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Interaction between Serum Albumin and Mercaptoundecahydrododecaborate Ion (An Agent for Boron-Neutron Capture Therapy of Brain Tumor). I. Introductory Remarks and Preliminary Experiments

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The interaction between bovine serum albumin and dodecahydrododecaborate $B_{12}H_{12}^{2-}$ or mercaptoundecahydrododecaborate $B_{12}H_{11}SH^{2-}$ anion has been investigated by equilibrium dialysis, equilibrium distribution in and out of a Sephadex gel, gel-filtration-, ion-exchange-, and ion-retardation-chromatographies. The results have been discussed with the following conclusions. 1) Both borates are strongly bound to the albumin through ion-pair formation with cationic sites on the protein molecules. 2) This binding of ionic character can be readily broken up by ion-exchange or ion-retardation chromatography. 3) The latter borate shows, in addition, another mode of binding which is resistant against ion-exchange and ion-retardation resins. 4) This binding of covalent character is due to the formation of disulfide linkage between the boron cage of $B_{12}H_{11}SH^{2-}$ and the albumin.

Sodium mercaptoundecahydrododecaborate, $Na_2B_{12}H_{11}SH$, is an agent currently used for the boron-neutron capture therapy of brain tumor. This compound administered to a patient by a direct intra-arterial infusion (internal carotid) is incorporated selectively into malignant tissues and gives high and long-lasting concentration in tumors, while the incorporation into brain or blood is quite or tolerably small. Little uptake by the normal brain is believed to be due to the so-called Blood-Brain Barrier. The irradiation of thermal neutron

$$\begin{aligned} {}^{10}_{5}\mathrm{B} + {}^{1}_{0}\mathrm{n} &\longrightarrow {}^{7}_{3}\mathrm{Li} + {}^{4}_{2}\mathrm{He} + 2.79\,\mathrm{MeV}\ (6.1\%) \\ &\longrightarrow {}^{7}_{3}\mathrm{Li} + {}^{4}_{2}\mathrm{He} + 2.31\,\mathrm{MeV}\ (93.9\%) \\ &\qquad\quad \big\lfloor\!\longrightarrow {}^{7}_{3}\mathrm{Li} + \gamma + 0.48\,\mathrm{MeV} \end{aligned}$$

produces high-energy alpha particles and Li nuclei, by which the tumor tissue is destroyed. The surrounding normal tissue is kept intact because the produced particles travel only about ten microns through a living tissue.[2]

Investigation of the interaction between the agent and serum albumin was attempted for two reasons. 1) The agent is conveyed to the target tumor by blood stream, some being bound to serum proteins and the other being free in solution. 2) The agent thus transported is captured by the tumor tissue and stays there long enough so that neutron irradiation can be made after the sufficient clearance of boron from the systemic blood. The capture and fixation are probably due to the interaction of the agent with proteins in or on tumor cells. Using serum albumin as a test material, one may gain some insight on the nature of protein-agent interaction.

A study along this line has already been carried out by Soloway, *et al.*[3] with noteworthy results. These authors observed the following phenomena among others. 1) Both dodecahydrododecaborate anion, $B_{12}H_{12}^{2-}$, and its mercapto derivative, $B_{12}H_{11}SH^{2-}$, are bound strongly to bovine serum albumin (BSA) under physiological conditions. 2) Even extensive dialysis failed to break the binding. 3) By the passage through an anion-exchange resin (OH form) column, the $B_{12}H_{12}^{2-}$ anion was totally removed from the protein, whereas the

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2) H. Hatanaka and K. Sano, *Z. Neurol.*, **204**, 309 (1973).
3) A.H. Soloway, H. Hatanaka, and M.A. Davis, *J. Med. Chem.*, **10**, 714 (1967).

$B_{12}H_{11}SH^{2-}$ was largely unaffected and migrated with its protein component. The formation of disulfide linkage between the boron cage and BSA was suggested as a likely mechanism of the binding resistant to the anion-exchanger.

