A Two-Dimensional (Magnetic Field and Concentration) Electron Paramagnetic Resonance Method for Analysis of Multispecies Complex Equilibrium Systems. Information Content of EPR Spectra

Antal Rockenbauer,*,† Terézia Szabó-Plánka,‡ Zsuzsanna Árkosi,† and László Korecz†

Contribution from the Chemical Research Center, Institute of Chemistry, Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest, Hungary, and Department of Physical Chemistry, University of Szeged, P.O. Box 105, H-6701 Szeged, Hungary

Received February 1, 2001. Revised Manuscript Received May 17, 2001

Abstract: A two-dimensional simulation method has been developed for the interpretation of electron paramagnetic resonance (EPR) spectra consisting of a multitude of strongly overlapping signal components. The set of EPR spectra for complex equilibrium systems is analyzed simultaneously as a function of metal and ligand concentrations and pH. The formation constants of the various species are adjusted together with the magnetic parameters of the component EPR spectra. At most 10 EPR-active and 5 EPR-silent species can be involved to simulate a maximum of 36 experimental spectra, while the number of adjusted parameters is at most 100. Statistical parameters are suggested to give the confidence intervals for parameter estimation and to distinguish alternative speciation models. The efficiency of the program is demonstrated for the copper(II)−L-asparagine system, in which 10 species, including 3 pairs of isomers, are characterized with magnetic parameters and formation constants. On the basis of the magnetic parameters, a structural assignment is made for the detected species. The two-dimensional approach can also supply the formation constant of the EPR-silent species, as demonstrated for the copper(II)−glycyl-L-serine system.

Introduction

For any spectroscopic method applied in the liquid phase, the elucidation of coordination modes in a complex equilibrium system becomes increasingly difficult as the number of species involved rises and the overlap between component spectra is large. This is typically the case for studies in which electron paramagnetic resonance (EPR) spectroscopy is used to analyze the coordination of various bioligands, such as amino acids or peptides, with copper(II) and other metal ions. These ligands contain a great variety of donor groups in their backbones and side chains, which results in various coordination modes in aqueous solution, depending on the pH and on the concentrations of the ligand and the metal ion. Though some particularly stable complex may be predominant under specific conditions, the coexistence of various species in the solution is most typical. Therefore, it is frequently difficult to clarify even those features that are of fundamental importance in the possible biological effects of the various species, i.e. which of the competing donor groups bind to the central ion, and which positions these groups occupy.

The primary method for the identification of various species is pH potentiometry: this technique can furnish their overall compositions and formation constants. Occasionally, further information on the nature of the coordinating groups can be obtained by comparing the stabilities and other thermodynamic parameters of different complexes. The concentration distribution established by pH potentiometry for the various species can serve as a starting point for further studies, involving ultraviolet−visible (UV−vis), circular dichroism (CD), nuclear magnetic resonance (NMR), or EPR spectroscopy, which may allow a more detailed description of the coordination modes. The procedure either entails the decomposition of the measured curves to the contributions of the species previously identified by pH potentiometry, or simply utilizes the pH and concentration ranges in which one of the complexes is predominant. With most spectroscopic techniques, however, there are difficulties in distinguishing geometric or coordination isomers. In the case of UV−vis or CD spectroscopy, the analysis can be carried out with the help of the characteristic bands of model compounds selected by chemical analogy.[1] NMR relaxation studies frequently lead to contradictory results for isomeric equilibria even in the same system under similar experimental conditions,[2−4] presumably because the rapid interconversions of different isomeric species in the method of selective line-broadening might result in the loss of information from the separate isomers.

A number of authors have used EPR spectroscopy as a spectrophotometric device, in combination with pH potentiometry, to obtain the formation constants of complexes of different compositions.[5,6] In other cases the predominant complexes were investigated, and various isomers were identified by computer analysis of the superhyperfine (shf) pattern produced by the ligand nuclei in the high-field region.[7−10] Abundant information

† Hungarian Academy of Sciences.
‡ University of Szeged.

(1) Casella, L.; Gullotti, M. *J. Inorg. Biochem.* **1983**, *18*, 19−31.
(2) Henry, B.; Boubel, J.-C.; Delpuech, J.-J. *Inorg. Chem.* **1986**, *25*, 623−631.
(3) Kruck, T. P. A.; Sarkar, B. *Can. J. Chem.* **1973**, *51*, 3563−3571.
(4) Valensin, G.; Basosi, R.; Antholine, W. E.; Gaggelli, E. *J. Inorg. Biochem.* **1985**, *23*, 125−130.
(5) Kittl, W. S.; Rode, B. M. *J. Chem. Soc., Dalton Trans.* **1983**, 409−414.
(6) Gampp, H. *Inorg. Chem.* **1984**, *23*, 1553−1557.
(7) Goodman, B. A.; McPhail, D. B.; Powell, H. K. J. *J. Chem. Soc., Dalton Trans.* **1981**, 822−827.
(8) Goodman, B. A.; McPhail, D. B. *J. Chem. Soc., Dalton Trans.* **1985**, 1717−1718.

10.1021/ja0102888 CCC: $20.00 © 2001 American Chemical Society
Published on Web 07/13/2001

on the coordination modes may also be provided by the *g*-factor and the metal hyperfine (hf) coupling constant, which are highly sensitive to changes in the ligand field, in the character of the metal−ligand bonds, and in geometric distortions. The literature typically furnishes only approximate values for the parameters that have been determined so far from the characteristic points of the spectra of predominant complexes. Exact determination of these parameters requires a more sophisticated calculation procedure, particularly when several species are present, since the relatively broad spectral lines of the various complexes overlap strongly. To analyze such systems, we have developed a computer program[11,12] which can decompose the overlapping fluid-phase spectra throughout the whole field region, resulting in the *g* values, the metal and ligand coupling constants, and the linewidth data, together with the relative concentrations of the various complexes for up to four species. With this program, we have succeeded in giving a consistent description of several copper(II)−amino acid and copper(II)−dipeptide systems,[12−14] including information even on minor complexes.

Frequently, however, more than four paramagnetic species coexist in solution, further increasing the number of parameters to be adjusted. At the same time, the information content of an individual spectrum is limited, and, if the number of adjusted parameters exceeds a critical value, the confidence intervals become too large, making any fitting procedure meaningless. To overcome this difficulty, we propose a method which significantly enhances the amount of information that can be extracted from the analyzed spectra and limits the number of adjusted parameters. Thus, we do not analyze the EPR spectra recorded under different conditions (pH and metal and ligand concentrations) separately but simulate the entire set of spectra simultaneously, using the same magnetic parameters for a given species. Furthermore, the relative concentrations are not regarded as free parameters but are computed via the solution of mass balance equations, in which the formation constants are adjusted (together with the magnetic parameters of the various species) to minimize the deviation between entire sets of experimental and calculated spectra. In this approach, we regard the concentrations of the components (the metal and ligand concentrations and the pH) as new coordinates of the EPR spectra recorded as a function of the magnetic field. For this reason, we use the terminology "two-dimensional EPR spectroscopy".

Below we discuss the information theory aspects of the method and the main features of the corresponding computer program and present the evaluation of the copper(II)−L-asparagine system as an illustrative example. We also demonstrate briefly the possibility of determining the formation constants for EPR-silent oligomers in the case of a copper(II)−dipeptide complex.

