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LUMINESCENCE STUDIES OF THE LIGAND EXCHANGE BETWEEN TWO PHENANTHROLINE COMPLEXES AND BOVINE SERUM ALBUMIN

H.-B. Lin* **and Q.-H. Shen**

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The interactions between bovine serum albumin (BSA) and two Cu(II) phenanthroline complexes were studied by fluorescence and UV-visible absorption spectroscopy. The obtained results confirm that the phen ligand (phen = 1,10-phenanthroline) is dissociated from the two complexes and moves into the hydrophobic cavity of BSA and that the M–L complexes (M = Co^{2+}, Cu^{2+}; L = Hlact, imda; Hlact = lactic acid, H_2imda = iminodiacetic acid) coordinate with the amino acids on the surface of the peptide in the solution. This mode of action significantly inhibits the denaturation of BSA. The calculated distance between the BSA and the two complexes suggests that the energy transfer from the excited state of BSA to a complex occurs with high efficiency.

Keywords: *ligand exchange, phenanthroline, bovine serum albumin, fluorescence spectrum.*

Introduction. Protein interactions have a fundamental function in many biochemical processes in both healthy and diseased states, including critical functions in signal transduction, immune reaction, cell cycle control, differentiation, and protein folding. To understand, probe, and manipulate biological systems effectively, studying the influence of small molecules or inhibiting protein interactions through selective recognition is necessary [1–5]. Small metal complexes can potentially combine flexibly in ligands and can be designed to access a wide and diverse range of coordination geometries, optical isomers, and electronic states. This flexibility can be used to enable or prevent coordination of the metal complex to protein side chains or substrates [6–9]. At present, many research papers have reported the interaction between the complexes and bovine serum albumins (BSA) [9, 10–14], most of which were conducted using fluorescence spectroscopy. Dynamic and static quenching constants, binding sites, binding force, and binding distance between the complexes and BSA have been calculated, and the results showed that the complex quenched the fluorophore of BSA by forming ground state complexes in a solution, thereby perturbing the environment surrounding tryptophan or tyrosine residues of BSA.

In a previous work, we studied the interaction between complexes and BSA with the use of IR spectroscopy and found that the ligands in the complexes could be replaced by the amino acids in BSA [15, 16]. In order to demonstrate whether the experiment result is correct, we investigated the interaction between BSA and complexes **1** and **2** via fluorescence, UV spectroscopy, and the dialysis method {**1** = $[Co(Hlact)_2(phen)]\cdot 2H_2O$[15], **2** = $[Cu(imda)(phen)]\cdot H_2O$ [17], Hlact = lactic acid, phen = 1,10-phenanthroline, imda = iminodiacetic acid}, and further investigated whether or not the mode will have an impact on protein denaturation.

Experimental. All manipulations were carried out in open air. All chemicals were commercial analytical reagents and used without further purification. Nanopure-quality water was used throughout this work. The pH was measured using a potentiometric method with a digital pH meter. Infrared spectra were recorded as Nujol mulls between KBr plates using a Nicolet 360 FT-IR spectrometer. The concentrations of Co(II) and Cu(II) were determined using a GBC932B atomic absorption spectrometer. Fluorescence spectra were recorded on a Perkin-Elmer LS45 spectrophotometer with temperature maintained by a circulating bath.

UV absorption spectra were recorded on a Shimadzu UV-3000PC spectrophotometer.

UV-vis absorption studies. A series of 5.00 mL of complex **1** (0.04 mol/L) was added to six 10-mL volumetric flasks. Different quantities of BSA were also added. A buffer solution of Na_2HPO_4–NaH_2PO_4 with a pH value of 6.86 was added

*To whom correspondence should be addressed.

College of Chemistry and Environment, Fujian Province University Key Laboratory of Analytical Science, Minnan Normal University, Zhangzhou, 363000, China; e-mail: 526733412@qq.com. Abstract of article is published in Zhurnal Prikladnoi Spektroskopii, Vol. 84, No. 1, p. 169, January–February, 2017.

