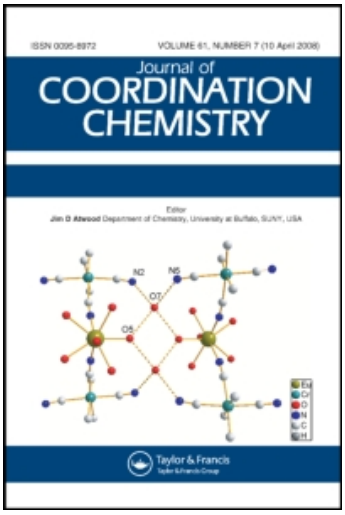

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Coordination mode in the binary systems of copper(II)/O-phospho-L-serine

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Results of equilibrium and spectral studies have shown that in the O-phospho-L-serine (Ser-P) with copper(II) system the complexes MHL, ML, ML_2 and $ML(OH)_x$ are formed; overall stability constants of the Cu(II) species have been determined. Coordination mode in the complexes was concluded on the basis of spectral investigation (NMR, ^{31}P NMR, ^{1}H–^{15}N NMR, IR, EPR and Vis). In the Cu/phosphoserine system at low pH, coordination mainly involves the phosphate group. At higher pH, the efficiency of $-PO_4^{2-}$ is low and coordination is by the amine and carboxyl groups. Differences in the coordination mode in the phosphoserine complex at low pH, compared to those of endogenic serine, in their reactions with copper(II) ions have been observed.

Keywords: Phosphoserine; Copper complexes; Stability; Coordination mode

1. Introduction

One of the most important processes of protein modification and signal transmission in cells is phosphorylation. It is assumed that about 30% of proteins encoded by the human genome contain covalently bound phosphate. Phosphorylation and dephosphorylation regulate almost every aspect of the cell metabolism and can modify the functioning of proteins [1]. Serine, with its $-CH_2OH$ side chain, is one of the most frequently phosphorylated amino acid residues. To understand the role of phosphorylation in biological processes, it is essential to characterize the site at which it occurs. This process may bring about a change in conformational behavior of the residue even if the phosphate is neutralized by Mg^{2+} [2].

Copper is a trace metal in humans and plays an important role. It is found in a number of enzymes crucial in, for example, electron transport and antioxidant defense. The role played by copper in lipoprotein metabolism is of increasing importance in understanding cardiovascular problems such as atherosclerosis [3]. Copper in trace amounts is essential to sustain life, but toxic accumulation of copper can be deleterious to human health (e.g. Wilson and Menkes disease genes [3, 4]). The concentration of copper in tissues of an organism is controlled by physiological conditions of the organism and the chemical state of the copper. Identification of

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copper chaperones provided understanding of the mechanism of copper transport [5]. The majority of Cu(II) ions in ceruloplasma are in the form of mixed complexes with amino acids, peptides, and other biomolecules [6]. Understanding of complexation processes of amino acids and their natural derivatives is important for explanation of the role of metals in living organisms [7].

This article presents results of potentiometric, spectral and theoretical studies of the complexation of phosphoserine with copper(II). The phosphorylated serine has three potential coordination sites (carboxyl, amine and phosphate). Determination of the mode of coordination between phosphorylated ligands and metal ions occurring in an organism is a first step to explain the function of the phosphate group in bioinorganic processes.

2. Experimental

O-phospho-L-serine (Ser-P) was purchased from Sigma. Copper(II) nitrate from Merck was purified by recrystallization from water. The concentration of copper ions was determined by inductively coupled plasma optical emission spectrometry (ICP OES). Potentiometric measurements were carried out using a Titrino 702 Metrohm equipped with an autoburette with a glass electrode (Metrohm 6.233.100) calibrated according to a literature method [8]. All potentiometric titrations were made under helium atmosphere at constant ionic strength $\mu = 0.1$ M (KNO_3), at $20 \pm 1^{\circ}C$, using as a titrant CO_2–free NaOH at a concentration of 0.1827 M. The concentration of Ser-P was 2×10^{-3} M, and the metal to ligand ratios were 2 : 1, 1 : 1 and 1 : 2. Determination of the stability constants of the complexes and protonation constants of ligands were made using the SUPERQUAD program [9] (determined ionic products using a value $pK_w = 13.78$), whereas the distribution of particular forms was obtained by the HALTFALL program [10]. The calculations were performed using 150–350 points taking into account only those parts of titration curves in which there was no precipitate in solution (an example of a titration curve, figure 1). The correctness of the model was confirmed by verification of the results obtained [11].

