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^{11}B NUCLEAR MAGNETIC RESONANCE STUDIES OF THE INTERACTION OF BOROCAPTATE SODIUM WITH SERUM ALBUMIN

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Abstract—The interaction between borocaptate sodium, $Na_2B_{12}H_{11}SH$ (BSH), and three types of serum albumin—bovine, human and dog (BSA, HSA and DSA)—has been investigated quantitatively using ^{11}B NMR. The ^{11}B chemical shifts and relaxation rates of BSH were studied with various concentrations of serum albumin (1–5%, w/v) at 295–310°K. Correction of the longitudinal relaxation rate (R_1) due to protein viscosity effects was accomplished. The corrected R_1 values were analyzed mathematically using a saturation function and linear regression. The linewidths of ^{11}B resonances, which are related to the spin-spin relaxation rates (R_2), were also measured. The binding fractions (P), the number of binding sites (NBS), and the binding constants (K_b) of BSH at various concentrations of the three types of serum albumin (1–5%, w/v) were determined at 295 and 310°K. We speculate that the nature of this interaction may be electrostatic.

Key words: borocaptate sodium; BSH; serum albumin; boron-11 NMR; binding

BNCT¶ [1] has been investigated as a method to treat brain cancers, such as glioblastoma multiforme, since the 1950s [2]. In this binary therapy, ^{10}B in the target drug absorbs thermal neutrons. The resultant unstable ^{11}B decomposes to ^{7}Li and releases high energy alpha particles [3]. The intensive radiation typically deposits the energy within a cell diameter (10–14 μm), causing local tumor cell necrosis. Since boron can be localized in abnormal and normal cells, one of the critical considerations of BNCT is the selectivity of the boron agent. BSH ($Na_2B_{12}H_{11}SH$) has been proposed as a boron localizer since the 1960s [4, 5] and is currently one of the only two agents nearing clinical trials in this country to treat glioblastoma multiforme.** The interaction between BSH and serum albumin is of interest since it is related to the pharmacokinetics of BSH. Early research suggested that a disulfide bridge might be involved [6]. However, some recent studies, using NMR techniques, indicated that there is no covalent binding [7, 8]. In the present study, we used a high field NMR spectrometer to achieve quantitative analysis of this interaction in terms of chemical shifts and relaxation rates of ^{11}B nuclei in BSH.

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¶Abbreviations: BNCT, boron neutron capture therapy; BSH, borocaptate sodium; P, the binding fraction; NBS, number of binding sites; K_b, the binding constant; f, the correction coefficient for viscosity effect; DSA, dog serum albumin; and HSA, human serum albumin.

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BASIC THEORY

NMR is a very powerful technique for studying protein binding effects [9]. Boron NMR is typically suitable to study the binding of boron agents. Both ^{10}B and ^{11}B have NMR activity, but ^{11}B has better sensitivity (16.5 vs 2% relative to ^{1}H) and higher natural abundance (80.4 vs 19.6%) [10]. Even though ^{10}B is the active nucleus for neutron capture in BNCT, the more sensitive ^{11}B is more appropriate in NMR studies, and the isotopic difference does not alter either the structure and binding or the pharmacokinetic effects of the agent. The spin quantum number of ^{11}B is 3/2 and that of ^{10}B is 3 [10]. The quadrupole interaction plays an important role in their NMR properties. The chemical shift of a quadrupole nucleus is very sensitive to its chemical environment. The 12 boron nuclei of BSH can be classified into four groups, as indicated in Fig. 1. Covalent binding of BSH to a large protein changes the chemical environment around the ^{11}B nuclei causing changes in chemical shifts. Previous research indicated that the formation of a disulfide bridge between BSH and serum albumin moves the ^{11}B chemical shift of the -SH attached boron, labeled as B1 in Fig. 1, 3 ppm downfield [7].