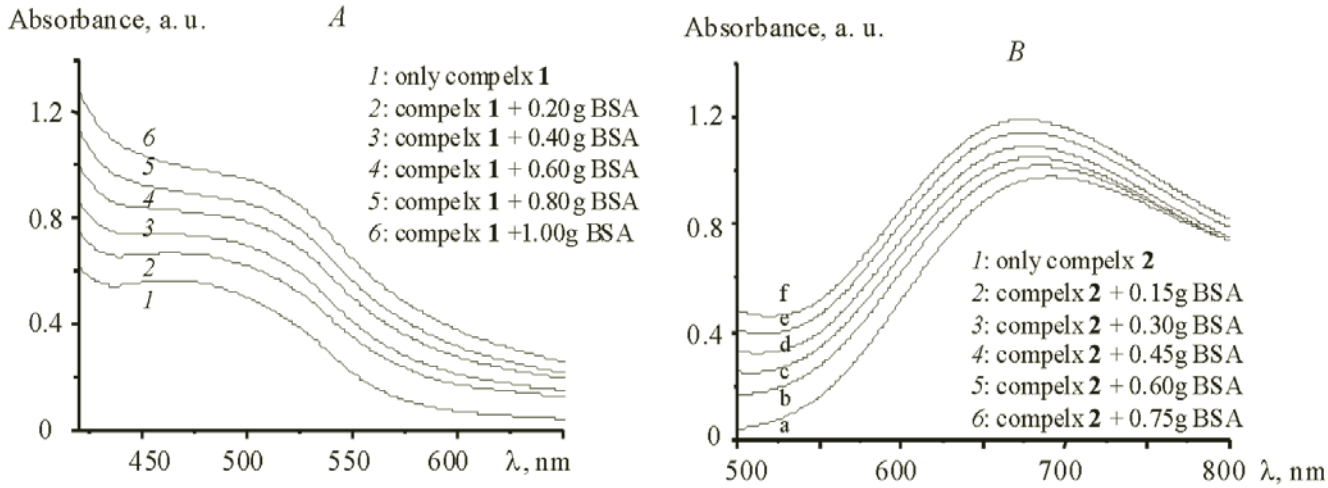

Fig. 1. UV-vis spectra of complexes **1** (A) and **2** (B) in the presence of BSA at different concentrations.

to the 10 mL flasks. The absorbance of different solutions in the visible region was then measured. The experiment on the interaction of complex **2** with BSA was carried out as for complex **1**.

Dialysis studies. BSA (0.10 g) and complex **1** (0.050 g) were dissolved in 50 mL of Na_2HPO_4–NaH_2PO_4 buffer solution (pH 6.8). The solution was transferred into the dialysis bag (M_w < 500), then dialyzed with 200 mL of Na_2HPO_4–NaH_2PO_4 buffer solution (pH 6.8). The dialysate was changed with the same volume everyday. The fluorescence, UV spectroscopy, and the measurements of copper ion concentrations in the dialysis bag were performed everyday. To compare the reactivity of the complexes with BSA, one more experiment was carried out similar to the above experiment, except that the dialysate used was 2.0 mmol/L $EDTANa_2$ buffer solution (pH 6.8, Na_2HPO_4–NaH_2PO_4). The experiments for the interaction of complex **2** with BSA were carried out as for complex **1**.

*BSA-complex **1** interaction with different quantities of β-CD.* A series of 5.00 mL of the solution of the BSA-complex **1**, which was dialyzed four times with 2.0 mM Na_2EDTA, was added to six 10 mL volumetric flasks. Different quantities of β-CD were also added. A buffer solution of Na_2HPO_4–NaH_2PO_4 with a pH value of 6.86 was added to the 10 mL flasks. The fluorescence of different solutions was measured.

Stability measurement. Two solutions of the BSA-complex **1,** which was dialyzed twice with 2.0 mM Na_2EDTA and free BSA, with the BSA concentration of 4.0 g/L for both were stored at room temperature, and the UV spectroscopy of the solutions was performed every day.