Measurements of ^{13}C, ^{31}P, ^{1}H–^{15}N NMR and IR were performed in D_2O, and the pD of the solution was adjusted using NaOD or DCl, taking into account that $pD = pH_{readings} + 0.40$ [12]. The concentration of the ligands used for NMR measurements was 0.1 M for ^{13}C and ^{31}P and 0.5 M for ^{1}H–^{15}N, at the M : L molar ratio of 1 : 75. NMR spectra were recorded on Gemini 300 Varian and Bruker Advance 600 MHz spectrometers using dioxane as an internal standard for ^{13}C NMR spectra and orthophosphoric acid for ^{31}P NMR spectra. The 2-D ^{1}H–^{15}N NMR spectrum was made by the HETCOR – long range method. Ligand concentration for the IR studies was 0.5 M and the ratio of M : L concentrations varied from 1 : 1 and 1 : 2. The IR spectra were recorded on an IFS 113v Bruker spectrometer, in the cells KRS-5. Samples for visible spectroscopic studies were prepared in H_2O at the M : L ratio 1 : 1 and 1 : 2 and at Cu(II) concentration of 0.05 M. The spectra were recorded on a Vega 400 Merck Vis spectrometer using a 1 cm^3 cell. EPR studies were carried out at 77 K using glass capillary tubes; the concentration of Cu(II) was 0.005 M in water : glycol (3 : 1), and the M : L ratio was 1 : 1 and 1 : 2. The spectra were recorded on an SE/X 2547 Radiopan instrument.

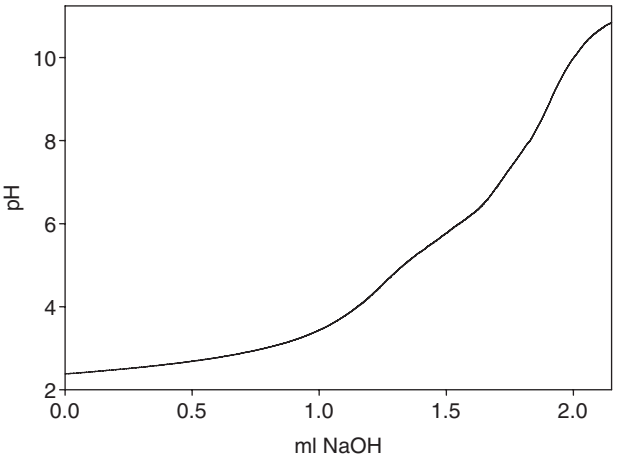


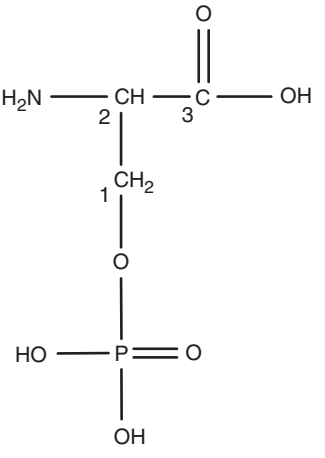
Figure 1. Experimental titration curve in Cu/Ser-P system ($c_{Ser\text{-}P} = c_{Cu} = 0.002$ M).

A full optimization of the isolated structures of each complex was performed with the GAUSSIAN 03 program (Ground State DFT and B3LYP/Set 6–311G level). As starting points, we used structures pre-optimized semi-empirically with HYPERCHEM-7.52 (PM3 hamiltonian) [13, 14]. The structure was minimized without protonation of the carboxylate at low pH.