The relaxation rates of a quadrupole nucleus such as ^{11}B are very sensitive to protein binding. The binding produces large changes in the quadrupole relaxation rate as a result of changes in electric field gradients and correlation times. The linewidths of boron resonances are inversely related to the spin-spin relaxation rate (R_2). During the titration of BSH with albumin, the ^{11}B linewidths, defined as $W^{b,e}$, contributed from the spin-spin relaxation rate

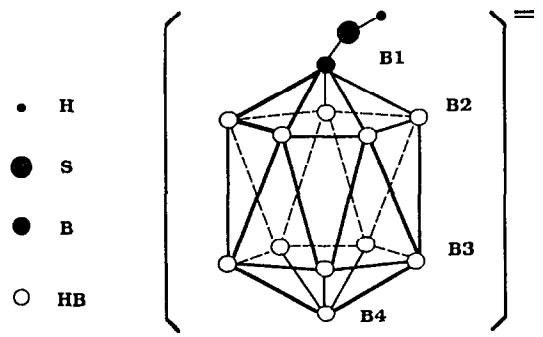

Fig. 1. Schematic illustration of the BSH anion. There is a net negative charge of 2 in the boron-hydride cage.

of bound BSH and the exchange rate between bound and unbound BSH, can be obtained by subtracting the ^{11}B linewidth of free BSH (W^u) from the observed linewidth of BSH (W^o) in albumin solution ($W^{b,e} = W^o - W^u$). When the bound and unbound BSH are under fast exchange, the linewidth $W^{b,e}$ decreases as temperature increases [11].

Under the so-called "extreme narrowing condition", the relationship between the longitudinal relaxation rate (R_1) and the correlation time is [12]:

$$R_1 = C\left(\frac{e^2Q_q}{h}\right)^2 \tau_c \qquad (1)$$

where C is a constant depending upon the spin quantum number and molecular symmetry, eQ is the electric quadruple moment, eq is the electric field gradient, which strongly depends on the symmetry of the electron distribution around the nucleus, and τ_c is the correlation time characterizing the fluctuation of the electric field gradient. The correlation time, and therefore the relaxation rate, is strongly dependent on the viscosity of the solution. The upper limit of the correlation time can be considered as the rotational diffusion time. According to Stoke's Law, the relationship of the rotational diffusion time with temperature and solvent viscosity is [13]:

$$\tau_c = \frac{MV\eta}{RT} \qquad (2)$$

where τ_c is the rotational correlation time, M is the

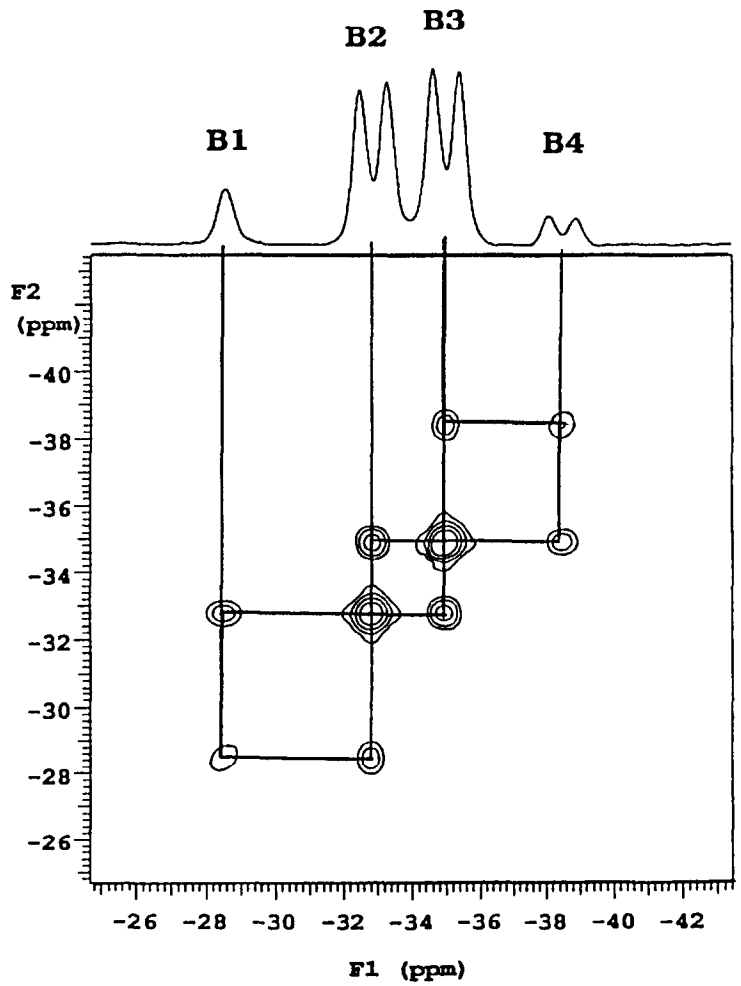

Fig. 2. ^{11}B COSY spectrum. The connections among the boron nuclei in BSH are indicated.

molecular weight, V is the partial specific volume, η is the viscosity of the solvent, R is the gas constant, and T is the absolute temperature. This equation indicates that τ_c is proportional to the viscosity of the solution. When more than 1% (w/v) of protein is used, the effect of viscosity on relaxation rate cannot be neglected.