Later workers,[2] however, manifested the suspicion that the sample employed by Soloway, *et al.* might have been contaminated with a large amount of disulfide species $B_{24}H_{22}S_2^{4-}$, which is known to be produced slowly by the oxidation of $B_{12}H_{11}SH^{2-}$ with atmospheric oxygen.[4] Another disadvantage of the Soloway's report[3] is that it contains only few numerical data in respect to the dialysis and ion-exchange chromatography. With these affairs in mind, the present authors made some preliminary observations with equilibrium dialysis, equilibrium distribution in and out of a Sephadex gel, gel-filtration chromatography, ion-exchange chromatography, and ion-retardation chromatography. Except for the last one, the data obtained are, at most, semi-quantitative because of complex nature of the interaction and poor accuracy of boron analysis; yet they seem serviceable to qualitative discussion.

Experimental

Material——Cesium dodecahydrododecaborate, $Cs_2B_{12}H_{12}$, was prepared from sodium borohydride and diborane by a conventional method.[5] The product was converted to cesium mercaptoundecahydrododecaborate, $Cs_2B_{12}H_{11}SH$, by a procedure developed in the authors' laboratory.[6]

$$B_{12}H_{12}^{2-} + \text{N-methylthiopyrrolidone} \xrightarrow{H^+}$$

$$H_2\uparrow + [B_{12}H_{11}\cdot(\text{N-methylthiopyrrolidone})\text{adduct}]^- \xrightarrow{OH^-}$$

$$B_{12}H_{11}SH^{2-} + \text{N-methylpyrrolidone}$$

High purities of these samples, especially the absence of disulfide species in the latter compound, were verified by elemental analyses, infrared (IR) spectra, and thin layer chromatographies using Baker-Flex Cellulose PEI as a plate, aqueous NH_4PF_6 solution as a developing solvent, and aqueous $PdCl_2$ solution as a detecting agent.

Commercially available BSA (Sigma Chem. Co. Cat. No. A4378) was used without further purifications.

Analysis——Protein content was estimated by nitrogen analysis with the sealed-tube combustion method.[7] The error was within $\pm 2\%$. Boron content was determined by the method of Ellis, *et al.*[8] with some modifications. A calibration curve was prepared for each series of experiments. The error was usually within $\pm 10\%$, but amounted to $\pm 15\%$ in case of high protein content. Sometimes, a negative value results from the calibration.

Equilibrium Dialysis

A 2.850×10^{-3}M solution of $Cs_2B_{12}H_{12}$ was prepared by dissolving the borate in a 1/15M phosphate buffer (pH 7.2). A two ml aliquot of the solution was sealed in a Visking tube and dialysed against 20 ml of the same buffer contained in a glass bottle with a cap. The capped bottle was continuously tumbled by fixing it to a disk rotating in a thermostatted water bath. The equality of borate concentrations, $[B]_i$ and $[B]_o$, in and out of the Visking tube was attained within four hours accompanied with no conceivable adsorption on the tube. Similar experiments were carried out with $Cs_2B_{12}H_{12}$ solutions and $Cs_2B_{12}H_{11}SH$ solutions that had been incubated in the presence of about 3×10^{-5}M BSA at 37° for 20 hr. After 24 hr equilibration at 25°, $[B]_i$ and $[B]_o$ were measured. The results are shown in Fig. 1, where the number of borate ions bound per BSA molecule, $\bar{\nu}=([B]_i-[B]_o)/(\text{concentration of}$

4) T. Nakagawa and K. Nagasawa, unpublished data; T. Nakagawa and K. Aono, J. Pat. Kokaikoho 49—30385 (1974).
5) H.C. Miller, N.E. Miller, and E.L. Muetterties, *Inorg. Chem.*, 3, 1456 (1964).
6) T. Nakagawa, T. Yoshizaki, and K. Aono, J. Pat. Kokaikoho 50-92897 (1975).
7) "Yūki Biryō Bunseki (Microanalysis of Organic Substances)," Nankōdō, 1969, p. 276.
8) G.H. Ellis, E.G. Zook, and O. Baudisch, *Anal. Chem.*, 21, 1345 (1949).

