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Drug-Biomacromolecule Interaction (XIII) Effect of Ionic Strength, pH and Temperature on Binding of Cephalothin to Bovine Serum Albumin

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약물과 생체고분자 간의 상호작용(제 13보) 세파로친과 소혈청알부민의 결합에 미치는 이온강도, pH 및 온도의 영향

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To investigate the protein binding characteristics of cephalothin, the effects of ionic strength, pH and temperature on the binding of cephalothin to bovine serum albumin (BSA) were studied by UV difference spectrophotometric method. With increasing ionic strength at constant pH and temperature, association constant decreased, but the number of binding sites sites was about 2 constantly. It may be deduced that the binding process is not only due to electrostatic forces. And the increased association constant at high ionic strength is explained by conformational changes of BSA from complex to subunits. The pH effect on the affinity of interaction indicated that the binding affinity of drug 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 alkaline region. The decrease in binding affinity of BSA to drug with increasing temperature was characteristic of an exothermic reaction. And the negative sign of ΔG° meant that the binding process occurs spontaneously under the experimental conditions. In cephalothin-BSA complex formation, since the net enthalpy change value and entropy change value are positive, it is assumed that hydrophobic bindings are predominant in this binding process.

Keywords—bovine serum albumin, cephalothin, protein binding, pH, ionic strength, temperature, difference spectra, interaction.

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 mainly contributed to drug-protein binding in blood.[2] Thus, to understand the behavior of drug in the circulatory system, it is nessary to examine the binding of drug to serum albumin.

In the process of binding of drug to serum albumin, various interactions are included. These interactions are mainly long range electrostatic forces, short range specific interactions such as hydrogen and hydrophobic bonds, and protein dispersion forces.[3] In order to allow meaningful interpretation of the binding process, several factors such as ionic strength, pH, temperature, buffer composi-

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tion and so on should be considered.

In this study, in order to investigate the protein binding characteristics of cephalothin, the effects of ionic strength, pH and temperature on the binding of cephalothin to bovine serum albumin (BSA) were examined quantitatively by UV difference spectrophotometric method.

EXPERIMENTAL METHODS

Meterials

Bovine serum albumin (BSA), fraction V was purchased from Sigma Chemical Co. and its molecular weight was assumed to be 69,000. The concentration of BSA solution was determied 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 cephalothin anhydrous was supplied by Yuhan Corp. All other chemicals were of analytical reagent grades. The water used was double distilled from the glass.

Apparatus

The ultraviolet difference absorbances were measured on the model SP1750 Pye Unicam Spectrophotometer using tandem cells. The temperatures during the binding study were controlled by using circulator (Technique Co.).

Difference Absorbance Measurement

The binding of the probe to BSA was determined by measuring the increase in difference absorbance 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 at a concentration of 1×10^{-3}M. The BSA solutions were prepared with phosphate buffer at pH 6.53-8.0. The concentration of BSA solution was 2.9×10^{-5}M and the concentration of cephalothin was 1×10^{-3}M.

Two 1 m*l*-portions of BSA solutions were pipetted into two compartments of tandem cells and two 1 m*l*-portions of buffer solutions were pipetted into two 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 Fig. 1.

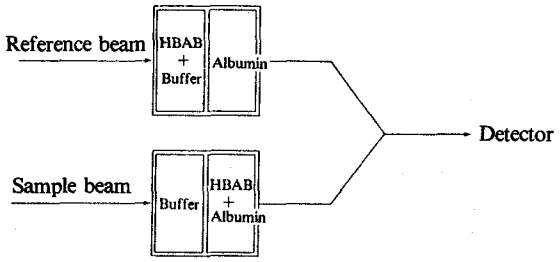


Figure 1—Tandem cell arrangement for difference spectrophotometry.

After drawing a base line, 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 μl of HBAB solution and difference absorbances were measured at 484 nm in each time. A separate titration of BSA solution was carried out in the presence of 1×10^{-5}M of cephalothin, individually.