Assuming that the extreme narrowing condition applies, the quadrupole relaxation rate is linearly related to the viscosity, as indicated in Equations 1 and 2. Since the flow times of pure solvent and those of the aqueous solutions of serum albumin as measured by an Ostwald viscometer are proportional to their viscosities (Eq. 3), the ratio (f) of the measured flow times between water and protein solutions can be used as a correction coefficient for the viscosity effect, as indicated in Equation 4:

$$F = B\frac{\eta}{\xi} \Rightarrow \frac{\eta^w}{\eta} = f = \frac{F^w\xi}{F\xi^w} \tag{3}$$

$$R_1^w = fR_1 \tag{4}$$

where F is the flow time, B is a constant related to the viscometer, ξ is the density of the solution, and w indicates pure water.

After correction for the viscosity, the observed ^{11}B longitudinal relaxation rate of BSH upon titration of albumin reflects the interaction between the drug and the protein, having contribution from both bound and unbound BSH under the exchange:

$$R_1^i = R_1^b P_1 + R_1^u(1 - P_i) \tag{5}$$

where R^b and R^u are the relaxation rates for bound and unbound BSH, respectively, R^i is the observed relaxation rate at the i% concentration of the protein, and P_i is the fraction of bound BSH at this concentration of the protein. If the relaxation rate of bound BSH can be obtained, the binding fraction P_i can be derived from this equation, and the concentration of bound and unbound BSH ($[B]^b$ and $[B]^u$) at the particular concentration (i) of protein can be obtained.

With known concentrations of bound and unbound BSH, one can evaluate the binding constant K_b ($mmol^{-1}$) and the NBS on serum albumin according to the protein binding equation [14]. Assuming there is only one type of binding site on the protein, the following equation can be used:

$$\frac{[B]_i^b}{[B]_i^u[SA]_i} = k(NBS) - k\frac{[B]_i^b}{[SA]_i} \tag{6}$$

where $[SA]_i$ is the concentration of serum albumin. Using linear regression of

$$\frac{[B]_i^b}{[B]_i^u[SA]_i}$$

versus

$$\frac{[B]_i^b}{[SA]_i},$$

K_b and NBS can be calculated from the slope and intercept.

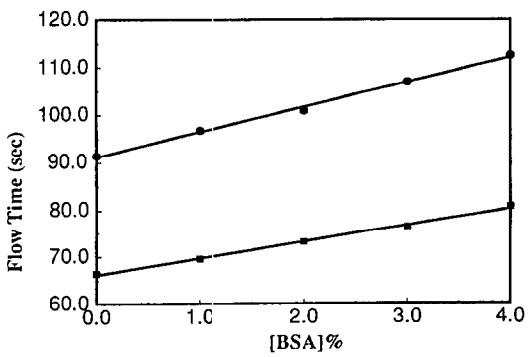

Fig. 3. Measured flow time versus the concentration of BSA. Key: (■) 310°K, and (●) 295°K.

EXPERIMENTAL PROCEDURES

All NMR studies were carried out on a Varian Unity 500 spectrometer (11.7 Tesla, 160 MHz for ^{11}B). A 3000 Hz sweep width and 8 k data points were used. The concentration of BSH was 200 μg B/mL at natural abundance (80.4% ^{11}B) in deuterated phosphate buffer at p[^{2}H] = 7.4. Quartz NMR sample tubes and baseline corrections were used to minimize the background ^{11}B signal from the borosilicate glass in the probe. It was determined that 200 μg B/mL is the minimal BSH concentration to produce adequate observable ^{11}B signals above the background.