Information Content of EPR Spectra

A basic question in spectroscopy is the number of independent parameters that can be determined from one spectrum; another relates to how the available information can be optimized if the spectra vary with external coordinates such as time, temperature, or concentration. The carriers of the spectroscopic information are the lines. In general, three parameters are necessary to characterize a line: the position, the width, and the amplitude or intensity. Each line therefore represents three information units. (Further parameters may be necessary occasionally to describe the line shape, e.g., a mixture of Gaussian and Lorentzian shapes, various distortion effects, etc.) EPR spectra are rather unique as concerns the available information: in contrast with optical or NMR (and in particular ^{13}C) spectra, the number of information carriers, i.e., the number of lines, may be much larger than the number of independent parameters that can be extracted. The large redundancy is related to the fact that the same unpaired electron simultaneously interacts with a large number of nuclei in the given molecule. The hf structure produced by this interaction is built up in a multiplicative way: each nucleus with spin I repeats the overall pattern of other nuclei $(2I + 1)$ times. Consequently, the number of lines increases multiplicatively with the number of nuclei giving separated hf splitting. In turn, the positions of all lines are determined by a few coupling constants, while their intensities can be given by simple rules. The linewidths in the primary hf multiplet can be given by three relaxation parameters as

$$W_M = \alpha + \beta M + \gamma M^2 \qquad (1)$$

where M is the magnetic quantum number of the metal ion. Mostly, the further shf structure of ligand nuclei does not require the use of additional relaxation parameters.

We shall focus our attention here on the EPR spectra of copper(II) complexes in liquid solution. If the isotopes ^{63}Cu and ^{65}Cu are present in natural abundance, and they are coordinated only to O donors, the number of hf lines is 8. The corresponding number is much higher if the O atoms are replaced by N atoms. It is 72 for 4 equivalent and 648 for 4 nonequivalent N donors. At the same time, the number of independent parameters is not more than 10: at most 5 hf coupling constants (copper and at most 4 nonequivalent N atoms), the isotropic *g*-factor, the 3 relaxation parameters (α, β, γ), and the line intensity, which is proportional to the concentration of the particular species. In contrast with the EPR hf lines, for the bands in an optical spectrum there is no trivial relation between the position, width, or intensity data. Consequently, while the simulation can strongly condense the information for EPR spectra, this is not feasible for optical spectra. If the system contains several species in equilibrium, each species can be characterized in the EPR spectrum by at most 10 parameters. This means that EPR spectra offer a mathematically tractable system even when the number of species is relatively high.

Obviously, the level of spectral resolution restricts the number of spectra that can be extracted from a superimposed spectrum. For copper(II) spectra recorded in the liquid state, the resolution is different in the low- and the high-field regions. At high field, a nice N shf pattern can occasionally be observed, whereas at low field, the resolution is rather poor. Throughout the field range of ca. 500 G, where the hf structure appears, typically around 10 lines are separated, which represent 30 information units. Since a species is characterized by 6−10 parameters, the separation of the signal components permits a good level of confidence if the number of components is not more than 3. In unfavorable cases, even the determination of a minor third component may be problematic. When there are 4 or more species, the procedure cannot give a reliable decomposition (unless the parameters of one or more complexes are known from other spectra and can be fixed[12−14]). Since the number of

(9) Pasenkiewicz-Gierula, M.; Froncisz, W.; Basosi, R.; Antholine, W. E.; Hyde, J. S. *Inorg. Chem.* **1987**, *26*, 801−805.
(10) Pogni, R.; Della Lunga, G.; Basosi, R. *J. Am. Chem. Soc.* **1993**, *115*, 1546−1550.
(11) Rockenbauer, A.; Korecz, L. *Appl. Magn. Reson.* **1996**, *10*, 29−43.
(12) Szabó-Plánka, T.; Rockenbauer, A.; Korecz, L. *Magn. Reson. Chem.* **1999**, *37*, 484−492.
(13) Szabó-Plánka, T.; Rockenbauer, A.; Korecz, L. *Polyhedron* **1999**, *18*, 1969−1974.
(14) Szabó-Plánka, T.; Rockenbauer, A.; Korecz, L.; Nagy, D. *Polyhedron* **2000**, *19*, 1123−1131.

species coexisting in solutions of various complex equilibrium systems is frequently higher than 3 (it should be borne in mind that geometric and coordination isomers generally produce different EPR spectra), reliable species identification may be possible if the information contents of spectra recorded at various concentrations and pH are added together. For this reason, the EPR intensity is regarded as a function not only of field, but also of the metal and ligand concentrations and the pH. This method will increase the amount of available information to an extent proportional to the number of spectra; the number of independent parameters will also be increased if the concentrations of all species in each spectrum are adjusted. To reduce the number of adjusted parameters, we compute the concentrations by solving the mass balance equations and treat only one formation constant for each species as an adjustable parameter. In this case, the total number of adjustable parameters for 10 species is not more than 100, which is very low relative to the amount of information available from a set of 20−40 appropriately different experimental spectra, recorded in solutions with equal metal ion and ligand concentrations or with a large ligand excess, in the pH range 2−13.

The Fidelity of EPR Spectra

Any distortion of the spectra will reduce the fidelity of decomposition. Our analysis will be restricted to spectra for which the modulation distortion is small, the saturation can be neglected, and the slow passage condition is fulfilled. For comparability of the total signal intensities of EPR spectra recorded at different pH values, we use narrow capillaries when the dielectric loss of the cavity is small. Though the unresolved ligand hf pattern can produce an overall line shape closer to the Gaussian than to the Lorentzian curve suggested by relaxation theory, we restrict ourselves to strictly Lorentzian curves, since this allows estimation of the N shf splittings,[11] which can provide important structural information. The lifetime of copper complexes in equilibrium is long enough for the spectra to be described as a superposition of the component signals, but extension of the method to free radicals[15,16] may necessitate inclusion of the broadening effect due to chemical exchange; alternatively, if the exchange is fast, the spectrum can be calculated from the average values of magnetic parameters assigned to the individual species. Molecular reorientation is considered to be fast, yielding isotropic spectra for all species. While the precise line positions in the metal hf splitting are calculated by solution of the Breit−Rabi equation, the ligand shf splittings are described by equidistant multiplets.

Statistical Parameters of Parameter Optimization

In principle, all hf lines can be simulated independently, giving their position, width, and intensity. The hf coupling constant, for example, can be determined by selecting any line pair. If the number of lines is large enough, this method can afford a statistical approach determination of both the average and the standard deviation of the respective parameters.

The concentration data relating to the *i*th species are determined by an iterative solution of the mass balance equation.[17] As usual, we have chosen the metal ion M, the free ligand L (not capable of further dissociation), and the proton H as components. Their numbers in a given species are denoted by *p*, *q*, and *r*, respectively. The equilibrium concentration of the species $M_pL_qH_r$ can be expressed in terms of its formation constant β:

$$[M_pL_qH_r] = \beta[M]^p[L]^q[H]^r \tag{2}$$

where [M], [L], and $[H] = 10^{-pH}$ are the equilibrium concentrations of the components in the solution.