3. Results and discussion

The values of successive protonation constants, $\log K_1 = 10.03$, $\log K_2 = 6.04$, $\log K_3 = 2.63$ and $\log K_4 = 2.12$, determined for Ser-P from computer analysis of titration data, correspond to protonation of $-NH_2$, $-PO_4^{2-}$, $-COO^-$ and $-PO_4H^-$ groups (scheme 1). The overall and successive protonation constants of O-phospho-L-serine (Ser-P) are given in table 1. The values of $\log K_{1-3}$ for Ser-P are consistent with literature data [15–17]; some deviations can be explained by different conditions of measurements. For the first time $\log K_4 = 2.12$ of Ser-P was determined and is close to $\log K_3 = 1.9$ for H_3PO_4 ($H_2PO_4^- + H^+ \leftrightarrows H_3PO_4$) [16].

The overall stability constants of the complexes formed in the Cu/Ser-P system are given in table 1. Computer analysis of the potentiometric titration data for the Cu(II)/Ser-P system of metal-ligand ratio 2 : 1, 1 : 1 and 1 : 2 revealed the formation of MHL and ML. At the ratios 2 : 1 and 1 : 1, hydrolysis takes place and additionally the ML(OH) and $ML(OH)_2$ species are formed (detected in previous potentiometric investigation [16, 17]). At the ratio 1 : 2, besides the MHL and ML, ML_2 forms. Computer analysis of the potentiometric titration data was performed taking into regard the protonation constants of Ser-P and the known constants for Cu(II) hydrolysis [18]. The stability constants of the Cu(II) complexes with Ser-P are generally consistent with literature data [16, 17]. The coordination mode in the complexes formed in the systems was concluded on the basis of analysis of d–d energy transitions in the Vis spectra and $g_{\|}$, as well as $A_{\|}$ values in EPR investigation (taking into consideration the relation of these values to the number of coordinated donor atoms [19–21]).



Scheme 1. The formula of O-phospho-L-serine.

The conclusions were supported with the analysis of the chemical shifts in the NMR spectra of the ligand in the complexes with respect to those of the free ligand interpreted according to our experience and critical analysis of the results for a number of systems with paramagnetic ions [22–25].

3.1. *Protonated MHL complexes*

The protonated complex Cu(HSer-P) starts forming in solution at pH 2. The species dominates at pH 4.5, binding 60% of the copper in solution (figure 2). Analysis of changes in the chemical shifts in the ^{13}C NMR spectra (taken in the pH range of complex domination) of the carbon atoms from phosphoserine: C(1) −0.279 ppm, C(2) +0.023 ppm and C(3) +0.080 ppm (table 2) indicates that the main site of metallation is the phosphate group from Ser-P. The participation of the phosphate group from Ser-P in coordination has been confirmed by a considerable change in the chemical shift observed in the ^{31}P NMR spectrum relative to the spectrum of the free ligand (−0.478 ppm, table 2). No significant shift in the signal position assigned to C(2) in the ^{13}C NMR spectra and no changes in the ^{1}H–^{15}N NMR spectra confirm that the amine is excluded from coordination at low pH (this group is protonated and blocked from coordination). Moreover, a relatively small change in the signal location assigned to C(3) from phosphoserine (table 2) suggests that the carboxyl group is not effective in metal binding. In order to confirm the conclusion that the carboxyl group is not involved in metallation, the IR spectrum was taken at the pH value at which the complex was dominant.

For the Cu(HSer-P) complex, the asymmetric stretching bands ν_{asC-O} from $-COO^-$ group was shifted only from 1625 to 1622 cm^{-1} (table 3). In spectra of the free ligand, the asymmetric stretching vibration assigned to the phosphate group of Ser-P was observed at about 1034 cm^{-1}. As a result of coordination this band disappeared, which indicates that the phosphate group is involved in metallation (table 3).

The conditions of the IR experiments prevented the observation of changes in far infrared bands assigned to the Cu–O and Cu–N bonds. The Vis and EPR spectral data

Table 1. Overall and successive stability as well as equilibrium constants of Cu(II) complexes and protonation constants of O-phospho-L-serine.