Data Treatment

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

$$\frac{V}{A}=nK-VK \qquad (1)$$

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 appearent binding constant of HBAB to BSA. The binding constant of cephalothin was calculated by using the equation derived by Klotz *et al.*[7]

Thermodynamic Analysis

Assuming no significant temperature dependence of enthalpy change occuring within the temperature range used, the standard enthalpy change ($\Delta H°$) for the association of 1 mole of ligand with 1 mole of macromolecule was estimated from eq. (1).

$$\log\frac{K_1}{K_2} = -\frac{\Delta H}{2.303R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (2)$$

Neglecting the electrostatic energy change of binding, the free energy change of binding ($\Delta G°$) is given as follows:

$$\Delta G° = -RT\ln K \quad (3)$$

and the entropy change ($\Delta S°$) is obtained by substituting $\Delta H°$ and $\Delta G°$ into the Gibbs-Helmholtz equation:

$$\left[\frac{\partial \Delta G°}{\partial T}\right]_P = -\Delta S° = \frac{\Delta G° - \Delta H°}{T} \quad (4)$$

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. These spectra have a maximum at 484 nm and the difference absorbance increases as the concentration of the probe increases. The decrease in difference absorbance of HBAB-BSA in the presence of cephalothin shows competition between HBAB and cephalothin for the same site or closely located sites on the BSA.

Effect of Ionic Strength

The difference absorbance titration curves at various ionic strengths are shown in Fig. 2. As the HBAB concentration increased, titration curves were concaved toward the abscissa since the binding sites of BSA at low concentration are saturated. The difference absorbances decreased in the presence of cephalothin. Scatchard plots for the binding of HBAB to BSA in the absence and presence of cephalothin at various ionic strengths are shown in Fig. 3.

Fig. 4 shows that the effect of ionic strength on the binding of cephalothin to BSA at 20 °C and pH 7.4. It shows that the apparent binding constants of probe and cephalothin decrease with increasing of ionic strength except high ionic strength ($\mu = 0.64$). This suggests that certain binding sites of BSA for cephalothin are located in an ionic region. It is consistent with the fact that acidic compounds are non-

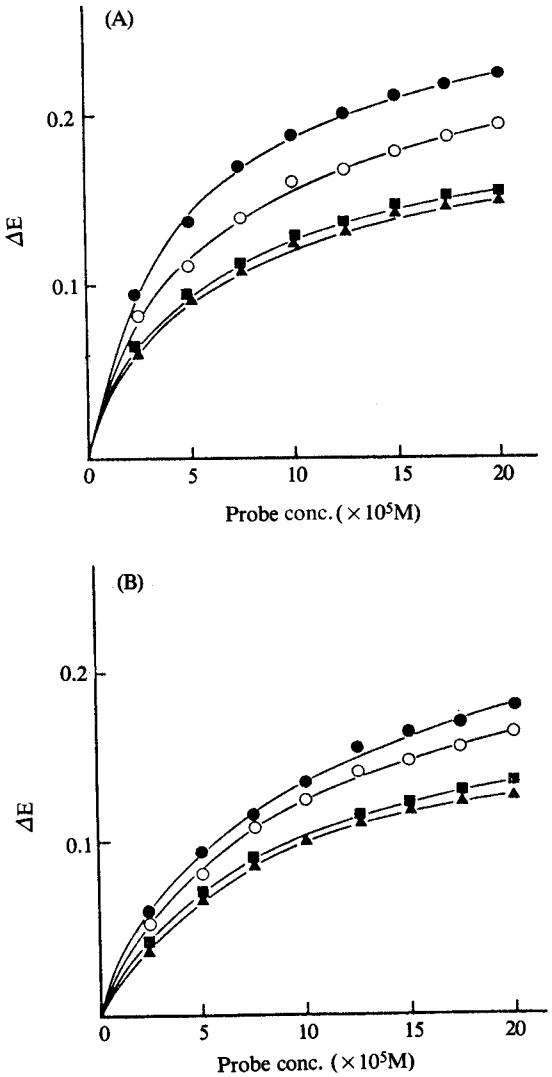
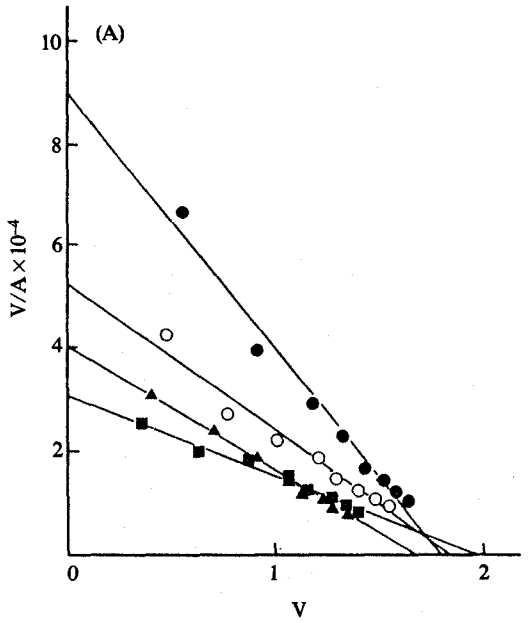