The chemical shifts of the four types of ^{11}B nuclei in BSH (see Fig. 1) were measured with variation of temperature and concentration of serum albumin. Saturated boric acid was used as an external reference for the chemical shift measurement. Since there are no significant changes in the chemical shifts during the titration and temperature variation, the chemical shift of B4 was used as internal standard, which is more convenient. The external reference was used to check for accuracy. Three types of serum albumin—human (HSA), dog (DSA) and bovine (BSA)—were used from 1–5% (w/v). The proteins were purchased from Sigma and used without further purification. The samples were stored at about 178°K, and all the measurements were conducted within 1 month from the sample preparation. The samples were brought to the detecting temperature for more than 2 hr before the measurement. Some samples were incubated for more than 4 hr at 310°K to check the temperature effect. The resonances of these nuclei were assigned by a ^{11}B chemical shift correlation (COSY) spectrum (Fig. 2). The coupling constants between ^{11}B and ^{1}H ($^1J_{^{11}B-^1H}$) were also measured.

The ^{11}B linewidth and longitudinal relaxation rates of BSH were studied at 295°K and 310°K as protein concentration was varied from 1 to 5% (w/v). Two to three samples were used at each concentration, and each sample was measured 2–3 times at each temperature. The signal intensities of the ^{11}B nuclei were measured with a standard T1 Inversion Recovery method (T1IR). The pulse sequence is: 180-τ-90-acquire. The $T_1(1/R_1)$ were calculated using a standard Varian NMR program from the observed