	Overall protonation constants			Successive protonation constants	
Reactions	$\log\beta$	$\log\beta^*$	Reactions	$\log K_{1-4}$	$\log K_{1-3}^*$
$L^{3-}+H^+ \leftrightarrows HL^{2-}$	10.03(1)	9.85	$Ser\text{-}P^{3-}+H^+ \leftrightarrows H(Ser\text{-}P)^{2-}$	10.03	9.85
$HL^{2-}+H^+ \leftrightarrows H_2L^-$	16.07(2)	15.63	$H(Ser\text{-}P)^{2-}+H^+ \leftrightarrows H_2(Ser\text{-}P)^-$	6.04	5.78
$H^2L^-+H+ \leftrightarrows H^3L^-$	18.70(2)	17.82	$H_2(Ser\text{-}P)^-+H^+ \leftrightarrows H_3(Ser\text{-}P)$	2.64	2.19
$H_3L+H^+ \leftrightarrows H_4L^+$	20.82(2)	N.D.	$H_3(Ser\text{-}P)+H^+ \leftrightarrows H4(Ser\text{-}P)^+$	2.12	N.D.
	Overall stability constants			Equilibrium constants	
Species	$\log\beta$	$\log\beta^*$	Reactions	$\log K_e$	
MHL	15.02(3)	14.88	$Cu^{2+}+H(Ser\text{-}P)^{2-} \leftrightarrows CuH(Ser\text{-}P)$	4.99	
ML	9.90(3)	10.04	$Cu^{2+}+Ser\text{-}P^{3}- \leftrightarrows Cu(Ser\text{-}P)^-$	9.90	
ML(OH)	2.29(4)	N.D.	$Cu^{2}++Ser\text{-}P^{3}+H_2O \leftrightarrows Cu(Ser\text{-}P)(OH)^{2-}+H^+$	16.07	
$ML(OH)_2$	−8.78(6)	N.D.	$Cu^{2+}+Ser-P^{3-}+2H_2O \leftrightarrows Cu(Ser-P)(OH)_2^{3-}+2H^+$	18.78	
ML_2	15.75(8)	15.90	$Cu^{2+}+2Ser-P^{3-} \leftrightarrows Cu(Ser-P)_2^{4-}$	15.75	

$\log\beta^*$ and $\log K^*$ from [17].

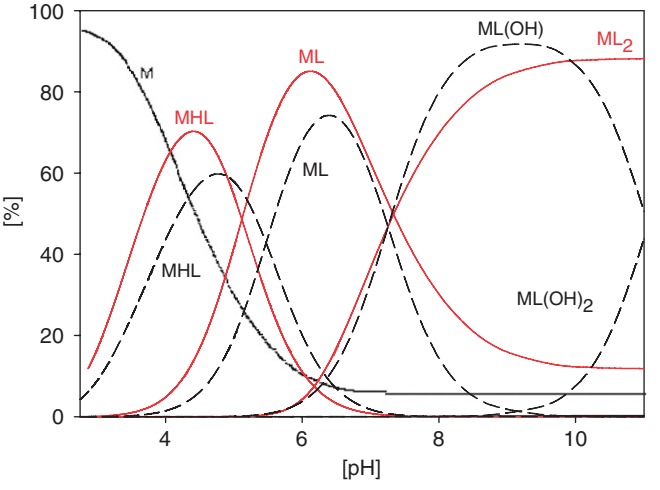


Figure 2. Distribution diagrams for the systems: (- - -) Cu/Ser-P (ratio 1 : 1) and (——) Cu/Ser-P (ratio 1 : 2); percentage of the species refers to relative concentration of the metal.

Table 2. Spectroscopic data of the investigated complexes.

		Vis		EPR		NMR differences between signal positions for the ligand in the complex in relation to the free ligand [ppm]			
	pH	λ_{max} [nm]	ε [dm^3 mol^{-1} cm^{-1}]	$g_{\parallel}$	A [10^{-4} cm^{-1}]	C(1)	C(2)	C(3)	P
Species									
Cu(HSer-P)	4.5	721	83.5	2.300	170	−0.279	+0.023	+0.080	−0.478
Cu(Ser-P)	6.0	710	98.0	2.308	170	−0.080	−0.011	+0.028	−0.102
Cu(Ser-P)$_2$	9.0	625	88.5	2.255	185	+0.032	+0.228	+0.321	−0.007
Cu(Ser-P)(OH)	9.0	648	93.5	2.241	180	–	–	–	–

Table 3. Observed infrared frequencies for investigated system [cm^{-1}].

