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# Drug-Biomacromolecule Interaction VIII

## Effects of pH and Ionic Strength on the Binding of Cefazolin to Bovine Serum Albumin Using Difference Spectra

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**Abstract** □ The effects of ionic strength and pH on the binding of cefazolin to bovine serum albumin (BSA) were studied by UV difference spectrophotometry. 2-(4′-hydroxybenzeneazo) benzoic acid as the UV spectrophotometric probe was used. As ionic strength at constant pH and temperature increases, the apparent bining constant decreased but the number of binding sites remained almost constant at 2. The constancy of the number of binding sites with increasing the ionic strength suggests that purely electrostatic forces between BSA and drug do not have great importance in the drug binding, even though there is a decrease in the apparent binding constant. Thus, the effect of ionic strength on the interaction between drug and BSA may be explained by the changes in ionic atmosphere of the aggregated BSA molecules and competitive inhibition by phosphate ions. In addition, the higher apparent binding constant at high ionic strength is explained by conformational changes of BSA from its aggregate forms into subunits. The pH effects on the affinity of interactions indicated that the binding affinity of cefazolin is higher in the neutral region than in the alkaline region. And at high pH value, the number of binding sites decreased from 2 to 1 because of the conformational changes of BSA in the alkaline region.

**Keywords** □ Difference spectra, Protein binding, Cefazolin, Bovine serum albumin, pH, Ionic strength, Absorbance difference.

The binding of drugs to plasma proteins may play an important role in the pharmacokinetic consequences and pharmacologic results of drugs.[1] Serum albumin is the major contributor to drug-protein binding in blood.[2] Thus, the binding affinity of drug to serum albumin is one of the most critical information about the drug efficacy.

In the process of binding of drug to serum albumin, various interactions are involved. These interactions are mainly long range electrostatic forces, short range specific interactions such as hydrogen and hydrophobic bonds, and proton dispersion forces.[3] In order to make a meaningful interpretation of the binding process, several factors such as ionic strength, pH, temperature, buffer composition, etc., must be considered. In this study, the effects of ionic strength and pH on the binding of cefazolin to bovine serum albumin (BSA) were examined quantitatively by UV difference spectrophotometric method at constant temperature (20°C).

## EXPERIMENTAL METHODS

### *Materials*

Bovine serum albumin(BSA), Fraction V was purchased from Sigma Co. and its molecular weight was assumed to be 69,000. The concentration of BSA solution was determined from the absorbance of the peak at 280 nm. The molar concentration was calculated on the basis

of $E_{1\%}^{1cm}=6.67$. The spectrophotometric probe, 2-(4′-hydroxybenzeneazo) benzoic acid(HBAB), was purchased from ICN Pharm. Inc.. Sodium cefazolin anhydrous was supplied by Chong Kun Dang Co.. All other chemicals were of analytical reagent grades. The water used was double distilled from the glass.

*Apparatus*

The ultraviolet absorbance differences were measured on the model SP 1750 Pye Unicam Spectrophotometer using tandem cells. The temperature during the binding study was controlled by circulator (Technique Co.).

*Measurement of Absorbance Differences*

The binding of the probe to BSA was determined by measuring the increase in the absorbance differences following the titration of the protein solution with the probe according to the method described in previous reports.[4,5]

The probe, HBAB, was dissolved in methanol to make the concentration of $1\times10^{-3}$M. The BSA solutions were prepared with phosphate buffer at pH 6.53-8.0. The concentration of BSA solution was $2.9\times10^{-5}$M and concentration of cefazolin was $1\times10^{-3}$M.

One ml quantity of BSA solution was pipetted into one compartment of each of tandem cells and 1 ml of buffer solution pipetted into each remaining compartments of the cells. These solutions were placed in the reference and sample beams in such a manner that a buffer and a BSA solution compartments were in tandem in each beam. Tandem cell arrangement is shown in Figure 1.

After a base line was established, the contents of buffer solution compartment in the reference beam and the BSA solution compartment in the sample beam were titrated simultaneously with successive additions of 5 $\mu$l of HBAB solution and absorbance differences were measured at 484 nm in each time. A separate titration of BSA solution was carried out in the presence of $1\times10^{-3}$M of cefazolin, individually.

