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A COMPARATIVE STUDY OF THE INTERACTION OF OCHRATOXINS WITH BOVINE SERUM ALBUMIN*

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Abstract—The interaction of different ochratoxins with bovine serum albumin (BSA) has been studied by equilibrium dialysis, solubility and spectrophotometric analyses. Spectrophotometric analyses revealed that the absorption maxima of ochratoxin B (OB) and ochratoxin C (OC) shift to longer wavelengths (365–377 nm for OB, 380–390 nm for OC) as a result of interaction. The spectra of ochratoxins α (Oα) and β (Oβ) were not altered in the presence of BSA. Equilibrium dialysis results indicate that 1 mole of BSA binds 1·03, 1·02, 1·93 and 3·24 moles of Oα, Oβ, OB and OC, with binding constants of $1{\cdot}5 \times 10^{5}$ M^{-1}, $5{\cdot}1 \times 10^{5}$ M^{-1}, 7·1 and $8{\cdot}9 \times 10^{5}$ M^{-1} respectively. Spectrophotometric titrations of the 1:1 molar ratio of BSA–ochratoxin complexes indicate that the pK values of OB and OC decrease on binding, whereas the pK values of Oα and Oβ remain unaltered. The dissociation of the phenolic hydroxyl group in the isocoumarin ring of ochratoxins A, B and C appears responsible for the binding of the second molecule of these toxins with albumin. The overall results suggest that both ionic and hydrophobic forces are important in the binding of ochratoxins with BSA.

OCHRATOXINS are a group of hepatic and nephrotoxic mycotoxins which contain a dihydroisocoumarin ring and are produced by several species of *Aspergillus* and *Penicillium*.[1–4] Ochratoxin A (OA), which causes glycogen accumulation in rat liver[5] and inhibition of mitochondrial respiration,[6] is the most toxic metabolite among this group. Ochratoxin C (OC), the ethyl ester of OA, has a similar toxic effect. However, ochratoxin B (OB), the non-chlorine-containing analog of OA, has been found to be less toxic.[7] The hydrolyzed products of OA and OB, i.e. Oα and Oβ, were found to nontoxic to test animals.[8–10] In a comparative study on the structural requirements for ochratoxin intoxication, we found that the dissociation of the phenolic hydroxyl group in the dihydroisocoumarin ring appears to be important.[9]

Ochratoxin A has been found to interact with albumin both *in vitro* and *in vivo*.[11, 12] Equilibrium dialysis and Sephadex gel filtration studies revealed that 2 moles of OA were bound to 1 mole of bovine serum albumin in a common binding site. In an attempt to reveal the mechanism of interaction of ochratoxins with proteins, we further examined the possibility of the interaction of other ochratoxins with bovine serum albumin. The modes of interaction of OB, OC, Oα and Oβ with bovine serum albumin are compared in this paper. For convenience of discussion, the structures of the different ochratoxins used in the present study are shown in Fig. 1.

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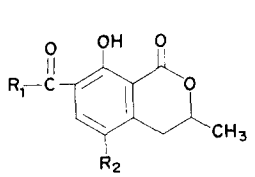

	R_1	R_2
OA	$-CH_2CH(COOH)NH-$	$-Cl$
OB	$-CH_2CH(COOH)NH-$	$-H$
OC	$-CH_2CH(COOEt)NH-$	$-Cl$
Oα	$-OH$	$-Cl$
Oβ	$-OH$	$-H$

FIG. 1. Structure of ochratoxins.

MATERIALS AND METHODS

Materials. Ochratoxin A and OB were produced by *Aspergillus ochraceus* in rice and were purified by Adsorbosil-5 column chromatography and Sephadex LH_{20} gel filtration as previously described.[11, 13] Ochratoxin C was prepared from OA through esterification with ethanol BF_3 according to Nesheim,[14] and was purified by passage through an Adsorbosil-5 column.[11] Ochratoxins α and β were prepared by hydrolysis of purified OA and OB, followed by passage through a Sephadex LH_{20} column.[11] The purity of these toxins was verified by TLC and spectrophotometric analysis, as well as by analysis of the phenylalanine content in the samples after acid hydrolysis. Bovine serum albumin was obtained from Sigma (Grade AA, crystallized and lyophilized, essentially free of fatty acid, cat. no. A7511) and used without further purification. Other chemicals were either reagent grade or chemically pure.