Figure 2—Difference absorbance titration curves of BSA with HBAB in the absence (A) and presence of cephalothin (B) at various ionic strengths (20 °C, pH 7.4). Key: ●, 0.04; ○, 0.16; ■, 0.36; ▲, 0.64

specifically bound to BSA by the electrostatic forces between acidic groups of the compound and cationic groups of BSA.[8]

The constancy of the number of binding sites with increasing the ionic strength suggests that purely electrostatic factors do not have major importance in the complex formation, even though there is a decrease in the apparent binding constant. Thus, the effect of ionic strength on the interaction



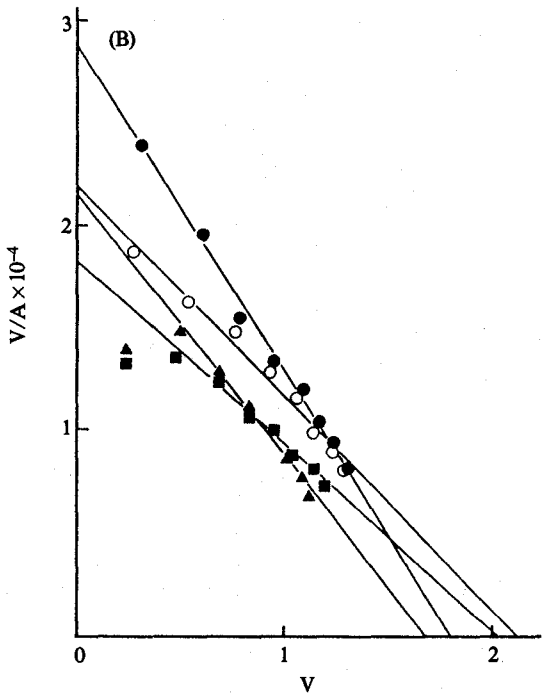


Figure 3—Scatchard plots for the binding of HBAB to BSA in the absence (A) and presence of cephalothin(B) at various ionic strengths (20 °C, pH 7.4).
Key: ●, 0.04; ○, 0.16; ■, 0.36; ▲, 0.64

between drug and BSA may be explained by the changes in ionic atmosphere of the association molecules.[9]

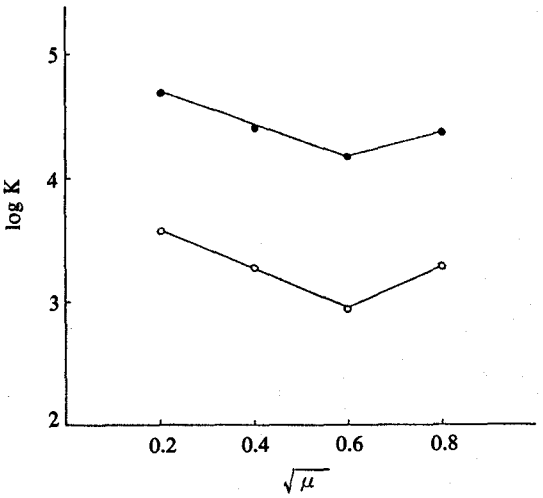


Figure 4—Effect of ionic strength on the binding of drug to BSA at 20 °C, pH 7.4.
Key: ●, HBAB; ○, Cephalothin

Table I—*Effect of Ionic Strength on the Binding Parameters at 20 °C and pH.*

μ	HBAB		Cephalothin	
	$K \times 10^{-4}$ (M^{-1})	n	$K \times 10^{-3}$ (M^{-1})	n
0.04	5.03	1.80	3.87	1.80
0.16	2.86	1.85	1.92	2.12
0.36	1.56	1.98	0.90	2.02
0.64	2.36	1.70	1.96	1.68

Whitlam *et al.*[10] and Cho *et al.*[11] explained that decreasing of the binding constants with increasing ionic strength is the result of phosphate buffer competition for the binding sites. These two reports are consistent with the result of this study to $\mu = 0.36$, but inconsistent at $\mu = 0.64$. This phenomena can be explained by the fact that at low ionic strength most of albumin molecules form heavy complexes by aggregation and at high strength dissociate to subunits.[12]

The resulting effects of ionic strength on the binding parameters are summarized in Table I.