BSA in the tube), is plotted against the logarithm of free-borate concentration $[B]_0$. The analysis by the Scatchard plot, $1/\bar{\nu}$ against $1/[B]_0$ or $\bar{\nu}$ against $\bar{\nu}/[B]_0$, seems invalid in the present system because there are plural modes of protein-borate interaction as detailed later. Another reason is the poor accuracy of boron analysis. Note that $\bar{\nu}$ is calculated from the difference between the two borate concentrations.

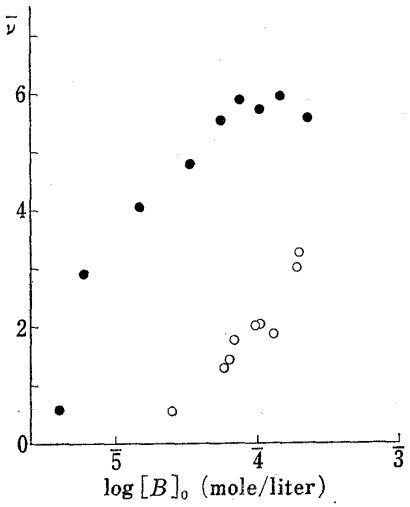

Fig. 1. The Number of Borate Ions Bound per BSA Molecule $\bar{\nu}$ plotted against the Logarithm of Free-borate Concentration $[B]_0$; determined by equilibrium dialysis
○: $Cs_2B_{12}H_{12}$, ●: $Cs_2B_{12}H_{11}SH$

A test of Soloway's experiments was tried with three solutions, (a), (b), and (c), which had been prepared by dissolving $Cs_2B_{12}H_{12}$, $Cs_2B_{12}H_{11}SH$, and none respectively in a BSA-containing isotonic physiological saline; NaCl 0.9 g, KCl 0.042 g, $CaCl_2$ 0.024 g, $NaHCO_3$ 0.01—0.03 g to make pH=7.8 in 100 ml water. After standing overnight at 4°, the solution (a) produced some precipitate that could be removed by centrifugation. The precipitate, which was assumed by Soloway, *et al.* to be denatured protein,[3] could be easily redissolved in the saline and showed no absorption maximum at 220—340 mμ. It turned out to be the $Cs_2B_{12}H_{12}$ crystallized out, as evidenced by the decrease in borate concentration after the centrifugation (*cp.* the third and fifth columns of Table I). A ten ml aliquot was transferred into a Visking tube from each of the centrifuged solutions and dialysed for 48 hours at 25° against 500 ml of the saline. The result is presented in Table I. The dialysed solutions were used for further experiments described later in "Ion-Exchange Chromatography."

Table I. Equilibrium Dialysis in Physiological Saline

Solution	Concns. of albumin $[P]$ and borate $[B]$, (mole/liter)								$\bar{\nu}$
	At preparation		After centrifugation		After dialysis				
					In tube		Out of tube		
	$[P]$ $\times 10^4$	$[B]$ $\times 10^2$	$[P]$ $\times 10^4$	$[B]$ $\times 10^2$	$[P]$ $\times 10^4$	$[B]_i$ $\times 10^4$	$[P]$ $\times 10^4$	$[B]_o$ $\times 10^4$	
a ($Cs_2B_{12}H_{12}$)	1.00	1.64	1.03	1.38	1.01	4.9	0	2.5	2.4
b ($Cs_2B_{12}H_{11}SH$)	1.00	1.80	1.06	1.78	1.09	8.2	0	3.1	4.7
c (None)	1.00	0	1.03	0	1.01	0	0	0	