Quantitation of ochratoxins. A combination of spectrophotometric and spectrophotofluorometric methods was used for ochratoxin quantitation.[11-13] A molar absorptivity of 6500 was used for quantitation of OB (at 318 nm), Oβ (at 322 nm) and OC (at 333 nm) when the concentration was greater than 1×10^{-5} M. For Oα, a molar absorptivity of 5800 (at 338 nm) was used. At lower concentrations (1×10^{-5} M and below), spectrophotofluorometric methods were employed. In each assay, the solution was adjusted to pH 2·0 with HCl before the fluorescence intensity was determined. A standard curve was used for comparison. The activation and emission wavelengths were set at 340 and 440 nm for Oα, 320 and 420 nm for Oβ, 316 and 470 nm for OB, and 340 and 475 nm for OC in such measurements.

Solubility studies for OC–BSA complexation. Since the solubility of OC in aqueous solution was considerably lower than that of OB or OA, a solubility method utilizing the principle of increase in solubility of the ligand in the presence of BSA was used in the study of the complexation between OC and BSA. Appropriate amounts of OC (total concentration, $6{\cdot}0 \times 10^{-5}$ M) in 0·5 ml ethanol were pipetted into a series of centrifuge tubes and the solvent was evaporated with a stream of nitrogen. Three milliliter sodium phosphate buffer (NaPB, 0·1 M, pH 7·0) or 3 ml BSA solution (0·6–3·6 mg in NaPB) was added to each tube, stirred for 30 min and then centrifuged. The spectra of the supernatants of the solution were determined. These solutions were then subjected to equilibrium dialysis or dialyzed against 0·16 M KCl for the spectrophotometric titrations.

Spectral analyses and spectrophotometric titrations. The spectra of various ochratoxin–BSA complexes were determined by the analysis of appropriate solutions in either a Beckman DU or a Beckman Acta spectrophotometer with a light path of 1 cm at room temperature (26–27°). Spectrophotometric titrations were carried out

in appropriate solutions containing 0·16 M KCl using a Radiometer automatic titrator with a Radiometer Titrigraph type SBR2 and SBU1 syringe buret (Copenhagen, Denmark) as previously described.[11] Titrations were performed at 365 and 377 nm for OB–BSA, 380 and 400 nm for OC–BSA, 390 nm for Oα–BSA and 375 nm for Oβ BSA complexes.

Equilibrium dialysis. Equilibrium dialysis was carried out at 6° and pH 7·0 in 0·1 M sodium phosphate buffer according to the method of Rosenberg and Klotz.[15] Details of this method have been described previously.[11] The total concentrations for BSA and ligands in the equilibrium dialysis experiments were generally $1{\cdot}0 \times 10^{-4}$ M, and $8{\cdot}5 \times 10^{-6}$ to $4{\cdot}0 \times 10^{-4}$ M respectively. At the very minimum, duplicate experiments were carried out for each concentration analyzed. The overall data, at $\bar{\nu}$ (mole of ochratoxin bound to 1 mole of BSA) below 2 for OB and OC and at $\bar{\nu}$ below 1 for Oα and Oβ were treated by the least squares method. A value of 65,000 was used for the calculation of the molar concentration of BSA.[16]

RESULTS

Effect of BSA on the solubility of ochratoxin C. The effect of BSA on the solubility of OC is shown in Fig. 2. The binding of OC by BSA was demonstrated by the increase of absorbance at 390 nm in the supernatant. The absorbance at 390 nm increased proportionally with the increase in BSA added to the experimental system until the protein concentration was greater than 2×10^{-5} M, at which point most OC appeared to be dissolved in the solution. Assuming most of the OC in the supernatant to be in the complexed form, the maximal binding sites for OC in the BSA molecule were estimated to be around 2·70.