Effect of pH

The difference absorbance titration curves at various pH are shown in Fig. 5. Scatchard plots for the binding of probe to BSA in the absence and pre-

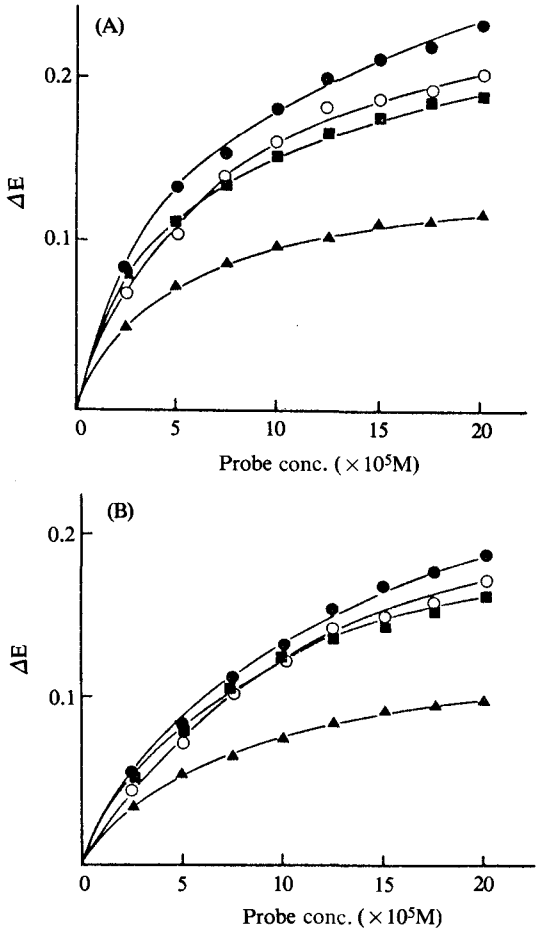


Figure 5—Differene absorbance titration curves of BSA with HBAB in the absence (A) and presence of cephalothin (B) at various pH (20 °C, μ=0.16).
Key: ●, 6.53; ○, 7.0; ■, 7.4; ▲, 8.0

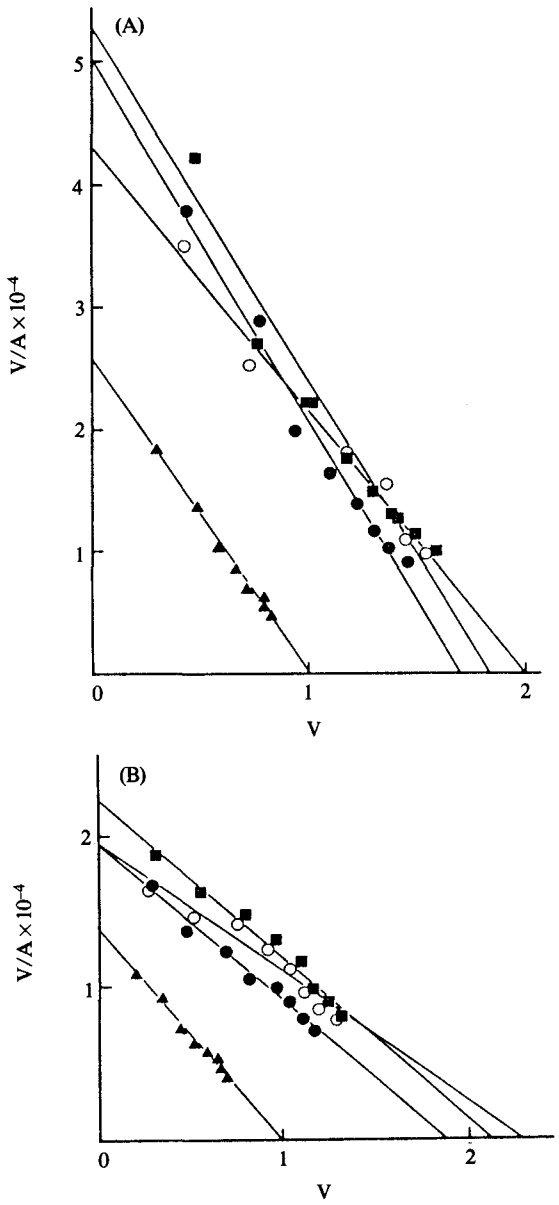


Figure 6—Scatchard plots for the binding of HBAB to BSA in the absence (A) and presence of cephalothin (B) at various pH (20 °C, μ=0.16).
Key: ●, 6.53; ○, 7.0; ■, 7.4; △, 8.0

sence of cephalothin at various pH are shown in Fig. 6. Fig. 7 shows the effect of pH on the binding of drug to BSA at 20 °C and μ=0.16. The apparent binding constants are slightly higher in the acidic region than in alkaline region.

