Metal ion interactions with antibiotic drugs: Part III—Complex formation of copper(II) with ampicillin

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Combined pH-metric and spectrophotometric study on the complex formation of Cu²⁺ with ampicillin,a-d-(−)-aminobenzylpenicillin, ampH⁺, at 37°C in aqueous medium at a fixed ionic strength, I = 0.1 M NaNO₃, indicates formation of complexes of the types: Cu([amp] = Cu(amp)₂, Cu([H⁻] amp), Cu([H⁻] amp)(amp)⁻ and Cu([H⁻] amp)⁻. Proton-ligand and metal-ligand co.istants are found to be: pK_HCOOH 2.50; pK_H₂COOH 7.05; pK_HCOOH(calc.), 13.37; log K_Cu(amp) 4.79; log K_Cu(amp) 7.70; pK_Cu(amp) 8.50; pK_Cu(amp) 9.81; pK_Cu(amp) 10.31; log β_Cu(amp) 3.71; log β_Cu(amp) 2.11; log β_Cu(amp) 12.42 and log K_Cu(amp) 22.08 ± 0.05. Ambidentate nature of the ligand and its possible modes of coordination with the Cu²⁺ at different pH values have been elucidated on the basis of electronic spectral measurements.

Penicillin group of pharmaceuticals function as broad spectrum antibiotics due to their ability to inhibit the protein synthesis on the ribosomes. The transfer of the genetic information for the synthesis of a specific protein requires the involvement of several enzymes. Most of these enzymes require one or other of the metal ions for their structural organisation and their activity. Metal ions are also known to stabilize the ribosome conformations. Complexation equilibria of metal ions with relatively simple biomolecules such as amino acids, small peptides, nucleic bases, nucleosides and nucleotides provided useful models to understand the nature of metal-protein, metal-nucleic acid interactions occurring in the biological systems. The aim of the present investigation is to study the complex formation of metal ions with a commonly used form of penicillin, viz., a-d-(−)-aminobenzylpenicillin (I), which is popularly known as ampicillin (hereafter, ampH⁺) in the absence and in the presence of biologically important ligands mentioned above, with a view to elucidating the molecular mechanism of action of this drug and the effect of metal ions thereon.

Materials and Methods

Ampicillin trihydrate, ampH⁺.3H₂O (99%) [M.W. 403.46; m.p. 198-200°C (dec.), [α]D 24° (C = 1, H₂O)] obtained from Aldrich Chemical Company, Inc. (USA), was directly used. It was stored in a cool and dry place away from light.

All the other reagents were of AR grade and their solutions were prepared in fresh doubly distilled, CO₂-free water. Aqueous solution of ampH⁺ (0.005 M) was preserved in a refrigerator for several days and electronic spectrum of this solution was run a number of times. The solution did not show any change in its UV absorption spectrum (267 nm, 260 nm). Equivalent weight of ampH⁺.3H₂O was determined potentiometrically and found to be 403.2 (Calc. 403.46). Yet, always freshly prepared solutions, obtained by dissolving weighed quantities of the drug were used for equilibrium and spectral measurements.

The following solutions were prepared for the pH metric study:

(i) 0.005 M HNO₃
(ii) (i) + 0.001-0.002 M Cu(NO₃)₂
(iii) (ii) + 0.005 M ampicillin in the form of ampH⁺
(iv) (iii) + 0.001-0.002 M Cu(NO₃)₂

The structural formula of the ligand species ampH⁺ is similar to that of ampH⁺(I), having its COO⁻ group protonated. Initial volume of each solution was 25 ml and a constant ionic strength of I = 0.1 M.
was maintained by adding requisite amount of NaNO₃. All the solutions were allowed to attain the equilibrium at 37 ± 0.1°C (thermostated) and titrated with a carbonate-free standard 0.1 M aqueous NaOH solution maintained at the same temperature.

pH measurements were made with a Systronics digital pH meter, type 335 (accuracy ± 0.01 pH units) using a special glass electrode (pH 1-14) in conjunction with an SCE. Analytical hydrogen ion and hydroxyl ion concentrations, [H⁺] and [OH⁻] respectively, corresponding to different pH values at the various stages of the titration were obtained as usual from the pH vs -log[H] and pH vs -log[OH⁻] plots of the titrations of free HNO₃, i.e., solution (i). Ionic product (pKw) of water at the experimental temperature was obtained from literature. All the titrations were carried out three times and pH, volume (of NaOH) data as were the averages of three titrations, were used for the calculation of the stability constants. Proton-ligand and metal-ligand constants were calculated with the aid of the SCOGS programme, run on a PC-XT computer system. Initial estimates of some of these constants, supplied to the computer as the input data, were obtained by analysing the pH titration curves according to the procedure of Irving and Rossotti.

### Results and Discussion

In the absence of metal ion, the ligand ampicillin, in the form of ampH₂⁺, exhibited two buffer regions corresponding to successive deprotonation of the -COOH and NH₃⁺ groups in the pH ranges 2-4 and 6-8 respectively. The wide difference between the pKCOOH and pKNH₃ values indicated the zwitterionic nature of the ligand, ampH⁺, in the intermediate pH values. At very high pH (pH > 12), another drawn out buffer region was observed, which might be due to the partial deprotonation of the amide (-CONH-) moiety of the ligand. Computer refined values of pKCOOH and pKNH₃; along with the estimated value of pKCONH are presented in Table 1.