*Data Treatment*

The fraction of HBAB bound was calculated using the method described in the previous report.[5] The Scatchard equation was applied to determine the binding parameters of the BSA-HBAB interaction.[6]

$$\frac{\overline{V}}{A}=nK-VK$$

where V is the number of moles of bound HBAB per mole of BSA, A is the concentration of free HBAB, n is the number of binding sites on the BSA molecule, and K is the apparent binding constant of HBAB to BSA. The binding constant

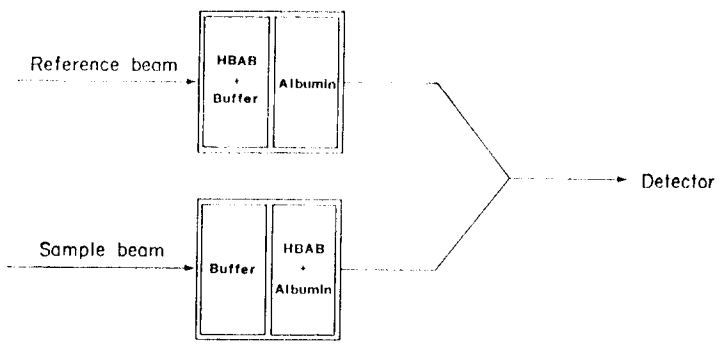


**Fig. 1**: Tandem cell arrangement for difference spectrophotometry.

of cefazolin was calculated by using the equation derived by Klotz et al.[7]

## RESULTS AND DISCUSSION

The difference absorption spectra were characterized by two positive peaks at 484 nm and 262 nm, respectively, and one negative peak at 345 nm in the wavelength range of 250~600 nm.[4] These spectra have a maximum at 484 nm and the absorbance difference increases as the concentration of the probe increases. The decrease in absorbance difference of HBAB-BSA in the presence of cefazolin shows competition between HBAB and cefazolin for the same sites or closely located sites on the BSA.

*Effect of Ionic Strength*

The absorbance difference titration curves at various ionic strengths are shown in Figure 2. The shapes of titration curves were convex at the higher concentration of HBAB because of the saturation of BSA binding sites. The absorbance differences decreased in the presence of cefazolin. Scatchard plots for the binding of HBAB to BSA in the absence and presence of cefazolin at various ionic strengths are shown in Figure 3.

Figure 4 shows the effect of ionic strength on the binding of cefazolin to BSA at 20°C and pH 7.4. It shows that the apparent binding constants of HBAB and cefazolin decrease with the increase in ionic strength up to $\mu=0.64$, and increase at higher ionic strength ($\mu=0.64$).

The number of binding sites of BSA is very slightly increased with the increase in ionic strength except higher ionic strength ($\mu=0.64$). This suggests that certain binding sites of BSA for drugs are located in the ionic region. It is consistent with the fact that acidic compounds are nonspecifically bound to BSA by the electrostatic forces between acidic groups of the compound and cationic groups of BSA.[8]

Zolla et al.[9] explained the effect of ionic strength on the interaction between drug and serum albumin in terms of changes in ionic atmosphere of the aggregated BSA molecules; competitive inhibition by other electrolytes; salting out effect. Whitlam et al.[10] and Cho et al.[11] explained that decreasing the apparent binding constants with increasing ionic strength is the result of phosphate buffer competing for the binding sites. These two explanations are consistent with the result of our study up to $\mu=0.36$, but inconsistent at $\mu=0.64$. This phe-

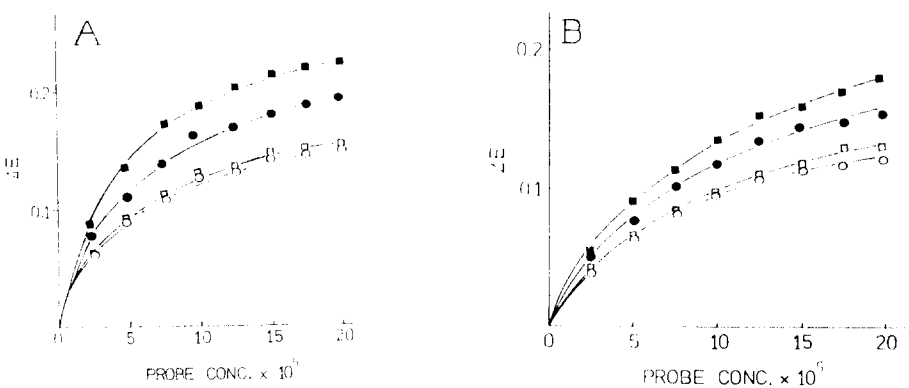


**Fig. 2**: Absorbance difference titration curves of BSA with HBAB in the absence (A) and presence of cefazolin (B) at various ionic strengths. (20°C, pH 7.4)
■, 0.04; ●, 0.16; □, 0.36; ○, 0.64.

