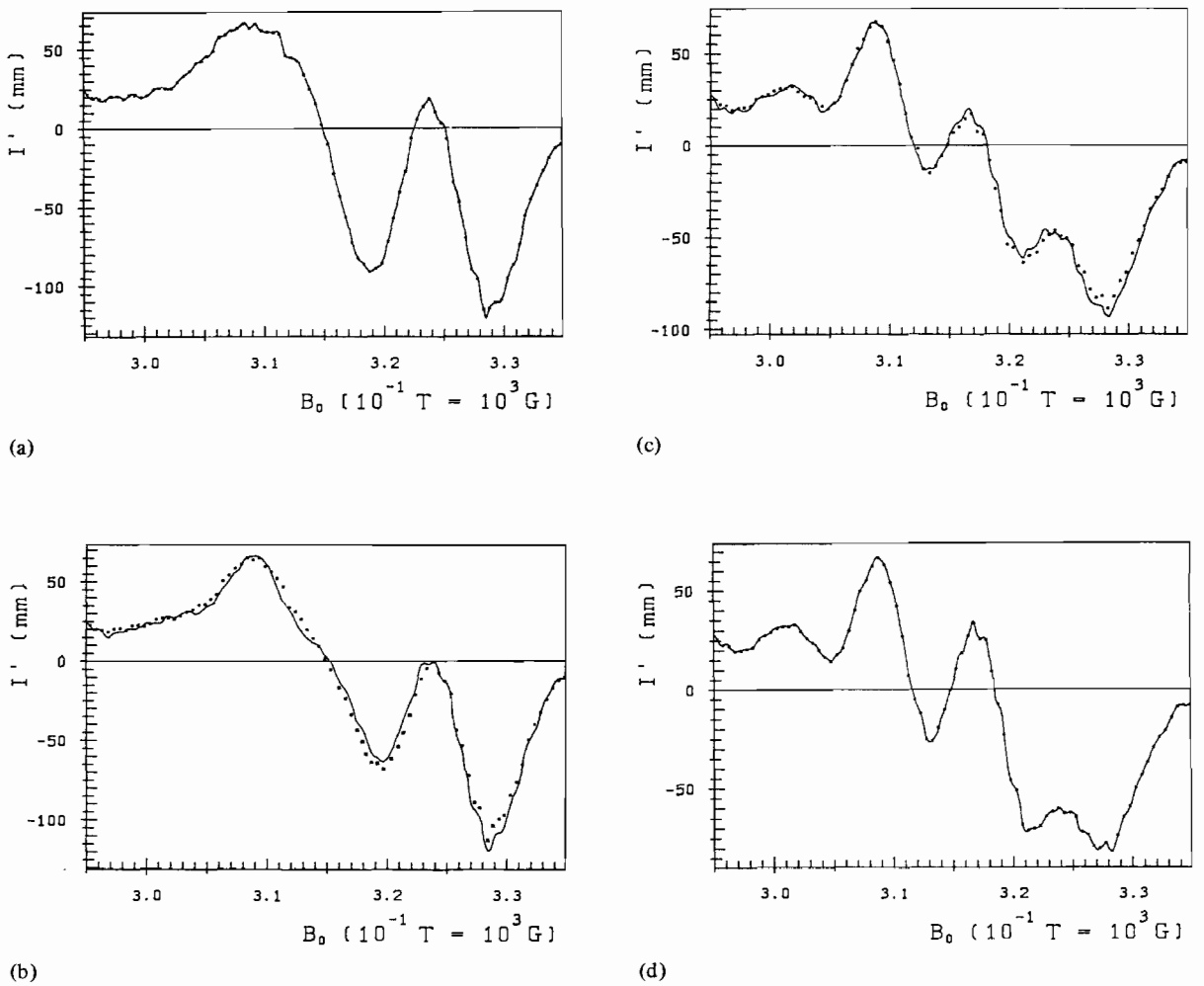

Fig. 11. Experimental (———) and calculated (■■■■■) spectra of different mixtures Cu(II)/GHL/HSA from 1/0/1 to 1/1/1 (RG = 16.000): (a) Cu(II)/GHL/HSA = 0.00125/0/0.00125; (b) Cu(II)/GHL/HSA = 0.00122/0.00028/0.00122; (c) Cu(II)/GHL/HSA = 0.00116/0.000926/0.00116; (d) Cu(II)/GHL/HSA = 0.00112/0.00114/0.00112.