In the following, each of the *n* species, denoted by the running index *i*, is characterized by the formation constant β_i. The values of *p*, *q*, and *r* for the *i*th species are denoted by the symbols p_i, q_i, and r_i. The equilibrium concentration of the *i*th species in the solution giving the *j*th spectrum is symbolized by c_{ij} and can be described according to eq 2. The sample giving the *j*th spectrum is characterized by three data: the measured pH and the total (or analytical) concentrations of the metal and ligand:

$$[M]_j(\text{total}) = \sum_i c_{ij}p_i \tag{3}$$

$$[L]_j(\text{total}) = \sum_i c_{ij}q_i \tag{4}$$

In the above mass balance equations, both the paramagnetic and the EPR-silent species are included. The program allows the use of at most 10 active and 5 EPR-silent species. (We applied an enhanced criterion of convergence to avoid false jumps in the error function of the spectrum fitting; see below.)

The superimposed spectrum $S_j(B)$ of the *j*th sample recorded as a function of field *B* can be built up by the coefficients of concentrations c_{ij} as

$$S_j(B) = c_{1j}F_1(B) + c_{2j}F_2(B) + ... + c_{nj}F_n(B) \tag{5}$$

where $F_1(B)$, ..., $F_n(B)$ denote the EPR spectra of the first to the *n*th species with unit concentration. Calculation of these component spectra in terms of the magnetic parameters was reported previously.[11]

During the parameter optimization procedure, the magnetic parameters of the component EPR spectra and the formation constants of the mass balance equations are adjusted simultaneously until the square deviation—between the experimental spectra $E_j(B)$ and the calculated spectra $S_j(B)$, summed over both the samples and the field points—achieves a minimum:

$$\text{SSQD} = \sum_j \text{SQD}_j = \sum_j \sum_k [E_j(B_k) - S_j(B_k)]^2/N \tag{6}$$

where *N* is the number of field points. For the *j*th spectrum, the quality of simulation can be given by comparing SQD_j with the variance,

$$V_j = \sum_k [E_j(B_k) - \bar{E}_j]^2/N \tag{7}$$

and the noise

$$\text{NOISE}_j^2 = \sum_k{}' [E_j(B_k) - \bar{E}_j]^2/N_w \tag{8}$$

where $\sum'$ denotes a summation restricted to N_w points in the wings of the spectra, outside the range where the EPR resonance is detected. Here the baseline is defined as $\bar{E}_j = \sum' E_j(B_k)/N_w$. In the event of a good simulation, SQD_j is small compared to V_j, and it can approximate the square of the noise. To

(15) Hustedt, E. J.; Cobb, C. E.; Beth, A. H.; Beechem, J. M. *Biophys. J.* **1993**, *64*, 614−621.

(16) Hustedt, E J.; Smirnov, A. I.; Laub, C. F.; Cobb, C. E.; Beth, A. H. *Biophys. J.* **1997**, *72*, 1861−1877.

(17) Beck, M. T.; Nagypál, I. *Chemistry of Complex Equilibria*; Akadémiai Kiadó: Budapest, 1990; pp 25−30.

characterize the quality of simulations, we introduce a noise-corrected regression parameter:

$$R_j = (V_j + \mathrm{NOISE}_j^{\,2} - \mathrm{SQD}_j)/V_j \tag{9}$$

This parameter attains unity if SQD becomes as small as the square of the noise.

When the baseline $\bar{E}$ is adjusted, the precision of its position is determined by the standard error on the mean, which can be given by the noise and the number N' of data points:

$$\sigma = \mathrm{NOISE}/N'^{\,1/2} \tag{10}$$

We can also define the precision of the best-fitting curve by σ in eq 10. Here, N' is equal to the number of digitized data points in the recorded spectra only if the noise is "white", i.e., when the signal filtering does not eliminate any frequency components smaller than the rate of data acquisition. Since this is often not the case, we carried out a statistical analysis of the noise by investigating the correlation integral defined as

$$K_{\delta k} = \sum_k{}' E(B_k)E(B_{k+\delta k})/N_\mathrm{w} \tag{11}$$

which gives the noise if $\delta k = 0$. The correlation integral is presumed to decrease exponentially with increasing δk value as

$$K_{\delta k} = K_0 \exp(-\delta k \tau_c) \tag{12}$$

where the correlation factor τ_c gives the extent of the correlation between the neighboring data points. The reduced number of independent data points can also be expressed in terms of τ_c:

$$N' = N/\sum_{\delta k=0} \exp(-\delta k \tau_c) \tag{13}$$

The precision of the parameter optimization, i.e., the confidence interval for the adjusted parameter Q_m, can be given by the variation in the error function when it is equal to the square of the standard error in eq 10. Since all the first derivatives are zero at the minimum in the error surface, the increase in SSQD can be expressed as

$$\Delta\mathrm{SSQD}(Q_m) = {}^1/_2[\delta^2\mathrm{SSQD}/\delta Q_m{}^2]\Delta Q_m{}^2 \tag{14}$$

and thus the confidence interval ΔQ_m of parameter Q_m can be given by the curvature of the error function:

$$\Delta Q_m = 6\sigma[\delta^2\mathrm{SSQD}/\delta Q_m{}^2]^{-1/2} \tag{15}$$

where we followed the usual practice of defining the confidence interval by 3σ, corresponding to a confidence level of 99.7%. In eq 15, only the diagonal elements of the curvature matrix are considered, but for certain parameter pairs the off-diagonal elements could significantly increase the confidence interval.[18] We take this effect into account by reducing the number N' of independent data in the definition of standard deviation. We presume that, if N_par parameters are to be optimized, then N_par times as many experimental data are needed to obtain the same accuracy as in the case of a single parameter adjustment. Accordingly, the confidence intervals are

$$\Delta Q_m = 6(\mathrm{NOISE})[N_\mathrm{par}/(N'N_\mathrm{sample}\delta^2\mathrm{SSQD}/\delta Q_m{}^2)]^{1/2} \tag{16}$$

When alternative models of complex speciation are compared, the level of improvement of the regression can reveal whether the inclusion of a new species results in a significant change in the fitting. By using the same principle as for the confidence interval in eq 16, the critical difference in regression for the overall set of samples can be defined as

$$\Delta R = 9(\mathrm{NOISE})^2[N_\mathrm{par}/(N'N_\mathrm{sample}V)] \tag{17}$$

where the overall variation V is given as the average of the variations V_j in eq 7. For the individual spectrum, the critical difference in regression is larger by 1 order of magnitude: $N_\mathrm{sample} = 1$ should be taken, and this increase is partially compensated, since N_par is reduced to the number of parameters corresponding to the species present in significant concentration in the respective sample. An analysis based on the ratio of SQD versus noise, or on the regression factors R_j, promotes a distinction between alternative equilibrium models. If one species is missing from the mass balance equations, then the regression factor becomes significantly smaller for solutions where the relative concentration of the missing species exceeds 10%. As the precision of the regression in eq 17 is inversely proportional to the number of spectra, the species assignment is not affected too critically by the signal/noise ratio S/N. As compared to the analysis of a single EPR spectrum, where S/N should be larger than 50, the two-dimensional approach can offer reliable assignments even when S/N does not exceed 10.