Equilibrium Distribution in and out of a Sephadex Gel

Hirose and Kano have developed a simple and rapid technique by which the protein-ligand binding can be examined with the use of a suitable Sephadex gel.[9,10] The principle is similar to that of the equilibrium dialysis; the ligand can diffuse into the gel cavities while the protein cannot, and thus, the cavities correspond to the outer compartment of a dialysis apparatus. The procedure consists of three steps. 1) A known volume of a suitable buffer solution containing the protein is mixed with the gel particles which have been swelled in a

9) M. Hirose and Y. Kano, *Biochim. Biophys. Acta*, **251**, 376 (1971). Note the hiatus of L/V in the right-hand side of eq. (8).
10) M. Hirose, *Seibutsu Butsuri*, **12**, 130 (1971). In Japanese. The notation β used in ref. 10) corresponds to β' in ref. 9), and β' in ref. 10) to β in ref. 9).

known volume of the same buffer. The concentration C of the resulted solution is calculated as the amount of the protein divided by the combined volume of the two buffer solutions. After equilibration, the protein concent ration C_o of the supernatant is measured. The ratio C_o/C is defined as α. The constancy of α is confirmed at several C's. 2) By using the ligand in place of the protein, a similar experiment is carried out to determine the ratio β'=(the concentration of the ligand in the supernatant)/(the concentration of the ligand in the combined solution). Here again, the constancy of β' is confirmed. 3) The concentration ratio β of the ligand is determined in the presence of a known amount P of the protein by the same procedure applied in 2). In contrast to 2), the value of β decreases with an increase in the ligand concentration. Of course, an additional series of experiments may be performed at a different P. Under a tacit assumption that the protein is not adsorbed onto the Sephadex surface (the ligand may or may not be adsorbed), the authors have shown (a) $\bar{\nu}$ is given by

$$\bar{\nu} = \frac{L}{P}\frac{\beta-\beta'}{\alpha-\beta'} \tag{1}$$

where L is the amount of the ligand used, and (b) the concentration of the free ligand outside the gel, $[L_o(\text{free})]$, is given by

$$[L_o(\text{free})] = \frac{L}{V}\frac{\beta'(\alpha-\beta)}{\alpha-\beta'} \tag{2}$$

where V is the total volume of the combined solution. It may be argued that $[L_o(\text{free})]$ is equivalent to $[B]_o$ in the dialysis experiment.

The results obtained after 40—50 min equilibration with a 1/15M phosphate buffer (pH 7.2) at 25° are shown in Fig. 2, where $\bar{\nu}$ is plotted against the logarithm of $[L_o(\text{free})]$ to allow direct comparisons with Fig. 1. The gel employed was Sephadex G-25 medium manufactured by *Pharmacia*.

Gel-Filtration Chromatography

The same lot of Sephadex was swelled in a 1/15M phosphate buffer (pH 7.2) and packed into 35 cm×1.5 cm columns thermostatted at 25°. Test solutions were prepared by dissolving

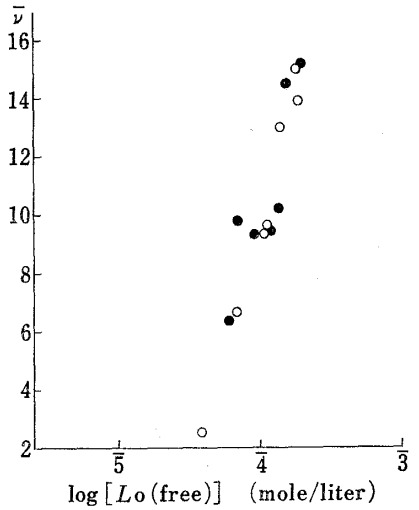

Fig. 2. The Number of Borate Ions Bound per BSA Molecule $\bar{\nu}$ plotted against the Logarithm of Free-borate Concentration $[L_o(\text{free})]$; determined by equilibrium distribution in and out of Sephadex gel