Decreasing of apparent binding constant of drug to BSA with increasing pH can be explained by the two mechanisms. One is that, since the BSA molecules in native state have a negative excess charge, electrostatic repulsion 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 over this pH region. They called the form at neutral pH (pH 6-7) the "N" form and the form at higher pH(around

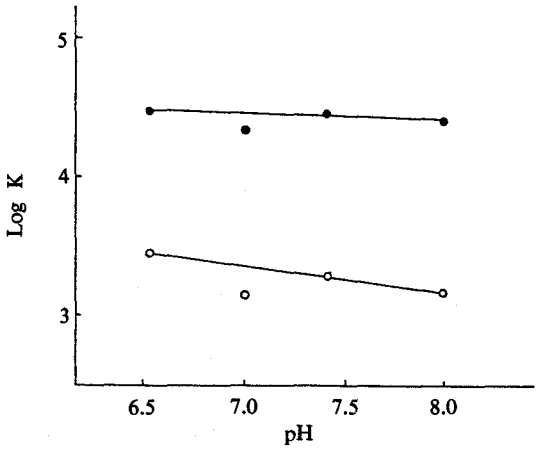


Figure 7—Effect of pH on the binding of drugs to BSA at 20°C and $\mu=0.16$.
Key: ●, HBAB; ○, cephalothin

Table II—*Effect of pH on the Binding Parameters at 20°C and $\mu=0.16$.*

pH	HBAB		Cephalothin	
	$K\times10^{-4}$ (M^{-1})	n	$K\times10^{-3}$ (M^{-1})	n
6.53	2.94	1.71	2.81	1.88
7.0	2.18	2.00	1.39	2.30
7.4	2.86	1.85	1.92	2.12
8.0	2.57	1.01	1.43	0.97

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 is seemingly dependent on pH and it may also occur to some extent with ionic strength of the buffer and buffer ion composition.[16] The decreasing number of the binding sites in alkaline region may be explained by this conformational changes of BSA molecules. Apparent binding constant of HBAB decreased to less extent than that of cephalothin. It suggests that HBAB-BSA interaction is less affected by pH than that of cephalothin.

Table II shows the binding parameters observed at various pH values.

Thermodynamic Analysis

The resulting energy and entropy effects are useful parameters for the interpretation of the

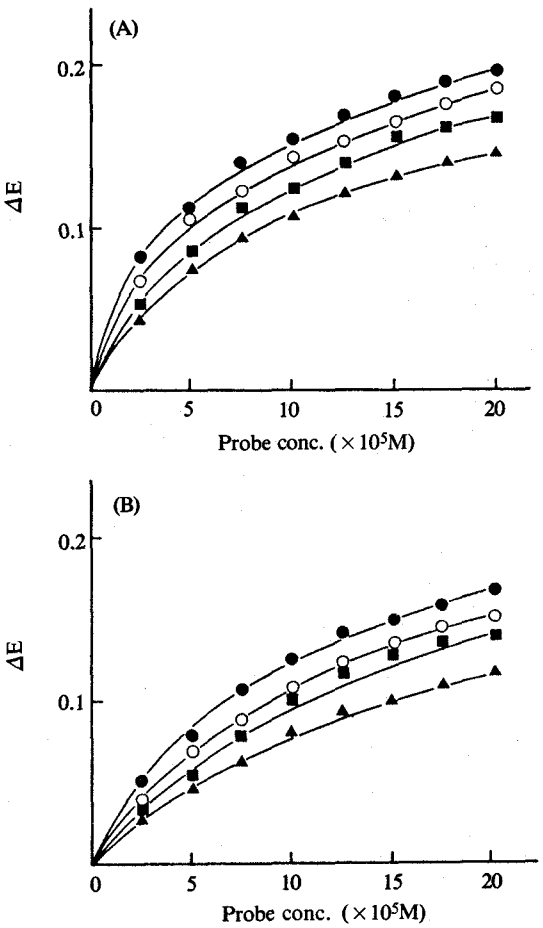


Figure 8—Difference absorbance titration curves of BSA with HBAB in the absence (A) and presence of cephalothin (B) at various temperatures (pH 7.4, $\mu=0.16$).
Key: ●, 20°; ○, 26°; ■, 30°; ▲, 37°