### Table 1—Proton-ligand and Cu²⁺-ligand constants with ampicillin at 37 ± 0.1°C in aqueous solution, I= 0.1 M(NaNO₃)

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
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<tbody>
<tr>
<td><strong>Proton ligand constants</strong></td>
<td></td>
</tr>
<tr>
<td>pKCOOH</td>
<td>2.50</td>
</tr>
<tr>
<td>pKNH₃</td>
<td>7.05</td>
</tr>
<tr>
<td>pKCONH</td>
<td>13.37 (calc.)</td>
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</tbody>
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<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>Cu²⁺-ligand constants</strong></td>
<td></td>
</tr>
<tr>
<td>log KCuHamp</td>
<td>4.79</td>
</tr>
<tr>
<td>log KCuHamp/H₂amp</td>
<td>2.91</td>
</tr>
<tr>
<td>log KCuamp/#Hamp</td>
<td>7.70</td>
</tr>
<tr>
<td>log KCuamp/Hamp</td>
<td>8.50</td>
</tr>
<tr>
<td>pKCuHamp</td>
<td>9.81</td>
</tr>
<tr>
<td>pKCuHamp/H₂amp</td>
<td>10.31</td>
</tr>
<tr>
<td>log KCuHamp/#Hamp</td>
<td>-3.71</td>
</tr>
<tr>
<td>log KCuHamp/Hamp</td>
<td>-2.11</td>
</tr>
<tr>
<td>log KCuHamp/#Hamp</td>
<td>-12.42</td>
</tr>
<tr>
<td>log KCuHamp/Hamp</td>
<td>-22.08</td>
</tr>
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*aLimits of error in the constants: ± 0.05 in log units. Other constants used: pKw = 13.62 (ref. 10), pKH⁺ = 6.29; pKONH = 13.05 (at 37°C).
Complex formation of Cu²⁺ ion with the ligand was observed in the pH range 3 < pH < 11. A plot of n (average number of protons liberated due to the reaction of Cu²⁺ ion with the ligand) vs pH (Fig. 1) clearly showed the stepwise nature of these reactions and release of 4H⁺ per Cu²⁺. It was further observed that 2H⁺ per Cu²⁺ were released between pH 3 and 7.5 in two discrete one-proton steps. The remaining 2H⁺ per Cu²⁺ were released in overlapping steps. As the initial buffer region corresponding to the Cu²⁺-ligand equilibria was observed at pH values much lower than the buffer region corresponding to the hydrolytic equilibria of Cu²⁺ (aq.) ions, so the hydroxo complexes, Cu(OH)⁺ and Cu(OH)₂ were not considered in calculating the metal-ligand constants.

Spectrophotometric measurements were carried out to elucidate the metal: ligand ratios in the complexes formed between Cu²⁺ and the drug. A 1:5 (Cu²⁺:ampH⁺) mixture showed λ max at 730, 670 and 530 nm at pH 5.0, 7.7 and 11.0 at which the n values were 1, 2 and 4 respectively. The Job’s method of continuous variation provided evidence of formation of 1:1 (Cu²⁺:ligand) complexes at n = 1 and 1:2 (Cu²⁺:ligand) complexes at n = 2 and 4. In the pH range 3 < pH < 7.5 the free ligand was predominantly present in the ampH± form. So the formation of 1:1 and 1:2 (Cu²⁺:ligand) complexes through the reaction of Cu²⁺ ion with ampH± with release of two protons in two successive one-proton steps, therefore, could take place according to the equilibria (1) and (2).

\[
\text{Cu}^{2+} + \text{ampH}^+ \leftrightarrow a\text{Cu(amp)}^+ + H^+ \tag{1}
\]

\[
K_{\text{Cu(amp)}}^{\text{Cu}} = \frac{[\text{Cu(amp)}]}{[\text{Cu}][\text{amp}]} \tag{1}
\]

\[
\text{Cu(amp)}^+ + \text{ampH}^+ \leftrightarrow \text{Cu(amp)}_2 + H^+ \tag{2}
\]

\[
K_{\text{Cu(amp)}}^{\text{Cu(amp)}} = \frac{[\text{Cu(amp)}_2]}{[\text{Cu(amp)}][\text{amp}]} \tag{2}
\]

The ligand species, amp⁻ in the Cu(amp)⁺ and Cu(amp)₂ complexes has the same structural formula (I) as that of the ampH± species with its -NH₃⁺ group deprotonated. In defining the equilibrium constants, charges are dropped for simplicity.