○: $Cs_2B_{12}H_{12}$, ●: $Cs_2B_{12}H_{11}SH$. V=2.5ml, P=26.98 nano moles for $Cs_2B_{12}H_{12}$, P=26.98 or 13.26 nano moles for $Cs_2B_{12}H_{11}SH$

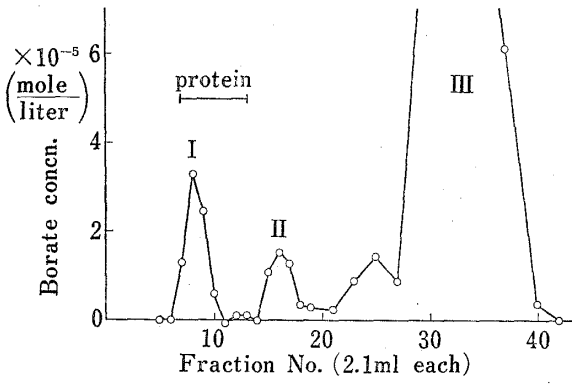

Fig. 3. A Typical Elution Curve obtained by Gel Filtration Chromatography of a BSA-$CsB_{12}H_{11}SH$ Sample

BSA: 7.0×10^{-5} mole/liter, $Cs_2B_{12}H_{11}SH$: 4.4×10^{-4} mole/liter, $\bar{\nu}$ in the region of peak I: 1.57

the samples (BSA, $Cs_2B_{12}H_{12}$, $Cs_2B_{12}H_{11}SH$, BSA+$Cs_2B_{12}H_{12}$, and BSA+$Cs_2B_{12}H_{11}SH$) in the same buffer and incubated at various conditions. After charging 0.5 ml of the test solution, the column was eluted with the buffer. Fig. 3 shows a typical elution curve obtained with a solution containing BSA and $Cs_2B_{12}H_{11}SH$. The position of peak I agrees with the elution range of BSA and this in turn agrees with that of the solution containing BSA alone. The position of peak III agrees with the elution range of the solution containing $Cs_2B_{12}H_{11}SH$ alone. Therefore, peak I can be ascribed to the bound borate and peak III to the free borate. The origin of peak II is not well understood at present. The disulfide species $Cs_4B_{24}H_{22}S_2$ does not seem responsible for this peak, because substantially the same elution pattern was observed also in BSA+$Cs_2B_{12}H_{12}$ systems. In both cases, different experimental conditions (amounts of albumin and borates, time and temperature of incubation, rate of flow, substitution of physiological saline for phosphate buffer) changed the areas of the three peaks in various degrees but with no or little shift of their positions. Also the ratio of borate to albumin in the elution range of peak I, *i.e.* $\bar{\nu}$, varied with the experimental conditions. Some experiments showed the following tendencies. A higher ratio of borate to albumin in the test solution, a longer time of incubation, and a larger rate of flow cause an increase in $\bar{\nu}$, if other conditions are equal. No prominent difference in $\bar{\nu}$ is observed between $Cs_2B_{12}H_{12}$ and $Cs_2B_{12}H_{11}SH$ under similar conditions. Analytical techniques of higher accuracy are needed to establish these interrelations.

Ion-Exchange Chromatography

As described in the last paragraph of "Equilibrium Dialysis," the dialysed solutions were employed to confirm the Soloway's observation for the behavior of ion-exchange chromatography. A two ml aliquot was taken from each of the inner solutions and charged into an anion-exchange column, in which the resin (Amberlite IRA 400, bed volume 10 cc) was in the OH form by the pretreatment with an NaOH solution followed by complete washing with water. The column was eluted with pure water and BSA-containing fractions were assembled into a test sample (20 ml) to measure pH, protein and borate concentrations, and to record ultraviolet (UV) and circular dichroism (CD) spectra. As seen in Table II, no borate was