As the number of local minima in the error function increases exponentially with the number of adjusted parameters, the fitting process with 100 parameters will certainly be trapped in false convergence if the starting values of the parameters are not sufficiently close to the best values. For this reason it is extremely important to carry out preliminary surveys to establish the appropriate pH and concentration regions where one of the species is close to predominance and the approximate values of its magnetic parameters can therefore be obtained. We carried out this preliminary parameter determination with the previously written program developed for analysis of the superposition of at most four species.[11] We found that, if the starting values are correctly chosen, i.e., the deviations of the parameters from the best values are not too large, the iteration process is remarkably stable: the best values obtained at convergence are independent of the starting values. This indicates that the information content of the overall set of spectra is much larger indeed than the number of adjusted parameters. The various iteration processes with which a search is made for the minimum of the error surface were reported earlier.[11,18]

A further advantage of the two-dimensional method is that it also allows detection of the silent (e.g., dimeric or tetrameric) species if a flow technique is applied that ensures identical sensitivity of the EPR recording for the entire set of samples. The high accuracy of signal simulation furnishes reliable overall concentration data for the paramagnetic metal centers. If the dimerization exceeds 10%, for instance, the reduction in the overall signal intensity can be considered significant. This reduction is proportional to the difference between the total and the silent copper concentration. To determine the formation constant of a silent species, we search for the minimum in the error function defined as

$$\mathrm{SQD}_\mathrm{conc} = \sum_j([\mathrm{Cu}_\mathrm{active}]_j - [\mathrm{Cu}_\mathrm{EPR}]_j)^2 \tag{18}$$

where $[\mathrm{Cu}_\mathrm{active}]_j$, i.e., the EPR-active copper concentration, is

(18) Rockenbauer, A. *Mol. Phys. Rep.* **1999**, *26*, 117−127.

computed from the mass balance equation, and $[Cu_{EPR}]_j$ from the experimental spectra recorded for the *j*th sample.

Results and Discussion

Speciation in the Copper(II)−L-Asparagine System. In the alkaline pH region, copper(II) induces proton loss from the amide group of asparagine. Accordingly, besides the usual α-amino acid complexes $[CuL]^+$ and $[CuL_2]$, formation of the species $[CuL_2H_{-1}]^-$ and $[CuL_2H_{-2}]^{2-}$ was suggested by the pH potentiometric data and visible spectral evidence.[19] In other works, the complex $[CuLH_{-1}]$ too was identified,[20,21] and formation of the complex $[CuLH]^{2+}$ was assumed.[22] Additionally, an isomeric equilibrium was previously suggested for the complex $[CuL_2]$,[7,8] and several modes of coordination may likewise occur for the other bis complexes.

All EPR spectra for the copper(II)−L-asparagine system could be described well when we assumed 10 superimposed components, which were assigned to the aqua complex, the above mono complexes $[CuLH]^{2+}$, $[CuL]^+$, and $[CuLH_{-1}]$, and the two isomers found for each of the bis complexes $[CuL_2]$, $[CuL_2H_{-1}]^-$, and $[CuL_2H_{-2}]^{2-}$. For this best model, the regression factor varied between 0.9826 and 0.9997; for a large majority of the spectra, it lay between 0.988 and 0.998. The critical difference in the overall regression defined by eq 17 in the analyzed set of spectra is 0.00012. The change for the individual regression factors can therefore be regarded as significant if it is larger than 0.001. If we omit one of the above species (i.e., the corresponding component spectrum), the decrease in the overall regression coefficient is significantly larger than 0.00012 (it varies between 0.00066 and 0.02199). Moreover, a systematic impairment of the spectral fit can be observed for a definite class of experimental curves, while for those spectra where the solution contains the respective species in negligible concentration (i.e., less than 5% according to the best model), the regression factor changes by less than 0.0005.

In highly acidic media, we should include the spectrum of the complex $[CuLH]^{2+}$ to obtain a satisfactory fit in the pH range 2−3: omission of this species decreases *R* by 0.001−0.005 (Figure 1). The EPR spectra offer even more convincing evidence of the existence of the complex $[CuLH_{-1}]$: in solutions with a metal ion:ligand ratio of 1:1 above pH 6, no acceptable fit was obtained without this species, as indicated by the considerable decrease in *R* (Figure 2). (The best fit above pH 7 was slightly poorer, which may be explained by the formation of a small amount of copper hydroxide, observed to precipitate at about pH 10.) The spectrum of the complex $[CuL_2]$ can be best described as a superimposed curve of two species, each with two N donors in equatorial positions. For the samples with excess ligand in the pH region 4−10.5, where the relative concentration of $[CuL_2]$ exceeds 60%, *R* was smaller by 0.0045−0.0065 if only one coordination mode was assumed for this complex (Figure 3). For the complex $[CuL_2H_{-1}]^-$, assumption of an isomeric equilibrium (now between molecules with two and three N atoms in equatorial positions, respectively) again results in the best description of the spectra. In the event of a single coordination mode, with two equatorial N donors, the impairment of *R* is between 0.001 and 0.0045 in the region

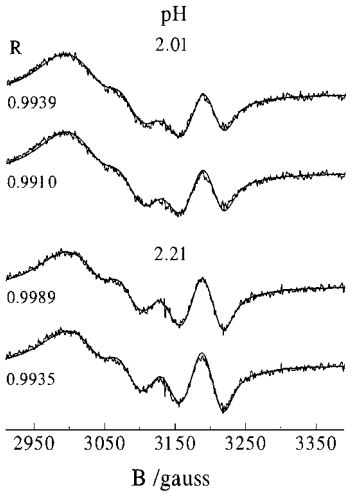

Figure 1. Experimental and calculated EPR spectra at excess L-asparagine. The complex $[CuLH]^{2+}$ is taken into consideration for the upper spectrum at each pH but omitted for the lower spectrum.

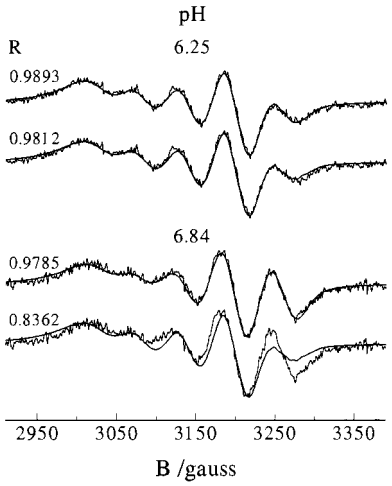

Figure 2. Experimental and calculated EPR spectra in a 1:1 solution of the metal ion and the ligand. The complex $[CuLH_{-1}]$ is taken into consideration for the upper spectrum at each pH but omitted for the lower spectrum.

pH 10.5−12, where this species is formed in more than 40% concentration. The other single coordination model assuming a 3N shf pattern yields an even worse fit: *R* is then smaller by 0.002−0.008 for the same experimental curves (Figure 4). The spectra of the complex $[CuL_2H_{-2}]^{2-}$ also reveal two kinds of coordination, with two equatorial N atoms in the first isomer and four N atoms in the second isomer. For the one-component model with 2N or 3N shf splitting, *R* decreases by 0.001−0.004 above pH 12; with the 4N model, the impairment is much larger (0.003−0.013). The poor agreement of the partially resolved shf splitting in the high-field region is particularly striking (Figure 5). A clear distinction between alternative models is not always possible. For the complex $[CuL_2H_{-1}]^-$, the two-component 3N + 3N model yields almost as good a spectral fit as that with the best, 2N + 3N model. Similarly, for the complex $[CuL_2H_2]^{2-}$, there was only a slight difference in R_j with the accepted 2N + 4N model and with the 3N + 4N model. The choice of the shf pattern in these cases was therefore based upon the g_o (and A_o) values (see below).