Results and Discussion. *UV-vis absorption studies of BSA and complexes **1** and **2**.* UV-vis absorbance was observed when different quantities of BSA were added to a fixed concentration of complexes **1** and **2**, as shown in Fig. 1. One can see from Fig. 1A, that when the BSA concentration gradually increases, the maximum absorption wavelength for the complex **1** increases gradually (Δλ = 48 nm) from 462 to 510 nm. From Fig. 1B it follows that when the BSA concentration gradually increases, the maximum absorption wavelength for complex **2** decreases gradually (Δλ = 18 nm) from 691 to 673 nm. These results show that when the concentration of BSA in both complexes gradually increases, groups from BSA, such as partial amino, hydroxyl, or carboxyl groups, can gradually replace the organic small molecule ligands of the complexes that previously interacted with cobalt or copper ions of the complexes. The ligand environment's cobalt and copper ions gradually changed, resulting in the shifted wavelength of maximum absorption in the visible region of cobalt and copper ions.

*Analysis of the interaction of complexes **1** and **2** with BSA.* The reactions of complexes **1** and **2** with BSA were investigated via dialysis. After the dialysis was carried out with only the Na_2HPO_4–NaH_2PO_4 buffer solution (pH 6.8) for five days, the fluorescence spectra of BSA enhanced gradually but did not change significantly after 3 days, indicating that the concentration of the quencher decreased gradually (Fig. 2A,B). The UV-visible spectral analysis (Fig. 2A,B, inset *a*) and the concentration of Co(II) and Cu(II) (inset *b*) showed the same result as the fluorescence spectral analysis. Thus, we assume that the complexes and BSA form a stable product in the solution and a dynamic equilibrium exists between the product and quenchers.

After adding 2.0 mM $EDTANa_2$ to the Na_2HPO_4–NaH_2PO_4 buffer solution (pH 6.8) and dialyzing for 5 days, the fluorescence spectrum of the solution in the dialysis bag (Fig. 2C,D) was quite similar to that when only Na_2HPO_4–NaH_2PO_4