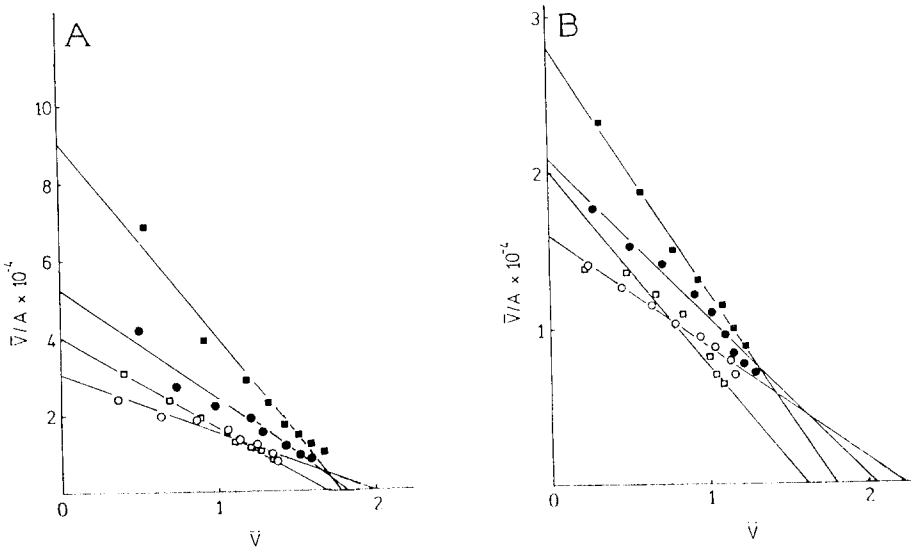


**Fig. 3**: Scatchard plots for the binding of HBAB to BSA in the absence (A) and presence of cefazolin (B) at various ionic strengths. (20°C, pH 7.4)
■, 0.04; ●, 0.16; ○, 0.36; □, 0.64.

nomenon can be explained by the fact that most albumin molecules aggregate at low ionic strength and dissociate into subunits at high ionic strength.[12] The resulting effects of ionic strength on the binding parameters are summarized in Table I.

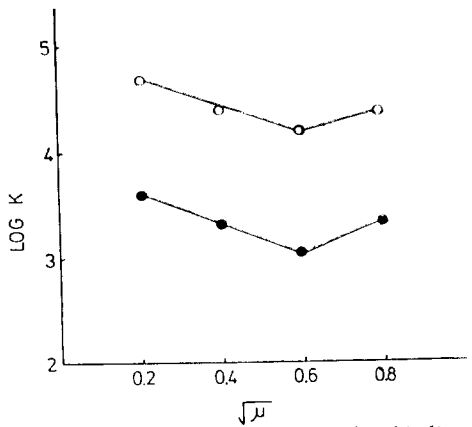


**Fig. 4**: Effect of ionic strength on the binding of drug to BSA at 20°C, pH 7.4.
○, HBAB; ●, Cefazolin.

*Effect of pH*

The absorbance difference titration curves at various pHs are shown in Figure 5. Scatchard plots for the binding of probe to BSA in the absence and presence of cefazolin at various pHs are shown in Figure 6. Figure 7 shows the effect of pH on the binding of drug to BSA at 20°C and $\mu$=0.16. The apparent binding constants are slightly higher in the acidic region than in the alkaline region.

The decrease in apparent binding constant of

**Table I: Effect of ionic strength on the binding parameters at 20°C, pH 7.4.**

| $\mu$ | HBAB | | Cefazolin | |
|---|---|---|---|---|
| | $K \times 10^{-4}$ ($M^{-1}$) | $n$ | $K \times 10^{-3}$ ($M^{-1}$) | $n$ |
| 0.04 | 5.03 | 1.80 | 4.08 | 1.79 |
| 0.16 | 2.86 | 1.85 | 2.11 | 2.06 |
| 0.36 | 1.56 | 1.98 | 1.07 | 2.24 |
| 0.64 | 2.36 | 1.70 | 2.19 | 1.66 |