Our (overall) formation constants for the various complexes do not differ from the corresponding literature pH potentiometric values to a higher extent than pH potentiometric data usually differ from each other (Table 1). The formation (micro)constants

(19) Gergely, A.; Nagypál, I.; Farkas, E. *J. Inorg. Nucl. Chem.* **1975**, *37*, 551−555.
(20) Lomozik, L.; Wojciechowska, A.; Jaskólski, M. *Monatsh. Chem.* **1983**, *114*, 1185−1188.
(21) Berthon, G.; Hacht, B.; Blais, M.-J.; May, P. *Inorg. Chim. Acta* **1986**, *125*, 219−227.
(22) Brumas, V.; Alliey, N.; Berthon, G. *J. Inorg. Biochem.* **1993**, *52*, 287−292.

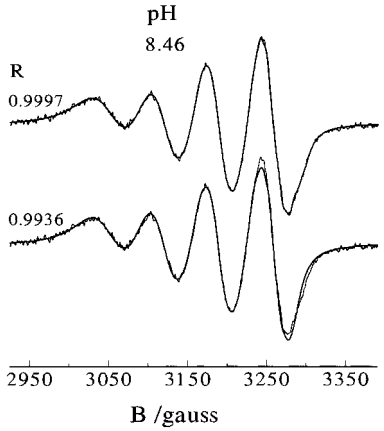

Figure 3. Experimental and calculated EPR spectra at excess L-asparagine. The spectrum of the complex $[CuL_2]$ is described as a two-component curve with the parameters in Table 2 for the upper spectrum, while it is taken as a one-component curve for the lower spectrum, where $g_o = 2.1258$, $A_o = 67.1$ G, α, β, $\gamma = 19.2, -7.6, 2.0$ G, respectively, $a_{No} = 9.7$ and 9.7 G.

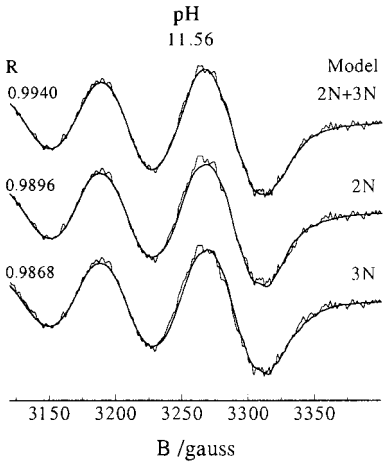

Figure 4. Experimental and calculated EPR spectra at excess L-asparagine. The spectrum of the complex $[CuL_2H_{-1}]^-$ is described as a two-component curve with the parameters in Table 2 for the uppermost spectrum, while it is taken as a one-component curve for the lower spectra. With the 2N model, $g_o = 2.1133$, $A_o = 74.0$ G, α, β, $\gamma = 22.3, -8.2, 1.8$ G, respectively, $a_{No} = 13.5$ and 10.7 G. With the 3N model, $g_o = 2.1165$, $A_o = 74.9$ G, α, β, $\gamma = 25.5, -10.5, 0.9$ G, respectively, $a_{No} = 11.9$, 11.9, and 6.2 G.

and the EPR parameters for every single species we identified are listed in Table 2. The corresponding concentration distribution curves are depicted in Figure 6, and the respective component spectra are in Figure 7.

Confidence Intervals of Parameters. Table 2 gives the confidence intervals for the various data. In highly acidic solution, two species which yield broad EPR lines and similar parameters coexist: the aqua complex and $[CuLH]^{2+}$; this makes decomposition of the spectra in this region less precise. This is reflected in the relatively large confidence intervals, particularly for the parameters of the complex $[CuLH]^{2+}$. The decrease in quality of the spectral fit when the complex $[CuLH]^{2+}$ was neglected (see in the previous section) could be reduced if the program was allowed to alter the parameters of the aqua complex much beyond the standard deviations of the independent measurements. When the complex $[CuLH]^{2+}$ was taken into consideration, a correlation between its parameters and those of the aqua complex was observed. The EPR parameters of the aqua complex were therefore kept near the averages of the "independent" values during the optimization.

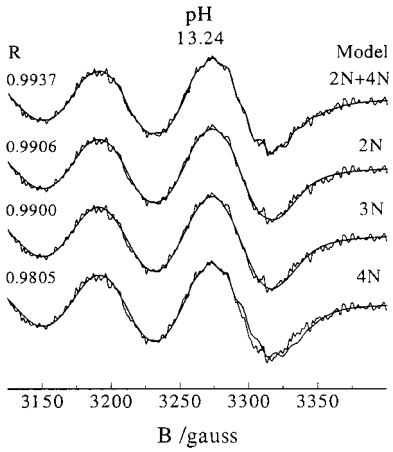

Figure 5. Experimental and calculated EPR spectra at excess L-asparagine. The spectrum of the complex $[CuL_2H_{-2}]^{2-}$ is described as a two-component curve with the parameters in Table 2 for the uppermost spectrum, while it is taken as a one-component curve for the lower spectra. With the 2N model, $g_o = 2.1142$, $A_o = 79.4$ G, α, β, $\gamma = 24.1, -6.6, 1.2$ G, respectively, $a_{No} = 13.0$ and 11.8 G. With the 3N model, $g_o = 2.1141$, $A_o = 79.32$ G, α, β, $\gamma = 22.2, -6.6, 1.2$ G, respectively, $a_{No} = 13.2$, 9.8, and 9.8 G. With the 4N model, $g_o = 2.1134$, $A_o = 79.1$ G, α, β, $\gamma = 18.4, -5.6, -0.2$ G, respectively, $a_{No} = 13.2$, 13.2, 11.4, and 11.4 G.

Table 1. Overall Formation Constants[a] Expressed as log β for the Copper(II)−L-Asparagine System

	method				
	pH potentiometry				ESR
complex	ref 19	ref 20	ref 21	ref 22	this work[b]
$[CuLH]^{2+}$				10.08	10.27
$[CuL]^{+}$	7.79	7.69	7.71	7.79	8.10
$[CuLH_{-1}]$		2.33	0.68		1.11
$[CuL_2H_2]^{2+}$			20.19		
$[CuL_2H]^{+}$			17.42		
$[CuL_2]$	14.29	14.38	14.20	14.14	14.52
$[CuL_2H_{-1}]^{-}$	3.84		3.94	4.17	4.00
$[CuL_2H_{-2}]^{2-}$	−8.16				−8.20

[a] The sums of the formation constants of the isomers for a given species. [b] For the proton complexes [LH] and $[LH_2]^+$, the log β values of 8.74 and 10.88, respectively, from ref 19 were used.

For the other component spectra of the resolved copper hf structure (Figure 7), the confidence intervals of g_o, A_o, the relaxation parameters α, β, and γ, and the formation constants are small even for the minor species. This holds for a large majority of the N shf coupling constants, too (Table 2).

EPR Parameters and Coordination Modes. The general statements that we applied to establish the coordination modes are given elsewhere.[14] Here, we wish to stress merely one point. The EPR spectra of copper(II)−L-asparagine complexes in frozen solution reveal a $d_{x^2-y^2}$ ground state,[23] which corresponds to elongated square-(bi)pyramidal geometry.[14] The EPR parameters are therefore sensitive to alterations in the equatorial coordination, whereas the weaker axial coordination has only a minor and indirect effect. This also holds for the wavelength of the nonresolved visible absorption band, which is the sum of the bands, in the sequence of increasing energy, of the $d_{z^2} \leftarrow d_{x^2-y^2}$, $d_{xy} \leftarrow d_{x^2-y^2}$ and $d_{xz,yz} \leftarrow d_{x^2-y^2}$ transitions for effective D_{4h} symmetry. It may be concluded from single-crystal polarized

(23) Szabó-Plánka, T.; Rockenbauer, A.; Gyõr, M.; Gaizer, F. *J. Coord. Chem.* **1988**, *17*, 69−83.