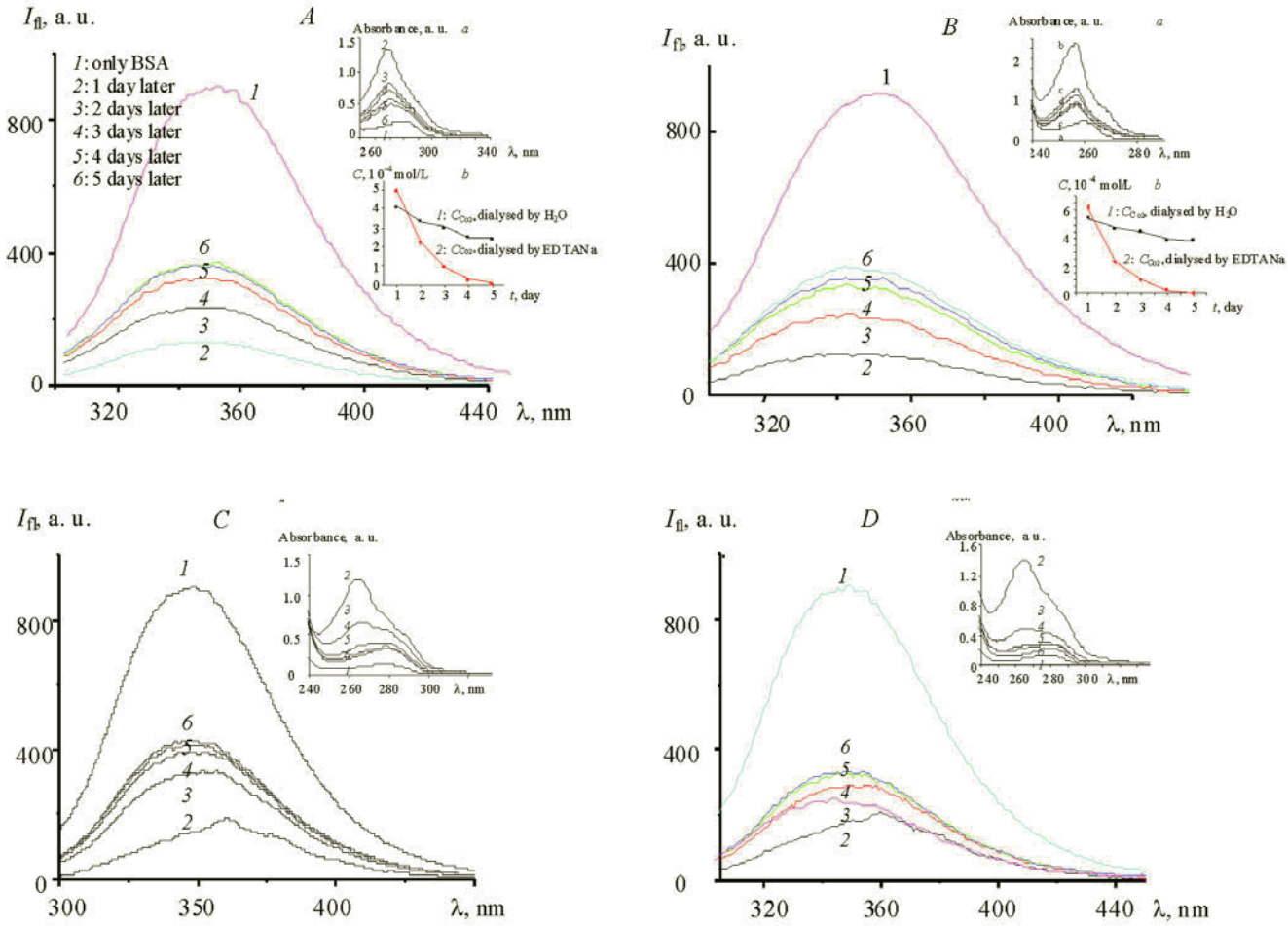

Fig. 2. Fluorescence, UV spectra (inset *a*) and concentrations of Co^{2+} and Cu^{2+} (inset *b*) of the solution dialyzed with (A, B) buffer solution (C, D) $EDTANa_2$ for 5 days, complexes **1** (A, C) and **2** (B, D).

buffer solution (pH 6.8) was used, indicating that the quencher for the BSA fluorescence almost did not react with $EDTANa_2$. However, the change in the concentrations of Co(II) and Cu(II) was quite different from that when only Na_2HPO_4–NaH_2PO_4 buffer solution (pH 6.8) was used (Fig. 2A,B, inset *b*). The former reduced the concentrations of Co(II) and Cu(II) by approximately four times, whereas the latter reduced the concentrations of Co(II) and Cu(II) by more than 60 times. The IR spectrum of the sediments that precipitated out from the solution in the dialysis bag after dialysing by 2.0 mM $EDTANa_2$ also showed that the *phen* in BSA exists (Fig. 3). These results showed that the quencher of the BSA fluorescence was different from complexes **1** and **2**, and it was probably the *phen* ligand.

Further, the reactions of complexes **1** and **2** with different concentrations of BSA were analyzed via UV-visible spectroscopy (Fig. 4). The absorption bands gradually red-shifted to 280 nm ($\lambda_{max,\ BSA}$ = 280 nm) with increasing BSA concentration. The value of λ_{max} was maintained at 273 nm when the dialysate was replaced for the fifth time with the Na_2HPO_4–NaH_2PO_4 buffer solution (pH 6.8) only (Fig. 2A,B, inset *a*). The results indicate that some new complexes were formed in the dialysis bag and that the concentration of the new complexes clearly did not change when the dialysis was carried out multiple times. This result is different from the case where $EDTANa_2$ was used (Fig. 2C,D, inset), in which λ_{max} was red-shifted to 278 nm when the dialysate was replaced three times. Then λ_{max} remained constant at 278 nm even though the dialysate was replaced two more times. These results indicate that other quenchers are present in the solution aside from complexes **1** and **2**. The quencher did not react with $EDTANa_2$; however, it blue shifted λ_{max} of BSA.

The mode of interaction between BSA and complexes **1** and **2** was established from the above results (Fig. 5). The hydrophobic functional domain of BSA attracted the *phen* ligand and repelled the Co(II) and Cu(II) ions and other