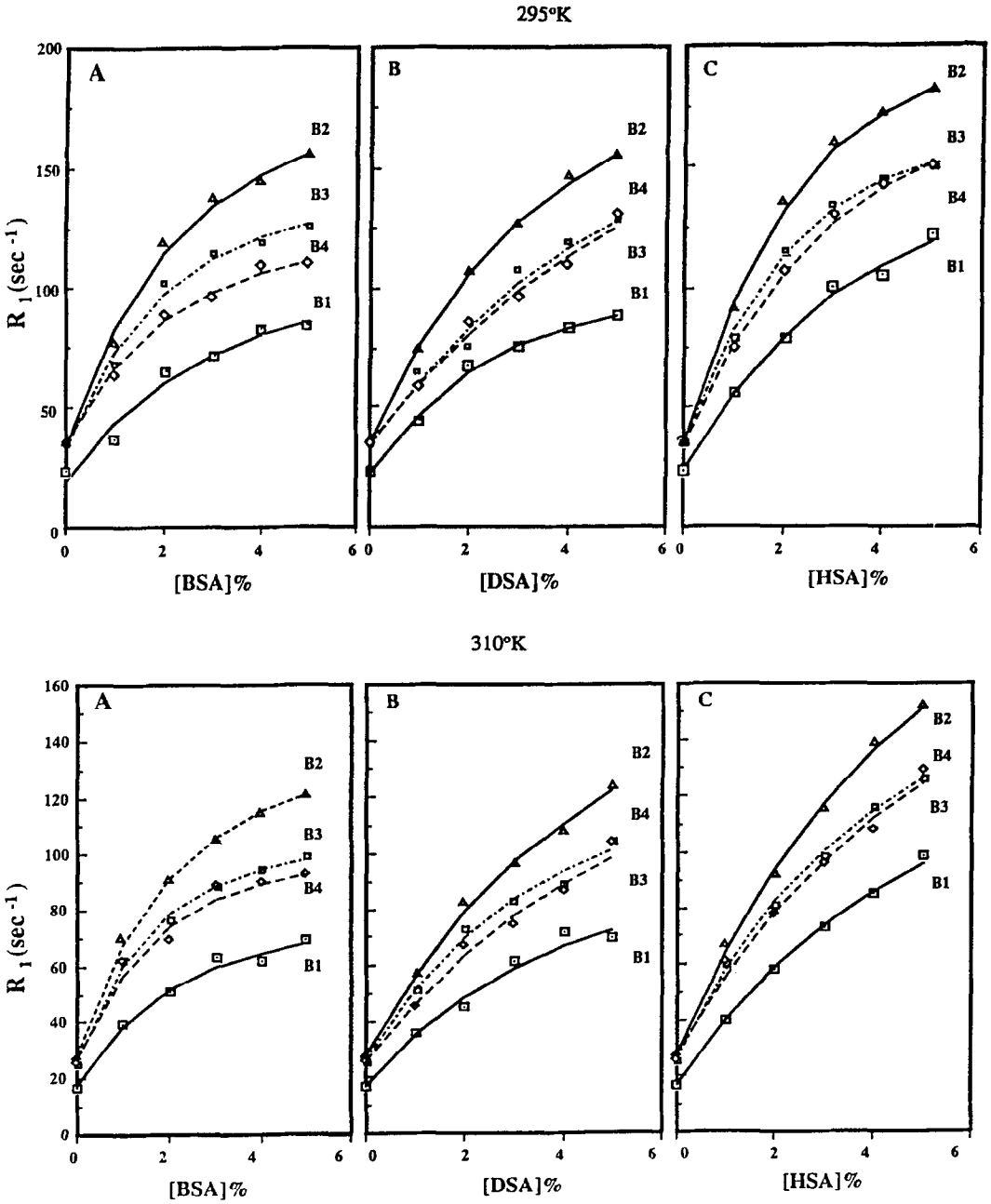

Fig. 4. Longitudinal relaxation rate versus the concentration of serum albumins. The points are the measured rates after viscosity correction, and the lines are those calculated using the fitting function (Eq. 7). The longitudinal relaxation rates of bound BSH are calculated with 300 iterations.

intensities. The equation is: $S(\tau) = (M_0 - M_{inf})\exp(-\tau/T_1) + M_{inf}$, where $S(\tau)$ is the measured signal at each delay time τ, M_0 and M_{inf} are the magnetizations when $\tau = 0$ and τ approaches infinity, respectively. The range of τ was between 1 msec and 1 sec. Each spectrum was used 5 or more times, and each time a slightly different baseline correction was applied. The calculated data were then averaged after discarding the highest and lowest values. The ^{11}B linewidth, $W^{b,e}$, was obtained by subtraction ($W^{b,e} = W^o - W^u$) as discussed above.

The flow times of water and 1–4% BSA aqueous solutions were measured using an Ostwald viscometer, which was immersed inside a water bath for temperature control. Two milliliters of each solution was transferred into the viscometer. The flow times of each sample were measured 5–6 times, and the data were averaged. Since the measured flow time is linear with BSA concentration, the flow time of 5% BSA was calculated from the regression line (Fig. 3). The densities of water and protein solutions were obtained from standard references

Table 1. ^{11}B chemical shifts of boron nuclei in BSH

B1	B2	B3	B4
−28.4 ± 0.08	−32.8 ± 0.04	−34.9 ± 0.03	−38.5 ± 0.00

Saturated H_3BO_3 = 0 as the external reference, and B4 is used as the internal reference. Values are means ± SD, N = 5.

[15]. Since the molecular weights of the three proteins are similar, the flow times measured from BSA were assumed for HSA and DSA. The viscosity correction coefficients, f_i, at different protein concentrations and the corrected relaxation rates were then evaluated using Eq. 1–4.

RESULTS AND DISCUSSION

The ^{11}B chemical shifts of BSH are listed in Table 1. The chemical shifts remain the same for various temperatures (295–310°K) and various concentrations of BSA, HSA and DSA (1–5%, w/v). Previous researchers observed that the ^{11}B chemical shift of BSH moved 3 ppm downfield when a disulfide bridge formed due to BSH reaction with cysteine [7]. However, the observation of no chemical shift change under our experimental condition indicates that there is a non-covalent binding, with no disulfide formed between BSH and these proteins. The observed $^1J_{^{11}B-^1H}$ is 126 Hz, and does not change with protein titration (1–5%, w/v) and temperature variation (295–310°K). This observation is also consistent with non-covalent binding.

Table 2 shows the subtracted linewidth, $W^{b,e}$, of B2 and B3 of BSH in the presence of 1–3% concentrations of HSA. The fact that $W^{b,e}$ decreases as temperature increases from 295 to 310°K indicates fast exchange between the unbound and bound BSH as discussed above. Similar behavior was observed during the titration of all three types of serum albumin, which indicates fast exchange in the BSH binding with all these proteins.

The longitudinal relaxation times of ^{11}B nuclei in BSH were measured at 295 and 310°K with the titration of serum albumin. After correction for the viscosity effect, using the correction coefficients (P_i) as discussed above, the observed R_1 values are plotted versus the concentration of the various albumins (Fig. 4).