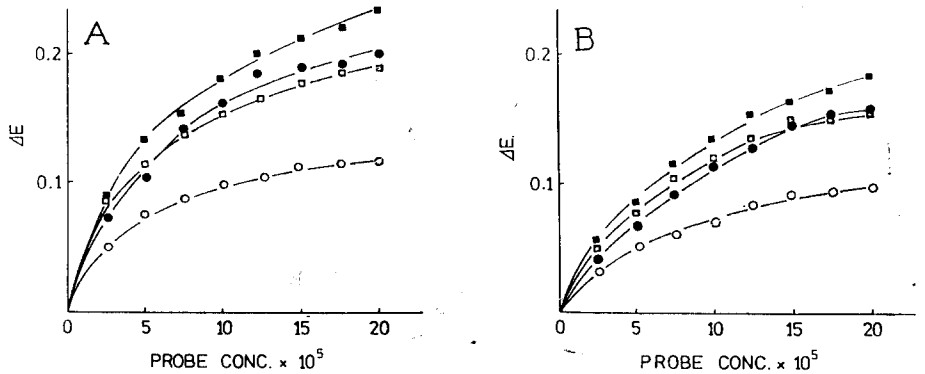


**Fig. 5**: Difference absorbance titration curves of BSA with HBAB in the absence (A) and presence of cefazolin (B) at various pHs. (20°C, $\mu$=0.16)
■, 6.53; •, 7.0; □, 7.4; ○, 8.0.

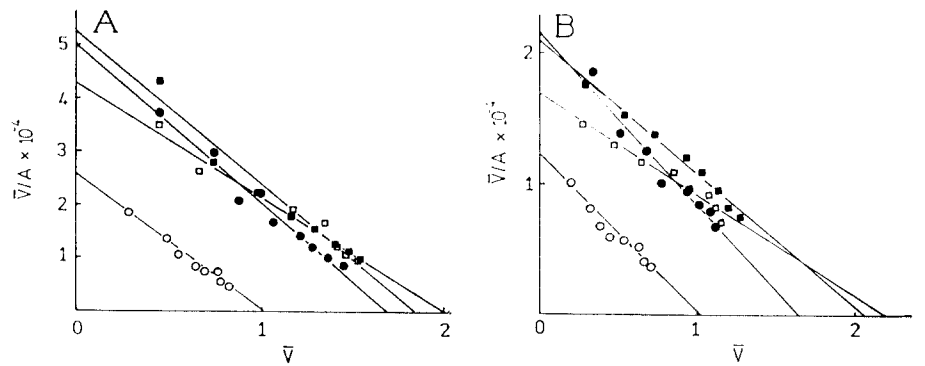


**Fig. 6**: Scatchard plots for the binding of HBAB to BSA in the absence (A) and presence of cefazolin (B) at various pHs. (20°C, $\mu$=0.16)
•, 6.53; □, 7.0; ■, 7.4; ○, 8.0.

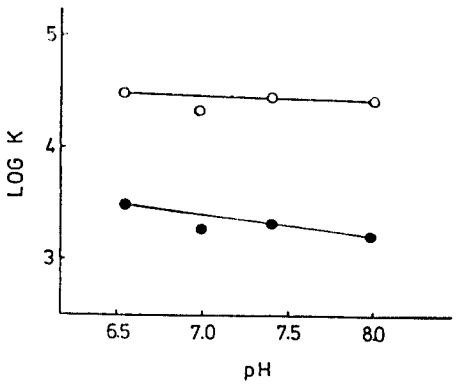


**Fig. 7**: Effect of pH on the binding of drug to BSA at 20°C, $\mu$=0.16.
○, HBAB; •, Cefazolin.

drug to BSA with the increasing pH can be explained by two mechanisms. One is that, since the intact BSA molecules have a negative excess charge, electrostatic repulsive forces affect the bindings between acidic drugs and BSA.[2] And the other is that, because of the hydrophobicity of substance in the binding process, drugs interact with BSA in less degree in the ionic than in the nonionic state.[13]

Leonard et al.[14] reported that conformational changes occur in serum albumin over pH 6-9. In 1974, Zurawski et al.[15] established that two conformational states exist in BSA molecule