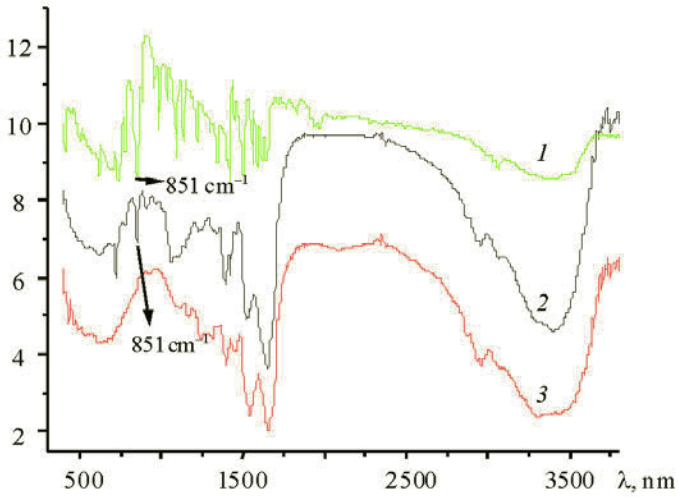

Fig. 3. IR spectra of which three complexes: 1) *phen*, 2) sediment from the BSA-complex solution dialysed with EDTA for three times, 3) BSA.

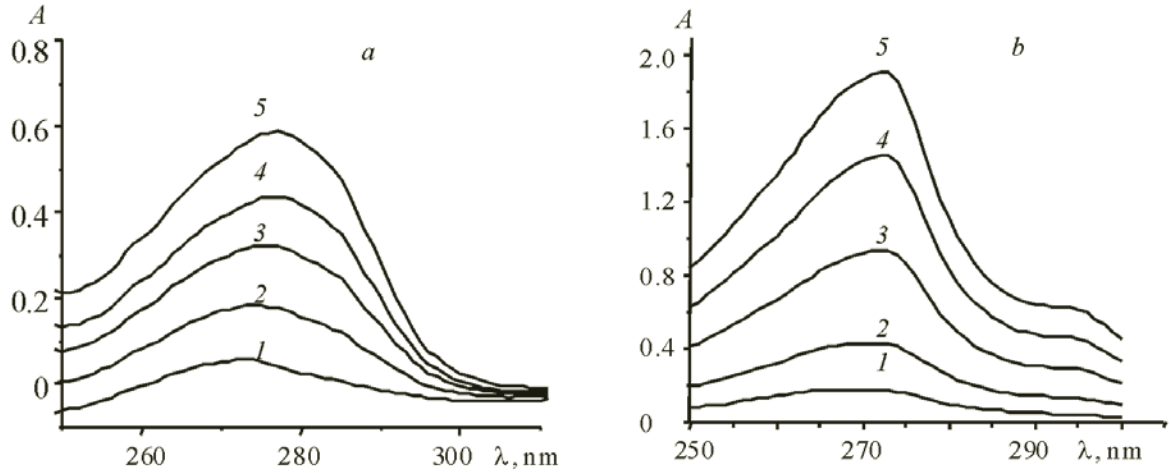

Fig. 4. UV spectra of BSA in the presence of complexes **1** (a) and **2** (b) at different concentrations.

hydrophilic ligands, such as Hlact and imda. The *phen* ligand inside the hydrophobic pocket of BSA perturbed the microenvironment close to the tryptophan or tyrosine residues, thereby quenching the intrinsic fluorescence of BSA [18]. The complex M–L (M = Co(II) or Cu(II), L = Hlact or imda) then coordinated to the amino acids on the surface of the peptide. BSA, and both complexes also interacted outside of the hydrophobic functional domain of the protein because of the hydrophobic interactions.

*Fluorescence studies of the BSA-complex **1** in the presence of β-CD.* As shown in Fig. 6, when the concentration of β-CD was increased gradually, the fluorescence intensity at 350 nm did not visibly increase, which indicated that the Van der Waals force between phen and BSA was stronger than that between *phen* and β-CD (Fig. 5).

Stability of the BSA-phen product. As shown in Fig. 7, the solution of the BSA-complex **1**, which was dialyzed four times with 2.0 mM Na_2EDTA, was analyzed via UV-visible spectroscopy to compare the stability of the BSA-*phen* product and free BSA at room temperature. The absorbance of the free BSA solution gradually increased every day, then BSA mostly precipitated after 5 days. However, the absorbance of the BSA-phen solution did not change significantly, and no precipitation was observed even after 30 days.