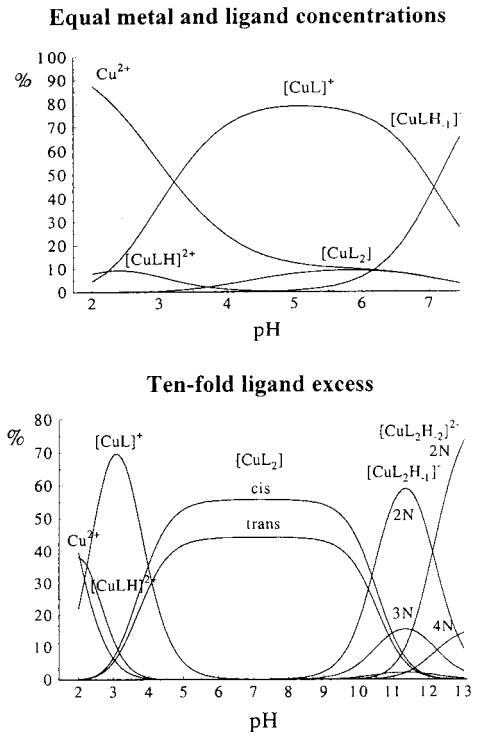

Figure 6. Concentration distribution in the copper(II)−L-asparagine system, calculated from the EPR spectroscopic formation constants. For the complex $[CuL_2]$ in 1:1 solution, the sum of the concentrations of the isomers is given. At excess ligand, the isomers are distinguished either by the number of equatorial N donors or by the arrangement of the equatorial donors of the same kind.

Table 2. EPR Parameters and Formation Constants Expressed as log β of the Copper(II)−L-asparagine Complexes, Together with the Corresponding Confidence Intervals in Parentheses

	parameters						
species	g_o	A_o/G	a_{No}/G	α/G	β/G	γ/G	log β
Cu^{2+}	2.1955	33.8		52.2	−1.8	0.6	
	(0.0011)	(1.0)		(2.4)	(1.2)	(1.2)	
$[CuLH]^{2+}$	2.1920	39.1		49.5	−1.8	0.9	10.27
	(0.0026)	(2.1)		(5.2)	(1.9)	(2.2)	(0.06)
$[CuL]^{+}$	2.1534	56.5	10.0 (0.5)	27.8	−6.4	0.7	8.10
	(0.0003)	(0.3)		(0.5)	(0.3)	(0.2)	(0.02)
$[CuLH_{-1}]$	2.1227	65.5	11.8 (0.9)	21.5	−9.2	3.6	1.11
	(0.0006)	(0.8)	6.7 (3.0)	(1.6)	(1.0)	(0.7)	(0.08)
$[CuL_2]$							
cis	2.1260	64.0	8.7 (0.3)	19.3	−8.1	1.3	14.26
	(0.0003)	(0.3)	8.7 (0.3)	(0.6)	(0.4)	(0.2)	(0.03)
trans	2.1248	72.8	10.6 (0.5)	20.4	−8.2	1.2	14.17
	(0.0005)	(0.6)	10.6 (0.5)	(0.9)	(0.8)	(0.4)	(0.04)
$[CuL_2H_{-1}]^{-}$							
2N	2.1143	72.3	11.7 (0.5)	23.6	−8.9	1.1	3.33
	(0.0003)	(0.4)	8.0 (1.0)	(0.7)	(0.4)	(0.3)	(0.05)
3N	2.1078	82.1	7.7 (2.2)	29.8	−14.3	0.9	3.90
	(0.0010)	(1.1)	7.0 (1.2)	(1.3)	(0.9)	(0.6)	(0.02)
			7.0 (1.2)				
$[CuL_2H_{-2}]^{2-}$							
2N	2.1154	79.1	13.2 (1.1)	20.2	−6.4	1.0	−8.29
	(0.0004)	(0.5)	11.4 (1.4)	(1.2)	(0.7)	(0.5)	(0.12)
4N	2.0969	75.7	12.4 (2.2)	18.0	−7.7	0.7	−8.95
	(0.0011)	(1.3)	12.4 (2.2)	(1.6)	(1.0)	(0.7)	(0.08)
			12.3 (2.3)				
			12.3 (2.3)				

and absorption electronic spectral studies[24] that the wavelength of the visible band is nearly equal to the wavelength of the $d_{xy} \leftarrow d_{x^2-y^2}$ band of medium energy and highest intensity. It is the

(24) Brown, D. H.; Mangrio, N. G.; Smith, W. E. *Spectrochim. Acta* **1979**, *35A*, 123−125.

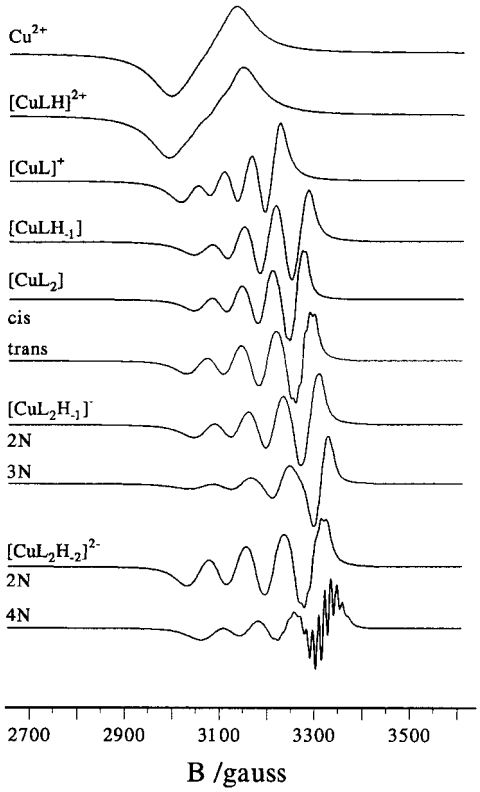

Figure 7. Calculated EPR spectra for all active complexes assigned in the copper(II)−L-asparagine system by using the data in Table 2 at a frequency of 9.4 GHz.

energy of this band that has the greatest effect on g_o.[14] This can explain the good correlation observed between λ_{max} and g_o in many equilibrium systems. The coordination by the O donors of water molecules, OH^- ions, and amide or peptide groups represents a weak ligand field and a high λ_{max} and g_o. When these atoms are substituted by carboxylate O, amino N, or deprotonated peptide N donors, an increasing extent of blue shift is observed,[25] which is accompanied by a decrease in g_o and, in the event of effective D_{4h} symmetry, by an increase in A_o.[14] (The effect of deprotonated amide N is similar to that of deprotonated peptide nitrogen.) Consequently, the g_o (and A_o) values afford useful information on the nature of the equatorial donor groups.

The EPR parameters for $[CuLH]^{2+}$ and the aqua complex are rather close to each other, which indicates a weak ligand field in the former species, as is expected for coordination of the weak O donors of water molecules, carboxylate group, and possibly amide group, while the amino group is protonated. The considerably smaller g_o and larger A_o values for the complex $[CuL]^+$ (Table 2) correspond to the much stronger ligand field represented by the "classic" glycine-like binding mode through the amino N and carboxylate O donors. As concerns the complex $[CuLH_{-1}]$, either the amide group or a coordinated water molecule may be deprotonated. As only negligible changes are expected in the ligand field, g_o and A_o in the second case, the considerable decrease observed in g_o and the increase in A_o (Table 2) suggest proton loss and equatorial coordination of the amide N.