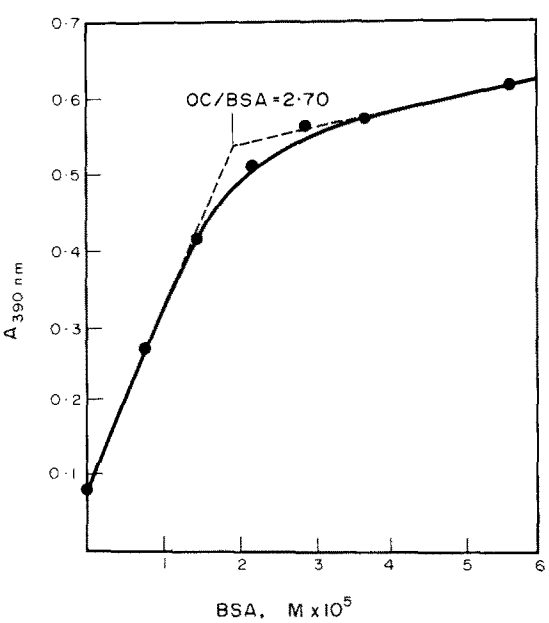

FIG. 2. Effect of BSA on solubility of ochratoxin C. The total starting OC concentration in each tube was $6{\cdot}0 \times 10^{-5}$ M. Total BSA concentrations added to each tube are indicated in the figure. The ratio OC/BSA was calculated directly from the total OC and BSA concentration in each tube.

Spectrophotometric analyses. The spectra of the OB and OB–BSA complexes at pH 7·0 are shown in Fig. 3. In presence of BSA, the band with a maximum absorption at 363 nm shifted to a longer wavelength at 377 nm. The shifting of spectra as a result of complexation of the toxin with BSA was pH-dependent, and the result is shown in Fig. 4. The spectra of the complex shifted to the longer wavelength when the pH of the solution gradually increased from acidic to basic values. However, at extremely alkaline pH, such as pH 11, the spectra shifted back to a shorter wavelength, which more or less resembled that of the free ligand. Since the lactone ring in ochratoxin may break down under more highly alkaline conditions, no experiments have been done at pH above 12·0. Shifting of spectra as a result of complexation with BSA was also observed for ochratoxin C. For the OC–BSA complex, the result of such shifting and the effect of pH on it are similar to the observations made for OB–BSA, except that the absorption maximum for OC–BSA was around 390 nm. Spectrophotometric analyses of Oα and Oα–BSA complex, and Oβ and Oβ–BSA complex, revealed that their spectra did not change significantly.

Spectrophotometric titrations. Spectrophotometric titrations for OB–BSA (1 : 1) and OC–BSA (1 : 1) complexes were carried out at 365 and 377 nm, and 385 and 400 nm respectively. The results of these titrations are shown in Fig. 5, *a* and *b*. The titration curves of OB–BSA and OC–BSA are qualitatively similar. When the OB–BSA titration was performed at 377 nm or the OC–BSA titration was performed at 400 nm, the titrations showed three distinct regions. Below pH 7·0, a specific group with

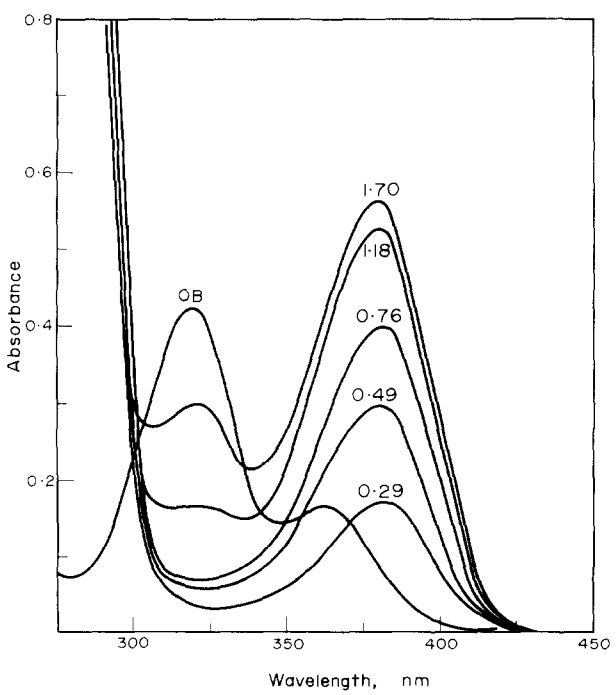

FIG. 3. Spectra of OB–BSA complexes. The number next to each curve indicates the molar ratio of OB to BSA. The spectra of the OB–BSA complex were measured against a dialysate OB solution to correct any absorption due to free OB at pH 7·0. The total BSA concentration was $1{\cdot}0 \times 10^{-4}$ M in each case. The concentration of OB (curve OB) was $6{\cdot}86 \times 10^{-5}$ M.