Similar patterns were observed from the plots of all three types of albumin. These plots also showed that the longitudinal relaxation rates increased more rapidly with the concentration of albumin up to about 3%, than they did thereafter. This behavior may indicate that the interaction between BSH and serum albumin is becoming saturated at the higher concentration of the proteins. To prove that the saturation occurs, a saturation function was used to fit the observed data:

$$R_1^i = R_{1,\,infinity}^b - ae^{-b[SA]_i} \qquad (7)$$

where R_1^i is the observed relaxation rate at albumin concentration $[SA]_i$, $R_{1,infinity}$ is the relaxation rate as $[SA]$ approaches infinity, and a and b are fitting coefficients. According to the binding equilibrium, as the concentration of serum albumin approaches infinity, BSH can be considered all bound to the protein; therefore $R_{1,infinity}$ corresponds to the longitudinal relaxation rate of bound BSH (R_1^b), and a is the difference of the relaxation rate between bound and unbound BSH. The larger the value of a is, the stronger is the binding. The meaning of b is not so obvious, but it indicates the extent of the binding saturation. The larger the absolute value of b is, the higher the saturation is. The calculated values of a and b are listed in Table 3. These values indicated that B1 is the least affected during the titration, which is consistent with non-covalent binding. The strongest effect was observed at B2, indicated by the high values of a and the highest position among all titration curves in Fig. 4. We speculate that this observation may result from an electrostatic and steric influence of the -SH group. The boron hydride cage carries a net negative charge of 2, which may act as the negative center for Coulombic interaction. The higher electronegativity of sulfur induces a negative charge toward the -SH group. The inductive electron withdrawing effect, which decreases with the distance from -SH, may influence the relative binding affinities of the boron nuclei. The chemical shifts of the ^{11}B resonances, that is, B1 is at the lowest field, followed by B2, B3

Table 2. Linewidth ($W^{b,e}$) of ^{11}B resonances in BSH versus temperature change

Temperature (°K)	$W^{b,e}$ (Hz) B2			B3		
	1% HSA	2% HSA	3% HSA	1% HSA	2% HSA	3% HSA
295	20 ± 2	46 ± 3	61 ± 3	16 ± 1	38 ± 2	57 ± 3
310	4 ± 3	16 ± 2	26 ± 7	2 ± 2	15 ± 3	20 ± 3

Values are means ± SD, N = 5.

Table 3. Binding parameters

A. Longitudinal relaxation rates of BSH bound to serum albumin

	$R_{1,infinity}$ (sec^{-1})					
	295°K			310°K		
	DSA	HSA	BSA	DSA	HSA	BSA
B1	103.1	146.9	99.7	92.0	138.0	73.7
B2	186.3	199.7	170.3	164.2	212.6	130.3
B3	158.3	165.3	134.8	122.1	162.7	102.8
B4	151.1	176.8	118.1	129.3	180.3	97.1

B. Saturation coefficients (a and b) of boron nuclei in BSH at different temperatures

	295°K						310°K					
	a			b			a			b		
	DSA	HSA	BSA	DSA	HSA	BSA	DSA	HSA	BSA	DSA	HSA	BSA
B1	79	123	80	−0.37	−0.30	−0.35	74	121	55	−0.28	−0.21	−0.48
B2	151	166	137	−0.32	−0.45	−0.45	135	185	102	−0.24	−0.22	−0.48
B3	145	165	102	−0.21	−0.46	−0.50	96	136	76	−0.30	−0.26	−0.56
B4	150	143	83	−0.18	−0.35	−0.48	120	152	71	−0.19	−0.20	−0.56

C. Binding constant (K_b) and the number of binding sites (NBS) of BSH on albumin

Temperature (°K)	Serum albumin	K_b ($mmol^{-1}$)	NBS
295	DSA	2.9	3.4
	HSA	3.1	4.2
	BSA	3.7	4.7
310	DSA	1.8	3.5
	HSA	1.3	3.4
	BSA	4.9	5.1