For the complex $[CuL_2]$, our analysis indicates an isomeric equilibrium, most probably between the cis and trans arrangements of the two equatorial amino N and two carboxylate O donors, as suggested by other authors[7,8] studying the shf

(25) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385−426.

structure of the high-field band in the second-derivative ^{63}Cu EPR spectra. The g_o values for the isomers are nearly identical, revealing that the same donor atoms are coordinated in equatorial positions. The copper coupling constants, however, differ significantly. This indicates that the donor atoms are arranged differently in the isomers, the lower A_o value corresponding to strong rhombic distortion. Since rhombic distortion is expected for the cis coordination, we assigned the spectrum with lower A_o to the cis isomer.

Proton loss from the above species is accompanied by a decrease in g_o. Apart from the complex $[CuL_2]$, however, the g_o values also differ significantly for the isomers of the complex $[CuL_2H_{-1}]^-$, suggesting different equatorial donor atom sets. For one of the isomers, the decrease in g_o is considerable; here, proton loss and equatorial coordination of an amide N are most probable (3N isomer). For the other species, the decrease in g_o is much less, suggesting the equatorial binding of only two N donors (2N isomer). Such coordination can arise in two different ways: binding of a deprotonated water molecule (i.e., a OH^- ion) or (in the event of proton loss from the amide group) axial ligation of one of the three N donors. If the amino N atoms remained in the equatorial positions, while a OH^- occupied an axial site, or if one of the equatorial carboxylate O atoms were replaced by a OH^- ion, an increase rather than a decrease in g_o would be expected. Replacement of an amino group by a deprotonated amide group is more probable: this corresponds to a moderately strengthened ligand field in the 2N isomer as compared to the complex $[CuL_2]$. It is the amino group of the deprotonated ligand which is likely to occupy an axial position, while coordination of ligand L is unaltered: it is most unlikely that ligand L would form a weak axial bond involving its strong amino N donor and two strong equatorial bonds involving the weaker amide and carboxylate O donors.

The further proton loss at high pH seems to proceed in two different ways. The very low g_o for the minor isomer of the complex $[CuL_2H_{-2}]^{2-}$ can be explained by the deprotonation of the second amide group and the equatorial binding of all four N donors (4N isomer). In contrast, for the major isomer g_o is unexpectedly high, equal to g_o for the 2N isomer of the complex $[CuL_2H_{-1}]^-$. In this case, coordination of a OH^- ion (a deprotonated water molecule) in the fourth equatorial position is more probable than proton loss combined with equatorial coordination of the second amide group. In principle, axial coordination of the deprotonated amide group(s) may also occur, but formation of a weak axial bond would be rather unlikely to induce proton loss from the amide group. The coordination modes suggested for the various L-asparagine complexes are shown in Figure 8.

Our data offer less information on the axial coordination. Literature pH potentiometric and calorimetric[19] studies have demonstrated that the complex $[CuL_2]$ of L-asparagine is more stable than the analogous α-aminobutyric acid complex, which is explained by the partial coordination of the amide group(s). Simultaneously, NMR relaxation studies[26] showed that the rate of ligand exchange for the first complex was considerably lower than that for the second species, indicating axial bonds in the asparagine complex. Moreover, there was a significant further reduction in the rate of ligand exchange on deprotonation of the bis L-asparagine complexes,[26] suggesting even stronger axial bonds. All these points were taken into consideration in connection with the suggested coordination modes in Figure 8.

Formation Constants of EPR-Inactive Metal Complexes. For the copper(II)−L-asparagine system, no oligomerization was revealed by pH potentiometry,[19−22] and our EPR measurements indicate no inactive metal complexes. We demonstrate this phenomenon by using other systems as examples. In moderately alkaline solutions of copper(II) and simple dipeptides in a ratio of 1:1, a species of composition $[Cu_2L_2H_{-3}]^-$ was identified by pH potentiometry.[27] The existence of this EPR-silent complex was similarly supported by our recent EPR analysis of the copper(II)−glycyl-l-serine and copper(II)−l-seryl-glycine systems.[28] For the former system, Figure 9 compares the pH dependence of the total copper(II) concentrations calculated from the data in ref 28, if the EPR-inactive dimer is neglected or taken into account. When the formation constant of the inactive species is optimized, the calculated copper(II) concentrations

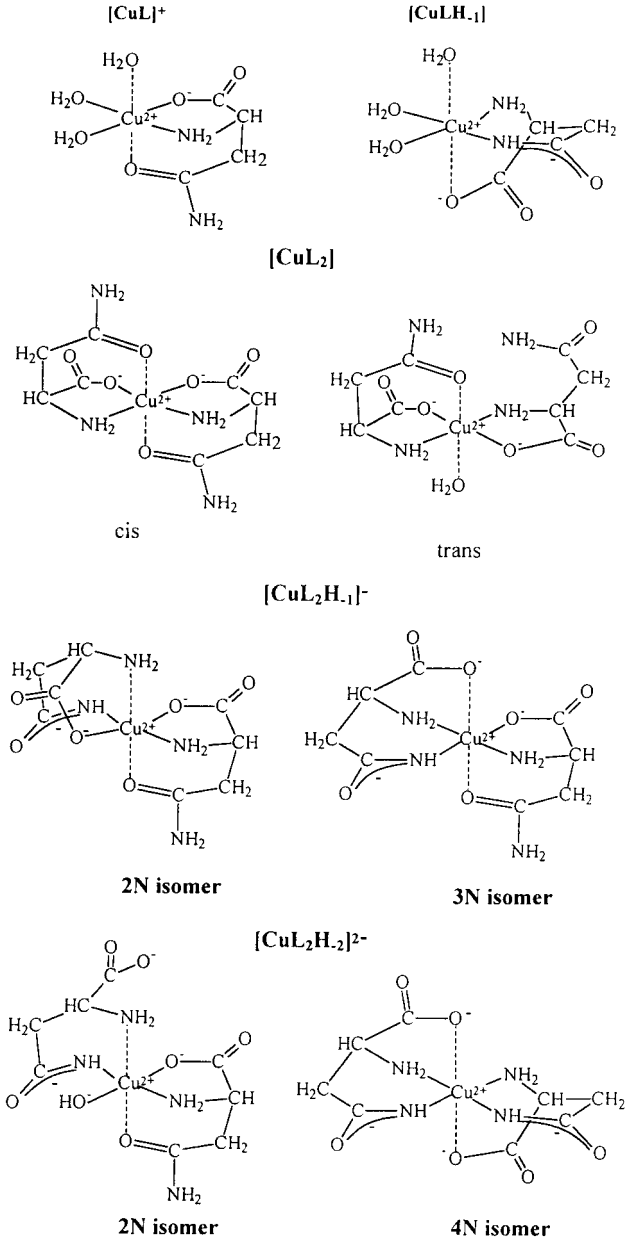

Figure 8. Coordination modes proposed for the various copper(II)−L-asparagine complexes.

(26) Nagypál, I.; Farkas, E.; Gergely, A. *J. Inorg. Nucl. Chem.* **1975**, *37*, 2145−2149.