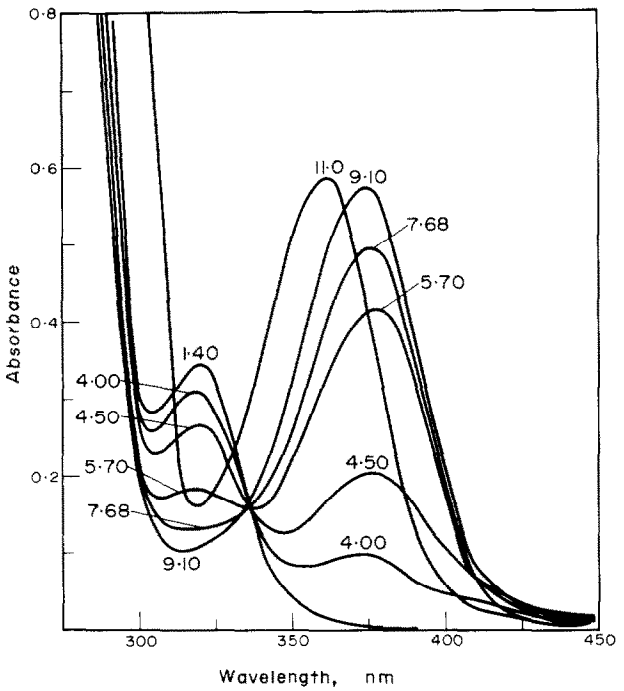

FIG. 4. Spectra of 1:1 OB–BSA at various pH values. The concentration of the 1:1 mixture of OB–BSA was $1{\cdot}50 \times 10^{-4}$ M respective to OA and BSA.

an apparent pK (estimated from the half-titration pH) around 4·65–4·75 was titrated. Above pH 7·0, another distinct specific group, with an apparent pK around 10·5–11·5, was titrated. Absorbance did not change appreciably between pH 8·0 and 10·0. The titration curves for OB–BSA and OC–BSA complexes at the wavelengths of 365 and 385 nm were similar to those at 377 and 400 nm, except that no deflection was observed above pH 10·0 and the absorbance continued to increase after this pH.

Contrary to the results obtained for the titrations of OB–BSA and OC–BSA, no significant difference was found between Oα and Oα–BSA (1:1), or between Oβ and Oβ–BSA (1:1). Only one apparent pK, having a value between 10·5 and 11·0, was found in all these titrations.

Determination of binding constants by equilibrium dialysis. The binding data, obtained in 0·1 M phosphate buffer at pH 7·0 and 6°, at different ochratoxin concentrations, were treated according to the method of Scatchard,[17] and are presented in Fig. 6. The lines in Fig. 6 were calculated from the individual experimental data using the least squares method, assuming that ochratoxin–BSA binding systems involve a single primary binding site. From the calculated lines it was found that 1·03, 1·02, 1·93 and 3·24 moles of Oα, Oβ, OB and OC were bound to 1 mole BSA in a strong primary site with the apparent equilibrium constants, K, of $1{\cdot}5 \times 10^6\ M^{-1}$, $5{\cdot}1 \times 10^5\ M^{-1}$, $7{\cdot}1 \times 10^5\ M^{-1}$ and $8{\cdot}9 \times 10^5\ M^{-1}$ for Oα BSA, Oβ BSA, OB–BSA and OC–BSA complexes respectively. For convenience of comparison, the binding data and absorption maxima of different ochratoxin–BSA complexes are summarized in Table 1.