	pH	$\nu_{s(C-O)}$	$\nu_{(-NH2)}$	$\nu_{as(-P-O)}$
Ser-P	4.5	1625	–	1034
Cu(HSer-P)	4.5	1622	–	–
Ser-P	6.0	1621	–	1023
Cu(Ser-P)	6.0	1618	–	–
Ser-P	9.0	1620	1584	1028
Cu(Ser-P)(OH)	9.0	1615	1615	1028
Cu(Ser-P)$_2$	9.0	1614	1614	1028

for Cu(Hser-P) ($\lambda_{max} = 721$ nm, $g_{\parallel} = 2.300$ and $A_{\parallel} = 170 \cdot 10^{-4}$ cm^{-1}, table 2) indicate that only oxygens are coordinated, as can be concluded on the basis of the literature data on coordination of Cu(II) ion with the O-donor ligands [26]. Serine, the endogenic analogue of the ligand studied, enters into coordination at low pH only via a carboxyl

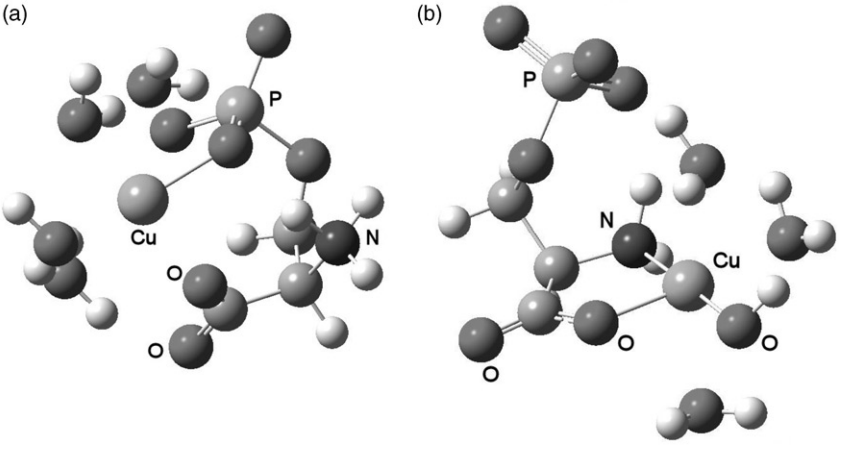


Figure 3. Optimized solution structure: (a) Cu(HSer-P); (b) Cu(Ser-P)(OH).

group [27]. The different mode of coordination in Ser-P and Ser is confirmed by significantly higher values of equilibrium constants of protonated species, $\log K_e = 4.99$ for Cu(HSer-P) ($\log K_e = \log \beta_{(CuHL)} - \log \beta_{(HL)}$) than the value for Cu(HSer) $\log K_e = 2.39$ [16], also point to a greater affinity for metal bonding of phosphate, compared to that of carboxyl, for Cu(II). This observation is in agreement with the values of the charge at the reaction centers (the oxygen from the phosphate group -0.63, the oxygen from the carboxyl group -0.42) calculated by GAUSSIAN for the free ligand. The different mode of the metal coordination by Ser-P and Ser has been confirmed by the difference in the spectral parameters, table 2, for the two complexes of the MHL type (for Cu(HSer): $\lambda_{max} = 759$ nm, $g_{\|} = 2.416$ and $A_{\|} = 134 \cdot 10^{-4}\,cm^{-1}$ [28]). The spectral values of Cu(HSer-P) are similar to those of the Cu(II) complex with phosphoric acid (at pH close to 4, ($\lambda_{max} = 708$ nm, $g_{\|} = 2.301$ and $A_{\|} = 168 \cdot 10^{-4}\,cm^{-1}$ [28]). Therefore, phosphorylation of serine leads to considerable changes in the coordination character of the bioligand at low pH. The conclusions on the solution structure were supported by DFT calculations (GAUSSIAN). The energy was optimized for the structure in which metal was coordinated only via the phosphate group from phosphoserine and water [figure 3(a)].