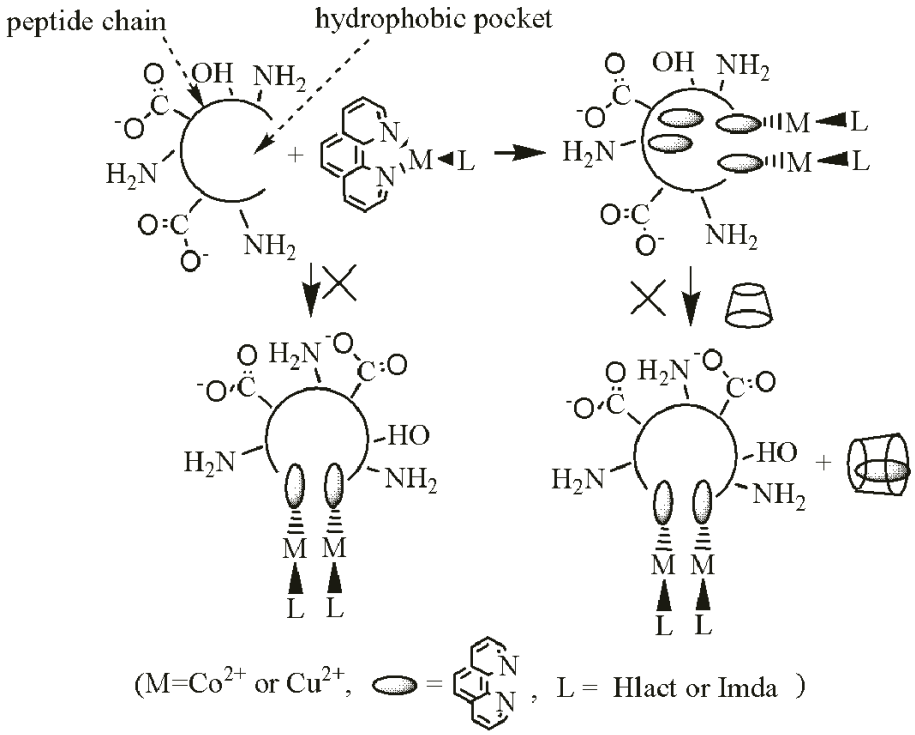

Fig. 5. Mode of the interaction between BSA and complexes **1** and **2.**

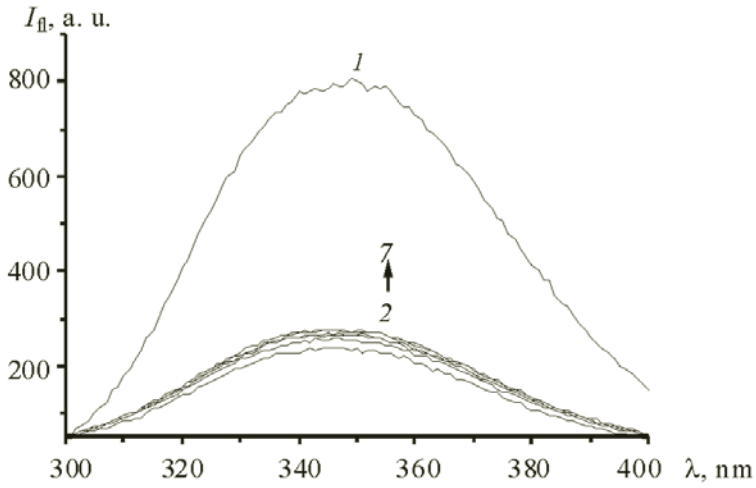

Fig. 6. Fluorescence spectrum of BSA-complex **1** in the presence of β-CD.

Energy transfer between two complexes and BSA. During Forster's resonance energy transfer (FRET), the donor fluorophore in its excited state transfers energy to an acceptor molecule through nonradiative dipole–dipole coupling [19]. The efficiency of this energy transfer can be used to estimate the distance (*r*) between the complex and fluorophore in a biomolecule. This energy transfer depends on the extent of overlapping of the emission spectrum of the donor with the absorption spectrum of the acceptor. Figure 8 shows that the fluorescence emission spectrum of BSA is overlapped with the absorption spectrum of the two complexes, thereby indicating the energy transfer from the excited state of BSA to the complex. The efficiency *E* of FRET is inversely proportional to the sixth power of the distance between donor and acceptor, i.e.,