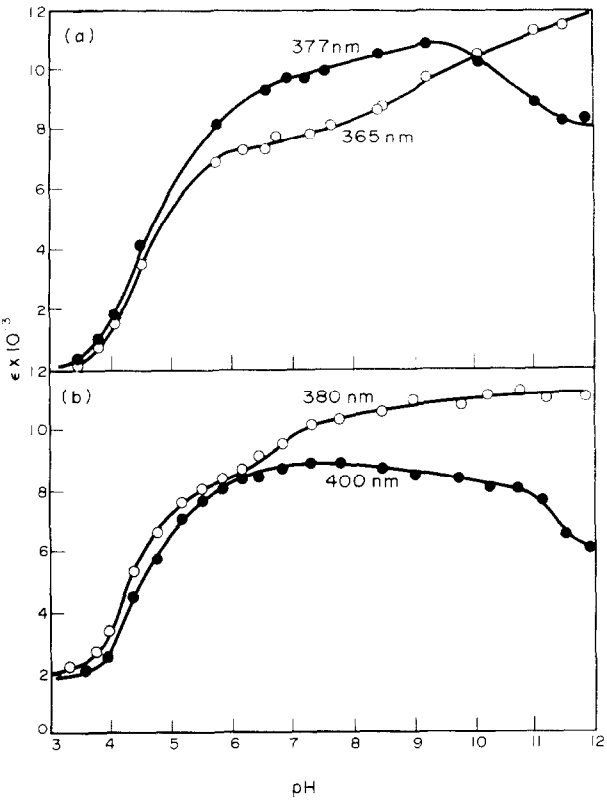

FIG. 5. Spectrophotometric titration of 1:1 molar ratio OB–BSA (a) and OC–BSA (b) complexes.

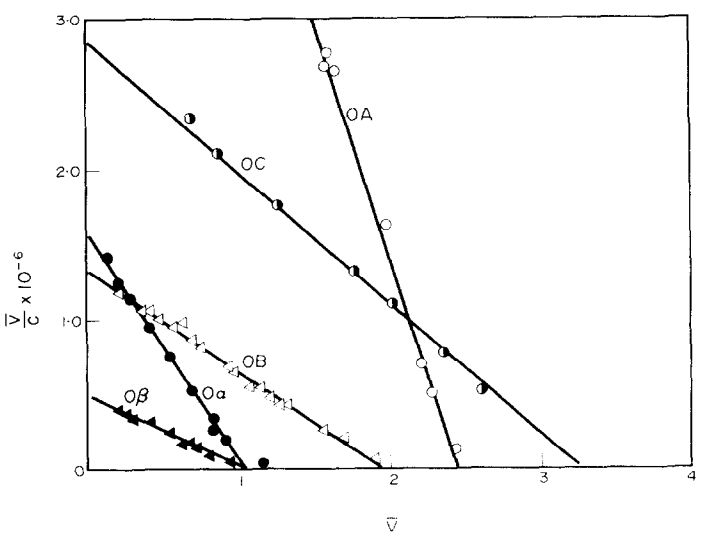

FIG. 6. Scatchard plots of binding of ochratoxins to BSA at pH 7·40 and 6·... Represents the moles of ochratoxin bound to 1 mole of BSA in a typical run. C represents the free ochratoxin concentration at equilibrium. The binding data for OA were obtained from a previous study.[4]

TABLE 1. SUMMARY OF BINDING PROPERTIES OF DIFFERENT OCHRATOXINS WITH BSA AT pH 7·0 AND 6

Ochratoxin	Absorption maxima		No. of binding sites	Equilibrium constant ($M^{-1} \times 10^{-n}$)	Standard free energy change ΔG (kJ mole)
	Toxin alone (nm)	With BSA (nm)			
A*	333,375 380	390 395	2·47	3·17	−34·73
B	318,365	377	1·93	0·71	−31·25
C	333,375 380	390	3·24	0·89	−31·71
α	333	333	1·03	1·50	−30·01
β	322	322	1·02	0·51	−30·50

* Data obtained from Chu.[11]