\[
\lambda_{\text{max}} \text{ values of 730 and 670 nm of Cu}^{2+}\text{-ampH}^± \text{mixtures at pH 5.0 (}n=1\text{) and pH 7.7 (}n=2\text{) respectively (Fig. 2) were in good agreement with the calculated }\lambda_{\text{max}} \text{ values for square planar [Cu(NH₃)(O = C)(H₂O)]₂ and [Cu(NH₃)₂(O = C)]₂ geometries of Cu}^{2+} \text{ complexes with small peptide ligands. It was, therefore, concluded, that the amp⁻ ligand species in the Cu(amp)⁺ and Cu(amp)₂ complexes coordinated Cu}^{2+} \text{ using the amino nitrogen atom and the carbonyl oxygen atom of the amide bond. With increase in pH (} > 8\text{), the 1:5 (Cu}^{2+}\text{:ligand) mixture gave another buffer region (8 < pH < 11), when the colour of the solution changed from blue to reddish violet.}\]

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Concentration distribution profiles (Fig. 1) of the ligand species amplified and the metal containing species, Cu²⁺, Cu(amp)⁺, Cu(amp)₂ and Cu(H₉amp)(amp)⁻ were in good agreement with the complexation equilibria (1) to (6). Cu(amp)⁺ complex comprised about 80% of total copper at pH 6. Both Cu(amp)⁺ and Cu(amp)₂ complexes dominated the pH region of biological importance, with the latter complex contributing nearly 60%. Amide deprotonated complexes, Cu(H₉amp) and Cu(H₉amp)(amp) were formed in significant amounts at pH > 7 and dominated the region between pH 9 and 11.

It is interesting to note that in the region of biological pH (7.0-7.5) nearly 50% of the metal ion remains in the Cu(amp)⁺ form and the remaining 50% in the Cu(amp)₂ form. The concentrations of the free ligand species, ampl, and amp⁻ are also comparable (30%) in this region.

At still higher pH values the concentration of the fully deprotonated 1:2 (Cu²⁺:ligand) complex, Cu(H₉amp)(amp)⁻, increased sharply, indicating the existence of the equilibrium,

\[
\text{Cu(H₉amp)} + \text{amp}^- \rightleftharpoons \text{Cu(H₉amp)(amp)}^- + \text{H}^+
\]

as an additional contributor to the formation of this complex. The Eq. constant, \(K_{\text{Cu(H₉amp)+amp}}\), could be calculated with the aid of the following relation,

\[
\log K_{\text{Cu(H₉amp)+amp}} = \log \beta_{\text{Cu(H₉amp)+amp}} + \log \beta_{\text{amp}} + \log \beta_{\text{amph}}
\]

using the computer-refined values of \(\log \beta_{\text{Cu(H₉amp)+amp}}\) and \(\log \beta_{\text{amp}}\), and the estimated value of amide deprotonation constant, \(pK_{\text{amp}}\) of the ligand, amp⁻ ion.

The colour of the titrated solution at pH > 11 gradually turned intense red violet (\(\lambda_{\text{max}}, \text{530 nm}\)) during the course of 2-3 days (Fig. 2). This was obviously due to some remarkable change of ligand field strength around the Cu²⁺ ion in the Cu(H₉amp)(amp)⁻ complex. The only possibility could be apical coordination of Cu²⁺ by the thiazolidine sulphur atom of the ligand, giving a distorted octahedral geometry (II) to the complex.

The slow increase in intensity of the red violet colour of the solution might be due to sterically hindered slow movement of the rest of the ligand molecule containing the β-lactam ring and the thiazolidine sulphur atom which provides axial coordination to Cu²⁺ by the sulphur atom. This colour change was irreversible and was accelerated by warming. The irreversible nature of this process indicates an ideal fit of the ligand molecule in its (N, N, S) terdentate mode of bonding with the Cu²⁺ ion, thereby conferring extra stability on the resulting complex. The high intensity of colour of the resulting solution could be assigned to the charge transfer due to delocalization of filled \(d\pi\)-electrons of Cu²⁺ ion over to the vacant \(d\pi\) orbitals of the sulphur atoms as shown in (III). A single band due to
\(2E^*_g \rightarrow 2T^*_g\) transition in the electronic spectrum of the \(\text{Cu}^{2+}\)-amp\(^-\) mixture ruled out the scope of any assumption of oxidation of \(\text{Cu}^{2+}\) to \(\text{Cu}^{3+}\) under this condition.

The utility of an antibiotic such as penicillin hinges on some sort of toxicity differential between the parasite and host. This in turn depends on exploitable differences in their metabolic patterns.\(^{19,20}\) In the case of \(\beta\)-lactam antibiotics, the cell wall appears to be the target that permits the selective inhibition of parasite in the presence of the host cells.

The present equilibrium study has revealed the ligational behaviour of the antibiotic ampicillin with respect to binding of metal ions. The modes of its coordination and its potential metal-binding sites have been elucidated. Coordination of the drug with the enzyme-bound metal ions may cause selective denaturation of the enzyme in some way, leading to inhibition of the parasite. The formation of enzyme-metal-drug complex may provide a possible mechanistic pathway in these processes. However, for elucidation of the nature of enzyme-metal-drug interactions, further equilibrium studies on the ternary systems, metal-[drug]L, with L= amino acids, and small peptides are needed, which are in progress.

**References**