mechanism of interaction. The difference absorbance titration curves of HBAB with BSA in the absence and presence of cephalothin at various temperatures are shown in Fig. 8. Scatchard plots for the bindings at various temperatures are shown in Fig. 9. Fig. 10 shows the effect of temperature on the binding at pH 7.4 and $\mu=0.16$. By the usual thermodynamic methods, the changes in standard free energy, enthalpy and entropy for the binding of 1 mole of drug have been calculated and listed in Table III.

It is generally believed that there are four types of binding, which can be considered in complex formation of ligands with macromolecule, i.e., ion-

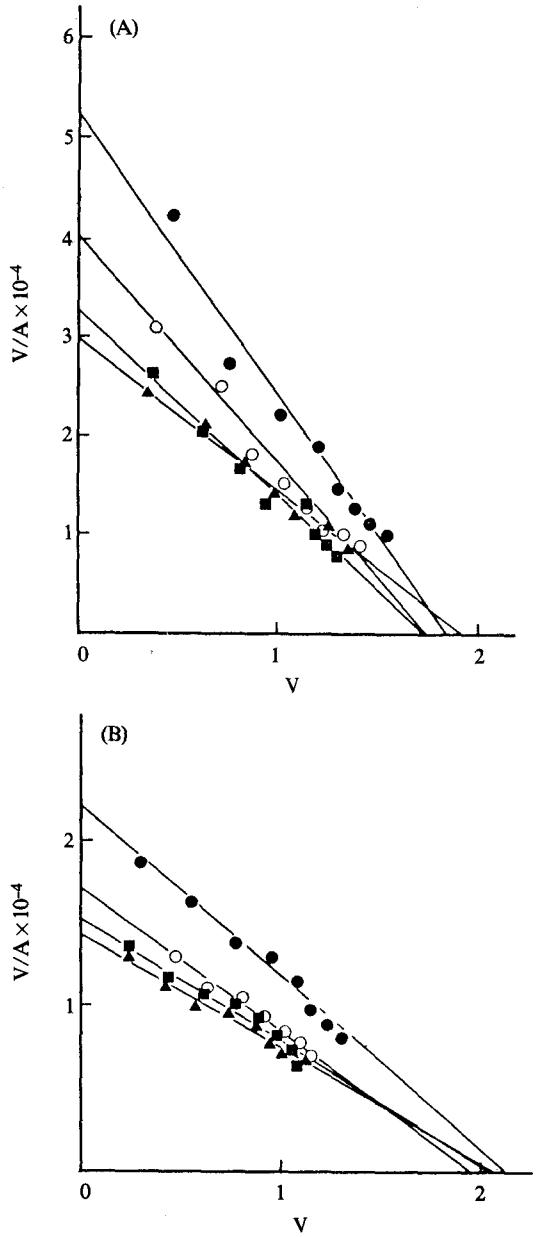


Figure 9—Scatchard plots for the binding of HBAB to BSA in the absence (A) and presence of cephalothin (B) at various temperatures (pH 7.4, $\mu=0.16$).
Key; ●, 20°; ○, 26°; ■, 30°; ▲, 37°

dipole, dipole-dipole, hydrogen and hydrophobic binding, and that the latter appears to play an important role.

The thermodynamic values are useful in considering the nature of the binding, in comparing the

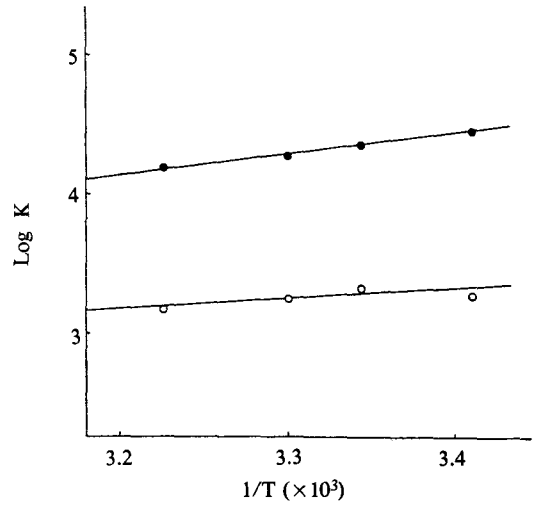


Figure 10—Effect of temperature on the binding of drugs to BSA at pH 7.4 and $\mu=0.16$.
Key; ●, HBAB; ○, cephalothin

binding of different ligand molecules by the same macromolecule, or in analyzing the effect of factors such as pH and ionic strength on the binding.[13]