D. Binding fractions (P_i) of BSH at various concentrations of serum albumin*

	P_i					
	295°K			310°K		
concn	DSA	HSA	BSA	DSA	HSA	BSA
1%	0.26 ± 0.01	0.31 ± 0.04	0.34 ± 0.03	0.23 ± 0.02	0.21 ± 0.02	0.42 ± 0.03
2%	0.46 ± 0.03	0.55 ± 0.07	0.63 ± 0.03	0.41 ± 0.03	0.35 ± 0.03	0.63 ± 0.02
3%	0.60 ± 0.03	0.70 ± 0.06	0.74 ± 0.05	0.54 ± 0.05	0.48 ± 0.03	0.79 ± 0.03
4%	0.71 ± 0.03	0.79 ± 0.03	0.83 ± 0.03	0.64 ± 0.05	0.59 ± 0.03	0.87 ± 0.03
5%	0.79 ± 0.02	0.85 ± 0.05	0.89 ± 0.03	0.74 ± 0.02	0.68 ± 0.03	0.93 ± 0.01

* Values are means ± SD. For 1–4%, N = 20; for 5%, N = 15.

and B4, are consistent with this electron polarization argument. Because serum albumins are rich in amino acid residues carrying an electropositive center (58 lysines and 24 arginines in HSA) [16], the electrostatic interaction can occur at these residues. The other effect from the -SH group is the steric bulk of the large S atom. This effect shields B1, making it less accessible to the binding sites. The combination of the steric bulk and inductive electron withdrawing effect makes B2 the most attractive to a positive electrostatic field, and B1 the least. This analysis further agrees with the electrostatic interaction between BSH and these proteins.

With the measured relaxation rate of unbound BSH (R_1^u) and the calculated rate of bound BSH (R_1^b), the binding fractions (P_i) were calculated using Equation 5. The concentrations of bound and unbound BSH were calculated from P_i and the initial BSH concentration. The binding constant K_b and NBS were then evaluated using Equation 6. Figure 5 illustrates the linear regression and Table 3 lists the binding parameters.