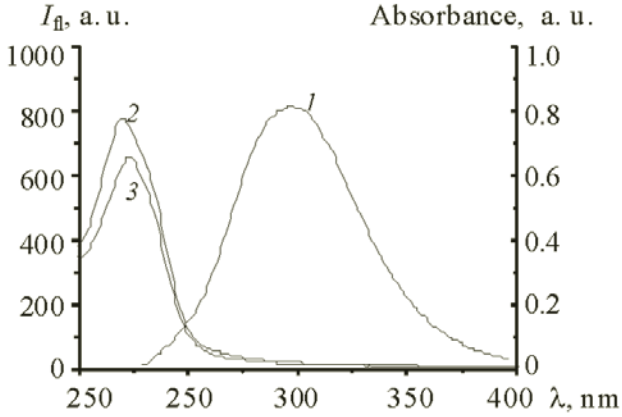

Fig. 7. Fluorescence emission of BSA (1) and UV absorption spectra of complexes **1** (2) and **2** (3).

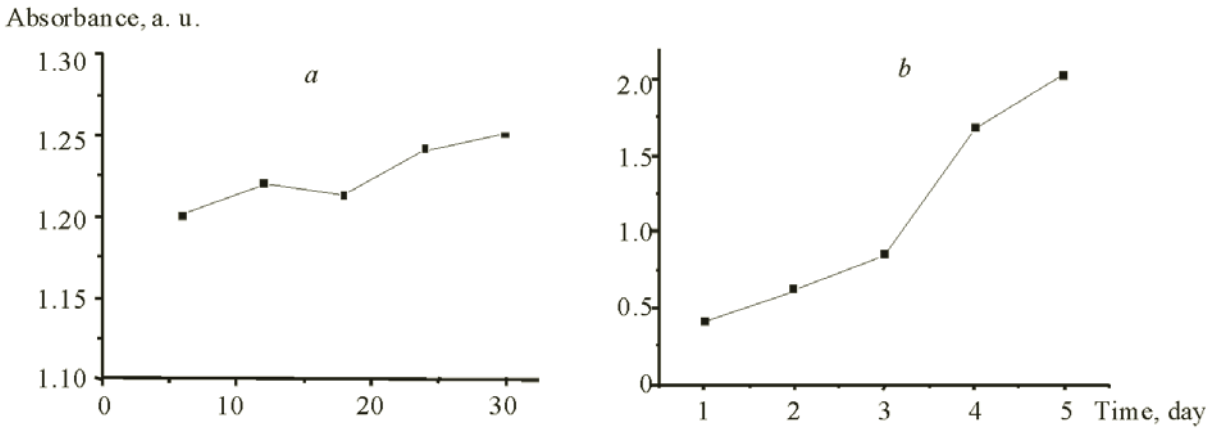

Fig. 8. Changes in UV absorption spectra with time for (a) the solution dialyzed with $EDTANa_2$ and (b) free BSA.

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r^6)\,, \tag{1}$$

R_0 is the critical distance when the transfer efficiency is 50% and is given by

$$R_0^6 = 9.79 \times 10^3 (k^2 n^{-4} \Phi_D J)^{1/6}\,, \tag{2}$$

where Φ is the fluorescence quantum yield of the donor, n is the refractive index of the medium, k^2 is the spatial orientation factor of the dipole, and J is the overlap integral for the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, which is given by equation

$$J = \int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda / \int_0^\infty F(\lambda) d\lambda\,, \tag{3}$$

where $F(\lambda)$ is the donor fluorescence intensity and $\varepsilon(\lambda)$ is the molar absorptivity of the acceptor at the wavelength λ. Given Eqs. (1) to (3) and the values for Φ = 0.15, k^2 = 2/3, and n = 1.36 [20], the values of r were calculated to be 2.13 and 2.19 nm for complexes **1** and **2**, respectively. These results show that the complexes are strong quenchers and are situated at close proximity to the BSA fluorophore (Fig. 7). This result is very similar to the interactions between BSA and the complexes of $Co(phen)_2Cl_2$ and $[Co(phen)_2(H_2O)_2][Co(nta)(phen)]_2 \cdot 12H_2O$ [21], and the values of r were calculated to be 2.21 and 2.25 nm, respectively.

Conclusions. The interaction of complexes **1** and **2** with BSA was studied via dialysis. The results show that when the complexes interacted with BSA, the ligand of phenanthroline moves into the hydrophobic cavity of BSA. This mode of action significantly inhibits the denaturation of BSA.

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