Table II. Data before and after Ion-Exchange Chromatography

	Before exchange (pH 7.8)				After exchange (pH 11.8)				
	$[P]^{a)}$ mole/liter $\times 10^4$	$[B]^{a)}$ mole/liter $\times 10^4$	UV$^{b)}$ λ_{max} mμ	UV$^{b)}$ OD at λ_{max}	$[P]$ mole/liter $\times 10^5$	$[B]$ mole/liter $\times 10^5$	UV λ_{max} mμ	UV OD at λ_{max}	$\bar{\nu}$
a ($Cs_2B_{12}H_{12}$)	1.01	4.9	278—280	0.479	1.03	0	290—292	0.190	0
b ($Cs_2B_{12}H_{11}SH$)	1.09	8.2	278—280	0.528	0.97	0.99	290—292	0.473	1.02
c (None)	1.01	0	278—280	0.483	1.09	0	290—292	0.025	

a) *cf.* Table I (In tube) *b*) Measured after ten times dilution.

detected in (a) and, of course, in (c), whereas a considerable amount was recovered in (b); the protein concentrations being similar among the three. The ratio of borate to BSA in (b) is 1.02 in contrast to 4.5 obtained in the Soloway's experiment. The discrepancy may be due to differences in the purities of $Cs_2B_{12}H_{11}SH$ samples and in experimental conditions, besides to the poor accuracy of boron analyses. The UV and CD spectra (not reproduced here) revealed that the BSA was denatured considerably by the elution process, during and after which protein molecules were exposed to a high pH (*cf.* Table II). Although the Soloway's argument seems qualitatively correct, the interaction with native BSA should be examined under more mild conditions.

Ion-Retardation Chromatography

When an electrolyte is passed through a column packed with an ion-retardation resin, anions are captured by cationic groups, *e.g.* $-N^+R_3$, on the organic polymer lattice of the resin, and cations by anionic groups, *e.g.* $-COO^-$, and thus the elution of the electrolyte is markedly retarded in comparison with a non- or weakly ionized substance such as a protein.

Protein solutions were prepared by dissolving BSA or ovalbumin (OvA, Sigma Chem. Co., Catalog No. A5503) in a 1/15M phosphate buffer (pH 7.4), and borate solutions by dissolving $Cs_2B_{12}H_{12}$ or $Cs_2B_{12}H_{11}SH$ in the same buffer. A test solution with known amounts of protein and borate was obtained by mixing a protein solution and a borate solution, deoxygened by pumping, charged with nitrogen gas, and incubated at 37° for 20 hr. Half ml of the test solution was poured into a column packed with a thoroughly washed ion-retardation resin (Bio Rad, Catalog No. 47050, Type AG11A8, Mesh 50—100, bed volume 7 ml) and eluted rapidly with pure water. Throughout the procedure, special care was exercised to minimize the entry of atmospheric oxygen. Protein-containing fractions, which flowed out at 5—10 min later after the begining of elution, were analysed to measure protein and borate contents. Fig. 4 compares the results obtained with four combinations of protein and borate. No recovery is found for $Cs_2B_{12}H_{12}$, in contrast with $Cs_2B_{12}H_{11}SH$.