**Table II: Effect of pH on the binding parameters at 20°C, $\mu$=0.16.**

| pH | HBAB | | Cefazolin | |
|---|---|---|---|---|
| | $K\times10^{-4}$ ($M^{-1}$) | $n$ | $K\times10^{-3}$ ($M^{-1}$) | $n$ |
| 6.53 | 2.94 | 1.71 | 3.01 | 1.63 |
| 7.0 | 2.18 | 2.00 | 1.89 | 2.18 |
| 7.4 | 2.86 | 1.85 | 2.11 | 2.06 |
| 8.0 | 2.57 | 1.01 | 1.54 | 1.01 |

over this pH region. They called the form at neutral pH(pH 6-7) the "N" form and the form at higher pH(around pH 9) the "B" form. Thus, the conformational change that occurs is the N to B or B to N transition. The N to B transition sems to be dependent on pH and it may also occur to some extent by ionic strength of the buffer and buffer ion composition.[16] The decrease in number of the binding sites in the alkaline region may be explained by this conformational changes of BSA molecules. The apparent binding constants of HBAB and cefazolin at various pH values are summarized in Table II.

## ACKNOWLEDGEMENT

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## LITERATURE CITED

1) Anton, A.H. and Solomon, H.V. (Ed.): *Drug-Protein Binding*, The New York Academy of Sciences, ANYAA9 226, 162-272 (1973).

2) Gibaldi, M.: *Biopharmaceutics and Clinical Pharmacokinetics* (2nd Ed.), Lea & Febiger, Philadelphia, p. 86 (1977).

3) Foster, J.F., and Sterman, M.D.: Conformational changes in bovine plasma albumin associated with hydrogen ion and urea binding II, Hydrogen ion titration curves. *J. Amer. Chem. Soc.* **78**, 3636(1956).

4) Kim, C.K., Yang, J.S. and Lim, Y.S.: Drug-Biomacromolecule Interaction IV: Binding of six cephalosporins to bovine serum albumin. *Arch. Pharm. Res.* **6**(1), 55(1983).

5) Kim, C.K., Lim, Y.S. and Yang, J.S.: Drug-Biomacromolecule Interaction VI: Binding of nalidixic acid and probenecid to bovine serum albumin. *Yakhak Hoeji* **27**, 257(1983).

6) Scatchard, G.: *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).

7) Klotz, I.M., Triwush, H. and Walker, F.M.: The binding of organic ions by proteins; Competition phenomena and denaturation effects. *J. Amer. Chem. Soc.* **70**, 2935(1948).

8) Moriguchi, I., Fushimi, S., Ohshima, C. and Kaneniwa, N.: Spectroscopic Studies on Molecular Interactions VI: Mechanism of metachromasy of 2-(4'-hydroxyphenylazo) benzoic acid by serum albumin. *Chem. Pharm. Bull.* **18**, 2447 (1970).

9) Zolla, L., Amiconi, G. and Catanese B.: Binding of organic ions by proteins I; Interaction of 1-benzyl-3-indazoleoxyacetate with serum albumin. *Biochem. Pharmacol.* **26**, 659(1977).

10) Whitlam, J.B., Crooks, M.J., Brown, K.F. and Pederson P.V.: Binding of nonsteroidal anti-inflammatory agents to proteins I; Ibuprofen-serum albumin interaction. *ibid.*, **28**, 675(1979).

11) Cho, M.J., Mitchell A.G. and Pernarowski M.: Interaction of bishydroxycoumarin with human serum albumin. *J. Pharm. Sci.* **60**, 196(1971).

12) Takada, K., Narimiya, O. and Muranishi S.: Biopharmaceutical study of the hepato-biliary transport of drugs III; Binding characteristics of bromophenol blue and amaranth to liver cytoplasmic Y and Z binding protein *in vitro*. *Chem. Pharm. Bull.* **23**, 729(1975).

13) Plaizier-Vercammen J.A. and De Neve R.E.:

Interaction of povidone with aromatic compounds III; Thermodynamics of the binding equilibria and interaction forces in buffer solutions at varying pH values and varying dielectric constant. *J. Pharm. Sci.* **71**, 552(1982).

14) Leonard, W.J., Vijai K.K. and Foster J.F.: A structual transformation in bovine and human plasma albumin in alkaline solution as revealed by rotatory dispersion studies, *J. Biol. Chem.* **238**, 1984(1963).

15) Zurawski, V.R. and Foster, J.F.: *Biochemistry* **13**, 3465(1974).

16. Elbary, A.A., Vallner J.J. and Whitworth C.W: Effect of albumin conformation on the binding of phenylbutazone and oxyphenbutazone to human serum albumin. *J. Pharm. Sci.* **71**, 241(1982).