3.2. *ML type complexes*

The Cu(Ser-P) forms in solution from pH about 4 and becomes dominant at pH close to 6.5, binding about 75% of the copper ions in solution (figure 2). The changes in positions of ^{13}C NMR signals assigned to the carbons of phosphoserine [C(1) -0.080 ppm, C(2) -0.011 ppm and C(3) $+0.028$ ppm, table 2], observed upon metallation, suggest that the main coordination site is the phosphate group, similar to the Cu(HSer-P) species. The involvement of phosphate in metallation is confirmed by the change in the signal coming from the phosphorus atom in the spectrum of ^{31}P NMR with respect to that in the spectrum of the free ligand at -0.102 ppm (table 2). Insignificant shift in the signal assigned to C(2) and no changes in the 2D ^{1}H–^{15}N NMR spectrum exclude amine coordination (figure 4).

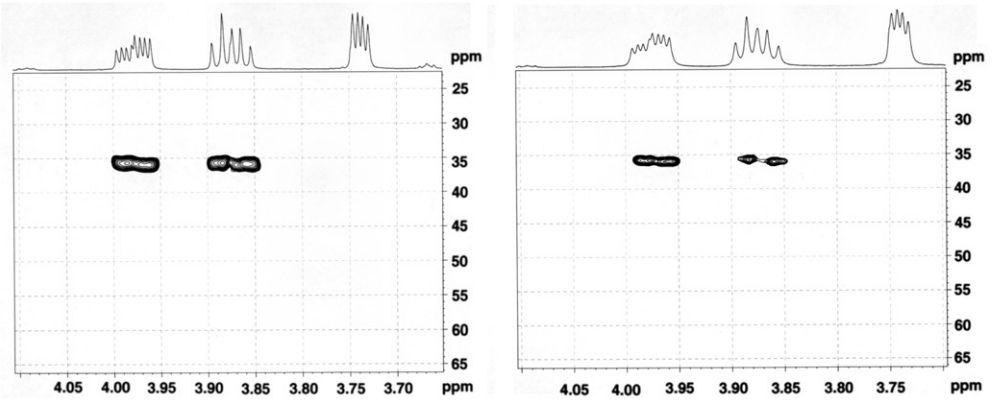


Figure 4. ^{1}H–^{15}N NMR spectra of Ser-P and Cu/Ser-P systems at pH = 6.5.

A relatively small change in the signal location assigned to C(3) from phosphoserine (table 2) suggests that the carboxyl group is also not involved in metallation, confirmed by the small shift of the IR asymmetric stretching vibrations ν_{asC-O} (from 1621 cm^{-1} to 1618 cm^{-1}, table 3). The asymmetric stretching band at 1023 cm^{-1}, characteristic of the phosphate disappears in the system with metal ions, which points to involvement of $-PO_4^{2-}$ in coordination (table 3). The Vis and EPR spectral data for Cu(Ser-P) ($\lambda_{max} = 710$ nm, $g = 2.308$ and $A = 170 \cdot 10^{-4}$ cm^{-1}, table 2) indicate the coordination mode in the ML complex is analogous to the protonated complex, including oxygen from the phosphate as the effective coordination site.

3.3. *ML_2 type complexes*

$Cu(Ser\text{-}P)_2$ formation (at Cu : Ser-P ratio 1 : 2) starts from pH 6.0 and dominates from pH = 8.5 binding 80% of the copper ions in solution (figure 2). The pattern of changes in the ^{13}C NMR positions of signals assigned to carbons from the ligands, C(1) + 0.032 ppm, C(2) + 0.228 ppm and C(3) + 0.321 ppm (table 2), and the lack of changes in the ^{31}P NMR spectrum (−0.007 ppm) indicates that $-PO_4^{2-}$ does not bind copper and points to amine and carboxyl groups as sites of metal bonding in ML_2. The involvement of the nitrogen in coordination with the paramagnetic copper(II) ion is manifested by a broadening of the 2-D ^{1}H–^{15}N NMR spectrum as a result of metallation. Analysis of the Vis and EPR spectral data ($\lambda_{max} = 625$ nm, $g_{\|} = 2.255$ and $A_{\|} = 185 \cdot 10^{-4}$ cm^{-1}, table 2) indicates $\{N2, O2_{carboxyl}\}$ coordination, analogous to that in CuL_2 complexes of other amino acids [21, 26, 29]. Participation of the phosphate in coordination has been also excluded on the basis of the IR study. No differences in the position of the band assigned to the asymmetric stretching vibrations of the phosphate group are found in IR spectra of free phosphoserine and its complex. On the other hand, the asymmetric bands assigned to the uncoordinated $-NH_2$ group [30] (1584 cm^{-1}) undergo a significant shift (figure 5) in the spectra of the system with copper (1616 cm^{-1}), which confirms coordination of the copper(II) ions to the $-NH_2$ group.