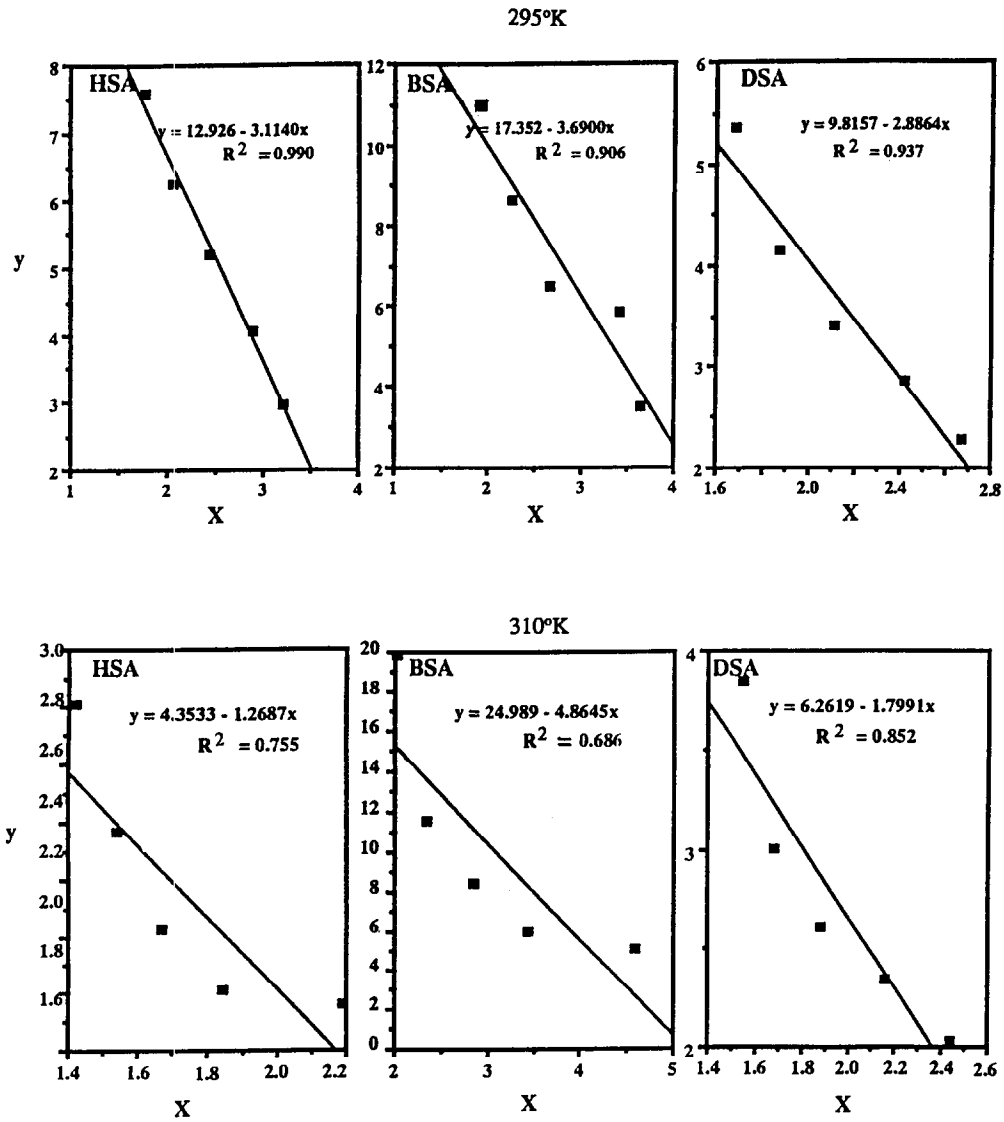

Fig. 5. Linear regression to evaluate the binding constant (K_b) and the number of binding sites (NBS) of BSH on serum albumins using Equation 6. Y is $\frac{[B]_i^b}{[B]_i^u[SA]_i}$; X is $\frac{[B]_i^b}{[SA]_i}$; R^2 is the regression coefficient.

The calculated P_i using NMR is slightly higher than that observed using microfiltration and equilibrium dialysis [8]. Table 4 lists the percentage of bound BSH in the presence of 3.4% BSA in aqueous solution at various temperatures using microfiltration [8]. A possible explanation is that the change of R_1 observed in NMR is a contribution from all possible interactions between BSH and the proteins, but the microfiltration detects only those BSH that are tightly bound. When the bound and unbound BSH are under fast exchange, the interaction affects the observed relaxation rate but may not be detectable by microfiltration. The resulting NBS and K_b are consistent with those obtained using microfiltration and equilibrium dialysis*. The observed NBS values were 2.4 to 4.8, and the K_b 1 − 4.1 (mmol^{-1}) at 37°. The range reflects some lot-to-lot variation of the BSA, and possibly the BSH*. However, there was no difference in side-by-side experiments using equilibrium dialysis or microfiltration membranes. The observed K_b of BSH–BSA binding at 310°K is higher than others, which may be caused by the larger error in linear regression ($R^2 = 0.686$). Other models, such as two-site binding of different binding strengths, were considered, but the data did not fit that model any better than the current one. Other studies, using equilibrium dialysis or microfiltration, also did not agree with the two-site model. It is possible that

*Hotz NJ, Bauer WF and Gianotto AK, Protein binding characteristics of borocaptate sodium and some related boron compounds. Presented at the *5th Int. Symp. NCT*, Sept. 14–17, 1992, Columbus, OH.

Table 4. Percentage of bound BSH in 3.4% BSA at different temperatures

Temperature (°K)	P_i*
278	71.5
288	69.5
293	65.3
298	55.5
303	54
308	47

* Measured using a microfiltration method from a previous study [8].

cooperative binding occurs at this temperature for this protein. However, there is no other evidence to prove it either from the NMR data or from other methods.

CONCLUSIONS

This study has indicated that the measurement of the changes in ^{11}B relaxation rates can be a useful tool to study quantitatively the protein binding parameters of boron-containing agents. The temperature and pH effects upon the binding can also be studied using this method. This method can be used during the titration of boron agents with different proteins and can also be used in the reversed titration, i.e. using constant concentrations of protein but varying the concentrations of BSH. The latter experiment could not be performed with the instrument due to the strong background from the borosilicate NMR probe.

The measured values of relaxation rates and chemical shifts, and the calculated binding parameters indicated that there is non-covalent binding, i.e. no formation of a disulfide bridge between BSH and the three types of albumin; the nature of this interaction may, in part, be electrostatic; and the bound and unbound BSH are under fast exchange at temperatures between 295–310°K. The number of BSH binding sites on these proteins is about 3–5, assuming there is only one kind of binding. The binding constant is about 3 ($mmol^{-1}$) at 295°K and 1.5 ($mmol^{-1}$) at 310°K, which means that the interaction decreases as temperature increases. This observation is common for non-covalent binding, because the molecular random diffusion increases at higher temperature.

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