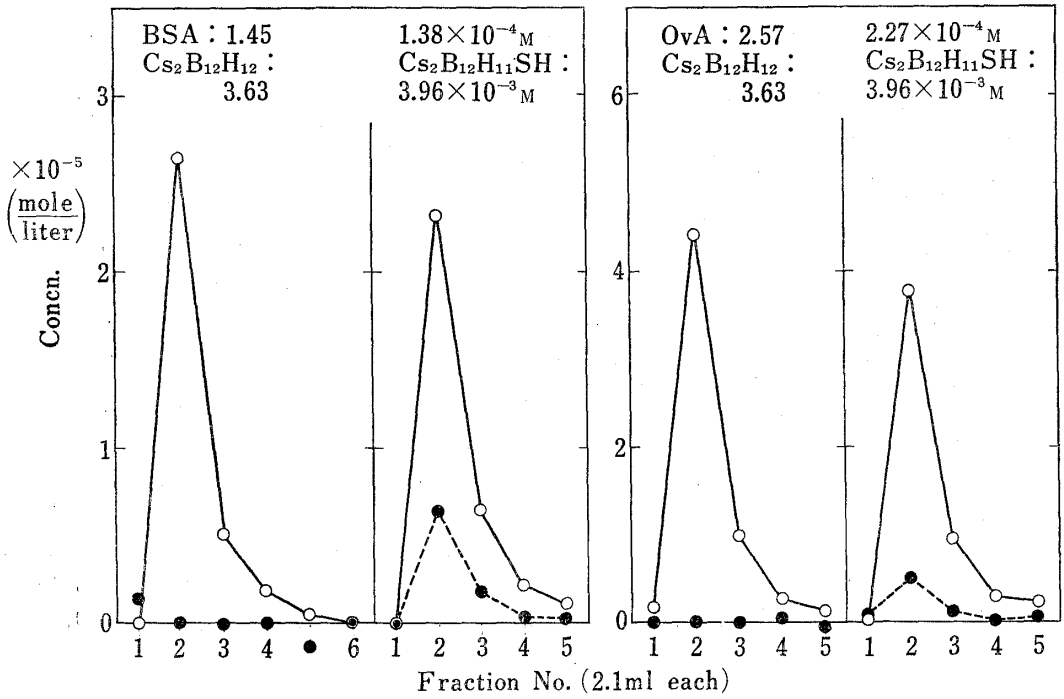

Fig. 4. Protein and Borate Contents in the Protein-containing Fractions of Ion-retardation Chromatography

—○—:protein, ---●---: borate

Additional experiments have been carried out to shed light on the nature of protein-borate interaction. A series of test solutions with different pH were prepared by adding NaOH or HCl to the buffer solution, and chromatographed after the incubation at 37° for 20 hr. The ratio of borate/protein (mole/mole) in the protein-containing portion of effluent, x_{obs}, was always negligible in the case of $Cs_2B_{12}H_{12}$. In the case of $Cs_2B_{12}H_{11}SH$, the ratio x_{obs} is very large at high pH where the denaturation of protein is noticeable as seen in Table III. For BSA, the ratio is considerably large also at low pH where the denaturation is recognizable. Several preliminary experiments indicated that the presence of sodium dodecyl sulfate (SDS) or concentrated urea affects the protein (BSA or OvA)–$Cs_2B_{12}H_{11}SH$ interaction (increases

Table III. The Ratio, $\varkappa_{obs}$, of $Cs_2B_{12}H_{11}SH$/BSA or $Cs_2B_{12}H_{11}SH$/OvA in Protein-Containing Effluent of Ion-Retardation Chromatography

pH	$[\theta]_{222}$ in CD	α-Helix Content %[a]	$\varkappa_{obs}$ mole/mole	
BSA 1.36×10^{-4}M, Borate 2.7×10^{-2}M in Test Solutions				
12.2	-0.424×10^4	10.6	10.7	
8.4	−1.730	43.3	0.46	
7·4	−1.736	43.4	0.32	
5.6	−1.751	43.8	0.35	
2.1	−1.238	31.0	0.90	
OvA 2.20×10^{-4}M, Borate 2.7×10^{-2}M in Test Solutions				
12.3	−0.322	8.1	3.66	
8.2	−0.854	21.4	0.16	
7.4	−0.855	21.4	0.10	
5.6	−0.840	21.0	0.09	turbid
2.8	−0.807[b]	20.2	—	precipitation

a) calculated by $-[\theta]_{222}/400$ (%), according to: T. Samejima, *Kagaku-no-Ryoiki*, **24**,34 (1970); K. Hamaguchi, K. Takesada, "Seibutsu Kagaku Jikkenho," Tokyo Univ. Press, Tokyo. 1969, p. 63; H. Hashizume, M. Shiraki, K. Imahori, *J. Biochem.*, **62**, 543 (1967).
b) supernatant

$\varkappa_{obs}$ in various degrees) at neutral pH. Systematic studies are required to elucidate the interrelation.