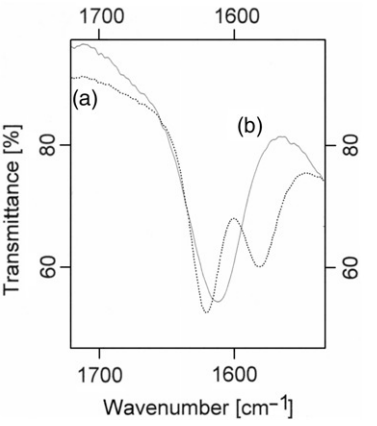


Figure 5. IR spectra of (a) phosphoserine and (b) Cu/phosphoserine (ratio 1 : 2) at pH = 9.0.

The coordination in the Cu(Ser-P)$_2$ complex leads to shift of the asymmetric stretching bands ν_{asC-O} from 1620 to 1614 cm^{-1} (figure 5), pointing to involvement of the –COO$^-$ group in coordination. In contrast to the situation at low pH, at high pH (MHL and ML complexes) the effect of phosphorylation of serine on the mode of coordination is not significant relative to that of the endogenic amino acids.

3.4. *MLOH type complexes*

Cu(Ser-P)(OH) starts forming in solution (at Cu:Ser-P ratio 1 : 1) from pH of 6 and dominates for pH 8.5–9.5 where it binds maximally to 90% of copper ions (figure 2). Analysis of the Vis and EPR spectral data for the complex Cu(Ser-P)(OH) ($\lambda_{max} = 648$ nm, $g_{\|} = 2.241$ and $A_{\|} = 180 \cdot 10^{-4}$ cm^{-1}, table 2) indicates $\{N, O_x\}$ coordination, similar to that in copper(II) complexes with endogenic amino acids [20, 21, 26]. Participation of the phosphate in coordination has been excluded on the basis of the IR study, where no differences in the position of the band assigned to the asymmetric stretching vibrations of the phosphate group were observed. Asymmetric bands assigned to the uncoordinated –NH$_2$ group [30] (1580 cm^{-1}, table 3) undergo a significant shift in the spectra of the systems with copper ions (at 1615 cm^{-1}), confirming coordination of the copper(II) to the –NH$_2$ group.

A shift of the asymmetric stretching band ν_{asC-O} from 1620 cm^{-1} to 1615 cm^{-1} (table 3), suggests involvement of –COO$^-$ in coordination. Thus, in basic medium the effective sites of coordination in the complex are oxygen of the carboxyl and the nitrogen from deprotonated amine. Conditions in which the hydroxocomplex is formed make it impossible to apply NMR for confirmation of the coordination mode. Coordination mode in Cu(Ser-P)(OH) was confirmed by calculations using the GAUSSIAN program and the solution structure obtained is presented in figure 3. As follows from the computer analysis of the titration data, from pH of 9 the Cu(Ser-P)(OH)$_2$ complex is formed, which dominates above the range of spectral observation (figure 2).

4. Conclusions

In the systems of copper(II) ions with phosphoserine formation of CuHL, CuL, $CuL(OH)_x$ and CuL_2 have been established. In acidic medium, the effective site of metal ion bonding is the phosphate, whose significance decreases with increasing pH as the effectiveness of the carboxyl and amine groups is raised. In Cu(HSer-P) and Cu(Ser-P), present below the physiological pH, the $-PO_4^{2-}$ group is involved in coordination. Thus, phosphorylation of the endogenic serine (which at low pH coordinates *via* carboxyl) results in changes in the mode of metal binding. Near the physiological pH value, a transformation in the coordination type is observed due to changes in the effectiveness of the competing donor groups. In $Cu(Ser\text{-}P)_2$ the carboxyl and amine groups of the ligands are involved in coordination, while the activity of the phosphate group considerably decreases. Thus, the $\{N2, O2_{carboxyl}\}$ coordination is similar to that in the serine complex $Cu(Ser)_2$.

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