DISCUSSION

Spectrophotometric analysis, solubility determination and equilibrium dialysis in the present study revealed that ochratoxin interaction with BSA was not confined to OA, but also included other currently known ochratoxins as well. Nevertheless, the data indicate that the mechanisms of interaction of different ochratoxin derivatives with bovine serum albumin vary considerably. Accordingly, the binding of different types of ochratoxins with BSA can be classified into two catagories. Ochratoxin B and OC appear to resemble OA in their mode of interaction with BSA, which is characterized by: (1) a significant red shift in the spectra of the ligand in the presence of BSA (Fig. 3 and 4), and (2) 2–3 moles of ligand bound to 1 mole of BSA. On the other hand, only 1 mole of either Oα or Oβ bound to 1 mole of BSA without bringing about a red shift in the spectra. These observations are comparable with the results obtained by others in the study of interaction of coumarin derivatives with human as well as bovine albumin.[18, 19] For example, the presursor coumarins, such as coumarin and 4-hydroxycoumarin, showed a single binding site for albumin, whereas all the other coumarins, such as warfarin, bishydroxycoumarin and acenocoumarin, showed two binding sites.[18,19] Since all ochratoxins contain an isocoumarin ring, the mode of binding of these toxins with BSA may be similar to that of anticoagulants. All these compounds may bind to the same general region in the albumin molecules.

Although the role of hydrophobic and ionic forces in the binding of OA was previously emphasized, data were not available at that time to distinguish which one was predominant.[11] Present data suggest that both hydrophobic and ionic forces are equally important. Since the phenolic hydroxyl group in the Oα and Oβ molecules did not dissociate until the pH had exceeded 10·5 and since only 1 mole of either of these ligands bound to 1 mole of BSA, the force involved in the interaction of these toxins with BSA at pH 7·0 may be confined to the coumarin nuclei, which are primarily hydrophobic in character. This hydrophobic binding site in BSA may be shared by all the ochratoxins and by other coumarin derivatives as well.[18, 19, 20] The second site for the binding of ochratoxins (i.e. in OA, OB and OC) in the same region of the BSA molecule may be ionic in character, because it was found that the pK of the toxins decreased on binding, and that the ionized form showed a red shift (Figs. 3–5 and reference 11). The red shift was pH-dependent and also correlated with the pH-dependent transformation of the BSA structure.[21] The second binding between ochratoxins and BSA occurred only when the pK values approximated neutral pH. Thus, when the pK increased, as in the case of OB as compared with OC and OA

(the pK of OB is 8·0, while pK values for OC and OA are 6·9 and 7·0 respectively), the binding became weaker. Observation of the similarity between the binding constants of the parent toxins, OA and OB, and those of the hydrolyzed products further suggests that the hydrophobic and ionic forces are of equal importance in the interaction. The involvement of ionic force,[18] hydrogen binding and hydrophobic force[19,20] in the interaction between anticoagulant drugs and serum albumin has been suggested. The third binding site in OC–BSA interaction is not identified at present. Since OC is very insoluble in aqueous solution, hydrophobic forces may play a significant role in the interaction.

The presence of both hydrophobic and ionic ochratoxin binding sites in BSA clarifies an earlier observation regarding the interaction of OA with BSA,[11] for it was noted that spectrophotometric (400 nm) determination using Job's continuous variation method suggested only one binding site, while analyses utilizing equilibrium dialysis techniques suggested slightly more than two binding sites. It now becomes apparent that only one of the binding sites, the one involving the interaction of phenolate ion with the cationic center of BSA, was determined by the spectrophotometric method; the other one, the one involved in the hydrophobic interaction, was detected only by equilibrium dialysis techniques.

Since it has been previously demonstrated that dissociation of the phenolic hydroxyl group in ochratoxins is necessary for their biological activity,[9] it is noteworthy that the dissociation of such groups is also essential for the binding of the second mole of biologically active ochratoxins to the albumin molecule (Table 1). If mycotoxin–protein interaction plays any role in the manifestation of biological activity by ochratoxin, the binding of ochratoxins with tissue proteins and enzymes *in vivo* may partially resemble the ochratoxin albumin interacting system. However, it must be pointed out that the synthetic racemic mixture of ochratoxin A (±) is less active than natural ochratoxin A (−). Until we learn more about the binding pattern of these optical enantiomorphs with albumin, the role of the steric effect on the binding of ochratoxins with proteins must not be overlooked. For instance, O'Reilly[19] has pointed out that while there is little difference in binding energy for (−)-5-warfarin albumin and (+)-5-warfarin albumin systems, the biological activity of the former is several times greater. The failure of ochratoxins α and β to induce toxicity in animals may be due, at least in part, to their ineffectiveness in the protein bindings. The toxin may either be failing to reach the target tissue or may fail to react with tissue or cellular protein.

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