The reverse mode of operation (adding HSA to an equimolar mixture of copper(II)–GHL, 0.0005 *M*; reference being solvent + equivalent amounts of HSA in the sample) is illustrated in Fig. 9. The peak at 610 nm [17] decreases linearly with the amount of HSA added and a new peak is observed at 535 nm. If a multiple of the copper(II)–GHL spectrum is subtracted from the experimental spectra (multiplication factor = (0.0005 *M* – $HSA_{Concentration}$)/0.0005 *M*) for each measured point, the resulting spectra (Fig. 10) have always the same shape and λ_{max} of 535 nm with absorbance increasing linearly with the multiplication factor. These spectra do not differ from the spectra for copper(II)–HSA (1:1) within experimental error (reference being solvent + equivalent amounts of HSA in the sample; see Fig. 10 [17, 24]).

These observations would lead to the conclusion that GHL cannot compete with HSA in complexing copper(II). To prove this hypothesis some ESR spectra were recorded.

ESR Spectroscopy on the System Cu(II)–HSA and Cu(II)–GHL–HSA at pH 7.4

If the above assumption is right, addition of GHL to an equimolar aqueous solution of copper(II)–HSA should not alter the ESR spectrum. In contrast to this expectation one finds that the addition of GHL leads to a significant change in the spectra (Fig. 11). Each spectrum can be computed as a mixture of the spectrum for copper(II)–HSA (1:1) and copper(II)–GHL–HSA (1:1:1); the concentration of copper(II)–GHL–HSA to be taken into account is equal to the GHL concentration until equimolar amounts of GHL are added (Fig. 11).

These findings are only consistent if we assume a ternary complex with the same optical absorption spectra as the binary complex copper(II)–HSA (1:1).

The concentration of binary complexes between copper(II) and GHL seems to be negligible up to equimolarity in copper(II)–GHL–HSA containing solutions. The copper(II)–GHL complex at pH 7.4 has approximately the spectrum shown in Fig. 6 ($M_1H_0L_1$) with characteristic high intensities; only a few percent of this spectrum would alter the shape of the spectra for the ternary system in a characteristic way*. This result is in contrast to that of Lau and Sarkar [17], obtained by equilibrium dialysis, namely that at an equimolar ratio of copper(II), GHL and HSA 40% of total copper(II) should be bound to GHL [17]. This discrepancy might be due to the difficulties in obtaining significant results by the dialysis method in the narrow range Cu(II)/GHL/HSA from 1/0/1 to 1/1/1.

Conclusion

Copper(II) has a high affinity for GHL and forms a very stable complex $M_1H_0L_1$. Although related peptides bind copper(II) almost as strongly as GHL (*e.g.* GH and AH), most of these peptides display no biological effect or only low activities [4]. The lysyl residue seems to be essential, therefore, for the biological activity of GHL. Perkins *et al.* [19] suggested that two important functions of GHL explain its biological role: GHL at physiological pH has one copper binding site (N-terminal amino group, histidine peptide nitrogen and the imidazole nitrogen N^3 of histidine) and one cell membrane binding site (ϵ ammonium group of lysine). The cell membrane binding site remains free if copper is complexed by GHL.

The latter fact supports the postulated mechanism in its first two steps [3]. GHL binds to the membrane with its ϵ ammonium group (receptor function); after that, copper(II) is bound to the molecule and the complex may be easily released from the membrane. Our results are in full agreement with this mechanism.

In the ternary system, a ternary complex of high stability is formed. This is the reason that the released $M_1H_0L_1$ complex may be bound as a ternary complex in HSA containing plasma. The ternary complex seems to exchange copper(II) with ceruloplasmin (histidine may act as an intermediate carrier molecule [3, 17]).

In general our findings support the mechanism postulated by Pickart and Thaler [3] with the exception that copper(II) is bound as a ternary complex in plasma in the presence of HSA and GHL.

*It is known that the viscosity of the solution can influence the ESR spectra [25]. To prevent such errors we recorded the spectrum for copper(II)–GHL at pH 7.4 at different concentrations of glycerol in water (up to 44% by weight; the viscosity of the latter solution is 4.434 times higher than that of pure water at 20 °C [26]; the viscosity of a 0.00125 *M* BSA solution is 5.707 times higher than that of pure water at 25 °C [27]) but the spectra did not change within experimental accuracy.

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