It is well known that sulfhydryl groups of protein can be masked by N-ethyl maleimide (NEM). An aliquot of 0.1M phosphate buffer containing BSA was mixed with a buffered solution of NEM, and after 3 hr equilibration, the mixture was dialysed twice against the same buffer (volume ratio 1: 250). The dialysed solution was mixed with an equal volume of a buffered solution of $Cs_2B_{12}H_{11}SH$, and chromatographed after the incubation at 37° for 20 hr. The same procedure was applied to another aliquot of the BSA solution but in the absence of NEM. The treatment with NEM caused an increase in $\varkappa_{obs}$ as shown in Table IV.

Table IV. Effect of Masking Sulfhydryl Groups ofBSA on $\varkappa_{obs}=Cs_2B_{12}H_{11}SH$/BSA in BSA-Containing Effluent of Ion-Retardation Chromatography

Sample	Free SH eq./mole	Concn. of test solution		$\varkappa_{obs}$
		BSA	Borate	
BSA treated with NEM	0	1.34×10^{-4}M	5.89×10^{-3}M	0.453
BSA with no treatment	0.60	1.31	5.89	0.382

Discussion

The equilibrium dialysis and the equilibrium distribution in and out of the Sephadex gel reveal that both $Cs_2B_{12}H_{12}$ and $Cs_2B_{12}H_{11}SH$ are bound to BSA, although the considerable differences observed between Fig. 1 and 2 are not well understood. Far larger surface area of the gel in comparison with that of the dialysis membrane may cause larger adsorption of protein and/or buffer salts, and this, in turn, may bring about the discrepancy in some way. Another possibility, which seems more probable, is the insufficient (though sufficient for diffusion) time of equilibration in the gel-distribution procedure. Too large values are expected for $\bar{\nu}$, if this is the case. The existence of peak I in the elution curves of gel-filtration chromatography indicates that the binding force between borates and BSA is remarkably strong. The nature of the binding force must be solely ionic in the case of $Cs_2B_{12}H_{12}$, since the binding is completely destroyed during the course of ion-exchange or ion-retardation chromatography.

In contrast, a part of $Cs_2B_{12}H_{11}SH$ seems to be bound covalently, because some borate is recovered from the albumin-containing fractions of both chromatographies.

The formation of disulfide linkage between the boron cage and BSA proposed by Soloway, *et al.* is considered to be reasonable. Probably, the primary step may be expressed by

$$B_{12}H_{11}SH^{2-} + PS\text{-}SP \rightleftharpoons B_{12}H_{11}S^{2-}\text{-}SP + PSH$$

where PS–SP stands for an intra- or intermolecular disulfide junction in the protein. The amount of $B_{12}H_{11}S$–SP can be determined by measuring the boron content in the protein-containing fractions of ion-retardation chromatography with an elution rate rapid enough to ensure the negligible progress of backward reaction. The above-mentioned mechanism is supported by the findings 1) a denatured protein, with a larger number of reactive PS–SP groups exposed to an aqueous medium, shows generally an enhanced x_{obs} in the ion-retardation chromatography, and 2) the masking of sulfhydryl groups of a native protein increases x_{obs}, by virtue of a decreased degree of the backward reaction.

For more detailed discussion of the $Cs_2B_{12}H_{11}SH$–BSA binding of covalent character, the authors propose two models of interaction in Part II of this series of papers, and based on these models, explain in Part III the results of ion-retardation chromatography obtained for a number of systems with various concentrations of borate and albumin.