(27) (a) Gergely, A.; Farkas, E. *J. Chem. Soc., Dalton Trans.* **1982**, 381−386. (b) Farkas, E.; Tõzsér, J.; Gergely, A. *Magy. Kém. Foly.* **1986**, *92*, 49−54. (c) Farkas, E.; Kiss, T. *Polyhedron* **1989**, *8*, 2463−2467. (d) Shtyrlin, V. G.; Gogolashvili, E. L.; Zakharov, A. V. *J. Chem. Soc., Dalton Trans.* **1989**, 1293−1297.

(28) Szabó-Plánka, T.; Árkosi, Zs.; Rockenbauer, A.; Korecz, L. *Polyhedron* **2001**, *20*, 995−1003.

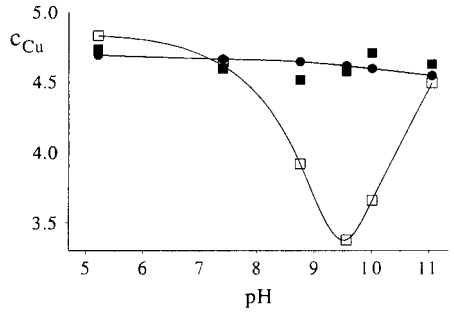

Figure 9. Analytical and calculated total copper(II) concentrations for 1:1 solutions in the copper(II)−glycyl-L-serine system, where ● denotes the analytical metal concentration, obtained from the initial concentration and the dilution ratio, changing together with the amount of NaOH solution added; ■ symbolizes the copper(II) concentration calculated from the mass balance equations by using the EPR spectroscopic formation constants (β_{pqr}) of the complexes $Cu_pL_qH_r$ from ref 28 (log $\beta_{111} = 9.26$, log $\beta_{110} = 5.74$, log $\beta_{11-1} = 1.81$, log $\beta_{11-2} = -7.79$, log $\beta_{11-3} = -20.41$, log $\beta_{12-1} = 4.72$, and for the inactive species log $\beta_{22-3} = -4.03$); □ denotes the copper(II) concentrations calculated from the previous data by neglecting the inactive dimer.

are close to the corresponding analytical concentrations, while omission of the "EPR-silent" species results in significantly decreased calculated total concentrations in the moderately alkaline pH region. For both glycyl-L-serine and L-seryl-glycine ligands, the EPR spectroscopic formation constants for the inactive species (log $\beta = -4.03$ and -3.99, respectively)[28] are in agreement with the pH potentiometric values of -3.80 and -4×10^{27}.

Conclusions

By analyzing the copper(II)−L-asparagine system at various pH values and metal and ligand concentrations, we have demonstrated the efficiency of our "two-dimensional" EPR method in the assignment and characterization of the numerous species formed in a complex equilibrium system. The variation of the regression factor offers a reliable device with which to decide between alternative speciation models. Identification of the various species and determination of their EPR parameters can still succeed if their relative concentration achieves 10−15%. Good confidence in the formation constants and the EPR parameters can be attained even for the isomer pairs present in the investigated systems.

In solutions of the copper(II)−L-asparagine system at a metal ion:ligand ratio of 1:1, we have suggested deprotonation and equatorial coordination of the side-chain amide group above pH 6. For the bis complexes, different coordination modes coexist: the cis−trans isomerism in the complex $[CuL_2]$. The isomers of the complex $[CuL_2H_{-1}]^-$ differ from each other in the position (equatorial or axial) of the amino group of the deprotonated ligand. Proton loss from the second side-chain amide group is a minor process; the major isomer of the species $[CuL_2H_{-2}]^{2-}$ is a mixed hydroxo complex.

For the copper(II)−dipeptide systems, where dimerization occurs in alkaline 1:1 solutions, our method can also afford reliable formation constants for the EPR-inactive species.

The method can be applied not only for the copper(II) complexes of other, not too large bioligands, but also for the complexes of other metal ions, e.g., for vanadium(IV), where the metal hf pattern is well resolved. It appears more problematic to study metals coordinated to bulky ligands such as proteins, where the slow molecular tumbling can produce a broad nonresolved hf structure. In this case, the application of lower EPR bands can improve the selectivity of spectrum decomposition at the cost of the less intensive relaxation broadening.

Experimental Section

Reagents and Solutions. The L-asparagine (denoted by HL in its neutral form) and other reagents from Reanal (Hungary) were of analytical grade and were used without further purification. The copper(II) concentration of the solutions was 5 mmol dm^{-3}, while the ligand:metal ion concentration ratio was 1:1 or 10:1. We used 0.2 M KCl as background electrolyte. The optimum concentrations and pH were chosen to meet the requirement that even the less stable complexes should form in as high a concentration as possible. Preliminary species distribution curves were calculated with the program PSEQUAD[29] from literature formation constants.[19−22] The pH was adjusted with HCl, and then NaOH to an accuracy of 0.01 pH unit, using a Radelkis OP208/1 pH-meter equipped with a Radiometer GK2401C combined glass electrode. The electrode was calibrated with IUPAC standard buffers from Radiometer. At a ligand excess, solutions of pH 13.1 and 13.24 were made by adding the calculated amount of 20 M NaOH solution.

EPR Measurements. A 10 cm^3 stock solution was titrated under an argon atmosphere. The sample was mixed by bubbling the gas through the solution. A Masterflex CL peristaltic pump ensured the circulation (14 cm^3 min^{-1}) of the solution through the capillary tube in the cavity. The EPR spectra were taken after circulation for 3 min at the chosen pH at room temperature (291 K) on an upgraded JEOL JES-FE3X spectrometer with 100 kHz field modulation, using a manganese(II)-doped magnesium oxide powder for the calibration of *g*. Further details of measurements were described previously.[14]

The parameters for the aqua complex were determined independently, in the absence of asparagine at 5 mM copper(II) chloride and 0−0.4 M potassium chloride, to decide whether interaction with the chloride ion could be revealed. The parameters of the aqua complex were found to be independent of the amount of the background electrolyte.

Evaluation of Spectra. For the experimental EPR spectra, we carried out background signal elimination by subtracting the "glass signal" which is dominated by the major perpendicular band at $g = 1.955$ due to the vanadium centers in the Pyrex tube, which also contained the manganese peaks of the manganese(II)−magnesium oxide external standard (see Figure 1 in ref 14). To compensate the minor frequency shifts in the course of measurements, the field scale was shifted to secure a perfect fit for the manganese lines of the external standard. Since the copper(II) chloride used to make the stock solution contained a natural mixture of isotopes, the spectrum of each species was calculated as the sum of spectra containing ^{63}Cu and ^{65}Cu weighted by their abundances in nature. The hf coupling constants and relaxation parameters given throughout the paper refer to the isotope ^{63}Cu. In accordance with literature ENDOR studies[30] which found a difference between the coupling constants for the sp^3 and sp^2 N atoms, we assumed only that N atoms of the same type were equivalent.

Acknowledgment. We thank the Hungarian Scientific Research Fund OTKA (Grant T-032929) for financial support. Our special thanks are due to Professor István Nagypál for helpful discussions regarding the pH potentiometric method and valuable comments on the manuscript. The assistance of Mr. Gy. P. Pálfy in the study of the copper(II)−L-asparagine system is gratefully acknowledged.

JA0102888

(29) Zékány, L.; Nagypál, I.; Peintler, G. *PSEQUAD for Chemical Equilibria*; Technical Software Distributors: Baltimore, MD, 1991.

(30) Iwaizumi, M.; Kudo, T.; Kita, S. *Inorg. Chem.* **1986**, *25*, 1546−1550.