The free energy of association for cephalothin-BSA complex is composed of 65% contribution from the negative enthalpy changes and 35% positive entropy changes (Table III). The negative enthalpic component is generally believed to result from van der Waals dispersion or dipolar forces, while the positive entropic component results from electrostatic and/or hydrophobic binding.[10,13]

In general, positive entropies were attributed to the formation of hydrophobic bonds[11,13,17] alalthough hydrogen binding cannot be overlooked. The results are interpreted on the basis of the "iceberg" concept which assumes that hydrocarbon groups, present both in the macromolecule and in the ligand molecule, are surrounded in aqueous solution with one or more layers of water molecules which are more highly ordered than the molecules in ordinary liquid water. Those layers are referred to as icebergs. The entropy changes occuring during the binding process are solely due to the disordering of the icebergs.[13] The complex formed will be accompanied by an iceberg which is less ordered compared to the icebergs of the two separate entities which results in a proportional gain in entropy. In

Table III—*Thermodynamic Data at pH 7.4 and μ=0.16.*

Temp. (°C)	HBAB $K\times10^{-4}$ (M^{-1})	n	$\Delta G°$ (Kcal/M)	$\Delta H°$ (Kcal/M)	$\Delta S°$ (e.u.)	Cephalothin $K\times10^{-3}$ (M^{-1})	n	$\Delta G°$ (Kcal/M)	$\Delta H°$ (Kcal/M)	$\Delta S°$ (e.u.)
20	2.86	1.85	-5.97	-6.62	-2.20	1.92	2.12	-4.40	-2.87	5.24
26	2.30	1.75	-5.97		-2.18	2.09	1.94	-4.54		5.60
30	1.89	1.75	-5.93		-2.28	1.81	2.04	-4.52		5.44
37	1.55	1.93	-5.94		-2.18	1.50	1.99	-4.50		5.28

1961, Molyneus *et al.*[17] calculated that the net enthalpy change associated with: (a) the heat needed to overcome any specific interactions (i.e., true hydration) between water and the macromolecule and between water and ligand molecule; (b) exothermic interaction between the dehydrated entities; and (c) exothermic interaction between the water and the bound system (i.e., reformation of true hydration), and is essentially constant with a value of -5 Kcal/mole.

By subtracting this values from $\Delta H°$ in Table III, the net enthalpy changes associated with the disordering of the water molecules in the icebergs around the polymer and the ligand molecule can be computed before the bindings and the reformation of hydrogen bonds in the icebergs around the complex. The net enthalpy change values are -1.62 Kcal/mole and 2.13 Kcal/mole for the binding of BSA with HBAB and cephalothin, respectively. These positive enthalpies, and the entropy values are responsible for the hydrophobic bonds. Klotz *et al.*[7] indicated that the molecular mechanism must be consistent with the thermodynamic features, but the thermodynamic parameter in themselves do not provide a diagostic criterion for distinguishing the types of forces involved in ligand binding by protein. Therefore, taking the effects of ionic strength and pH into consideration, it could be deduced that the binding process was not only due to hydrophobic bindings but also to exothermic reactions, such as van der Waals forces or hydrogen bondings.

Sakurai *et al.*[18] suggested that the absorption band at 484 nm and 350 nm may be originated in the HBAB molecules bound to metachromasy sites and nonmetachromasy sites, respectively. And Moriguchi *et al.*[8] suggested that HBAB, once bound to BSA by electrostatic forces, etc., is buried in such an environment of lower polarity in the interior of the protein molecule, and is converted into the hydrazone form which has an absorption maximum about 480 nm. So, at 484 nm, only hydrazone form of HBAB contributes to the bindings and the binding process is due to hydrophobic bondings. In Table III, however, because both enthalpy changes and entropy are negative, hydrogen bondings cannot be excluded.

ACKNOWLEDGEMENT

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