Method B: Compound (IIIb) (122 mg) was dissolved in 200 ml of hot water. The resulting solution was added to 150 mg of palladium on charcoal in 50 ml of water and hydrogenated for 6 hr at room temperature. The catalyst was removed by filtration and washed with 100 ml of hot water. The aqueous solution was evaporated to dryness and the residue was recrystallized from 200 ml of boiling water to give a white powder. 79 mg (70%). This sample was identified with the specimen obtained by the method A.

8-Hydroxyxanthosine (9-β-D-Ribofuranosyluric Acid) (Vb)——To a solution of (IVb) (150 mg) in 5 ml of hydrochloric acid which was cooled in ice–salt mixture, was added a cooled solution of 42 mg of sodium nitrite in 2 ml of water. After 5 hr stirring at 0°, precipitate began to separate; the reaction mixture was kept cold in an ice-box, and solid was collected by filtration; 132 mg was obtained. Yield 84%. Analytical sample was recrystallized from hot water to give white needles. mp >240° *Anal.* Calcd. for $C_{10}H_{12}O_7N_4 \cdot H_2O$: C, 37.74; H, 4.40; N, 17.61. Found: C, 37.82; H, 4.42; N, 17.75; UV absorption: λ_{max}^{pH1} 238 mμ, 288 mμ; λ_{min}^{pH1} 258 mμ; $\lambda_{max}^{H_2O}$ 241 mμ, 293 mμ; $\lambda_{min}^{H_2O}$ 225 mμ, 270 mμ; λ_{max}^{pH11} 251 mμ, 302 mμ; λ_{min}^{pH11} 280 mμ. Rf=0.10 (A), 0.59 (B).

This product was also identical with specimen by the Holmes's procedure[12] by paper chromatography and IR spectrophotometry.

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Studies on Synthetic Sweetening Agents. XII.[1] The Binding of Sodium Cyclamate with Bovine Serum Albumin

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It has been reported by some workers[3–7] that the distribution, metabolism, and elimination of a drug are affected by various kinds of proteins in plasma. Davis[3] has pointed out that the albumin fraction of plasma is primarily responsible for the properties of binding of a drug with plasma proteins.

In the previous paper,[8] the authors reported that albumin and casein were precipitated by the addition of sodium cyclamate (CHS–Na) from the aqueous solutions. However, nothing has been known about the interaction between CHS–Na and serum albumin.

The present report deals with physicochemical studies on the mode of binding of CHS–Na with bovine serum albumin (BSA) using the equilibrium dialysis method, and it was suggested that the binding of CHS–Na with BSA is predominantly a reversible one based on electrostatic force, by which an anonic form of CHS–Na may be bound with the positively–charged residues in a BSA molecule. Furthermore, it was found that the strength of binding of CHS–Na to BSA is considerably weak.

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Experimental

Reagents and Materials——As buffer solutions, the following two were used: Glycine–hydrochloric acid buffer of a pH of 3.7; phosphate buffers of pH of 4.6, 5.9, 7.0, 8.0, and 9.0. The sample of CHS–Na used was obtained by repeated recrystallization of pure reagent grade one and drying at 105° for 2 hr. BSA was purchased from Armour Laboratories, and stored below 5° over anhydrous calcium chloride. The molecular weight was assumed to be 70000. Acetyl–amino BSA was prepared according to the method of Tabachnick, *et al.*[9] An analysis for free amino groups using the Van Slyke method showed that 91% of the BSA amino groups, that is, 55 out of 60 lysine residues had been acetylated. The sample of acetyl-amino BSA was also stored under the same conditions as BSA.

Colorimetric Determination of CHS-Na——A sample solution containing CHS–Na was diluted with water and analyzed by the colorimetric mthod B, which had previously been reported by us.[10]

Equilibrium Dialysis Experiment——A seamless cellulose tubing (24/32 in size)[11] was cut to 90 mm in length, and tied up at one end to make a cellulose bag. Then the bag was immersed in distilled water for two days and placed between filter papers to eliminate water completely. The bag was attached to the lower end of a glass cylinder (15×100 mm) with the aid of a rubber ring. Eight ml of 0.8% BSA solution, which was dissolved in 0.1 M of each of buffers, was pipetted into the bag. The bag was inserted to a test tube (30×110 mm) containing 20 ml of CHS–Na solution ($0.5—4.0\times10^{-3}$ M) and fixed with the aid of a rubber stopper. The apparatus was kept in a cold chamber of approximately 6° for 72 hr, which was a sufficient period of time for attaining to an equilibrium between the solutions inside and outside the dialysis bag. As a control experiment, another bag containing 8 ml of a buffer in place of BSA solution was also immersed in the solution of each concentration of CHS–Na. Then the bag was removed and the solution external to the bag was subjected to an analysis for CHS–Na as described previously. The extent of binding of CHS–Na with BSA was calculated from the difference between the concentration of free CHS–Na in the sample tube and that in the control one.

Results and Discussion

As a preliminary experiment, the effect of concentrations of buffers on the binding properties of CHS–Na with BSA was studied. Glycine–hydrochloric acid buffer of a pH of 3.7 and phosphate buffers of pH of 5.3, 7.0, and 9.0 were used for this purpose. The results indicated that no significant difference was observed in the binding of CHS–Na with BSA within the range of 0.05 to 0.2 molar concentration of each buffer used (Fig. 1).

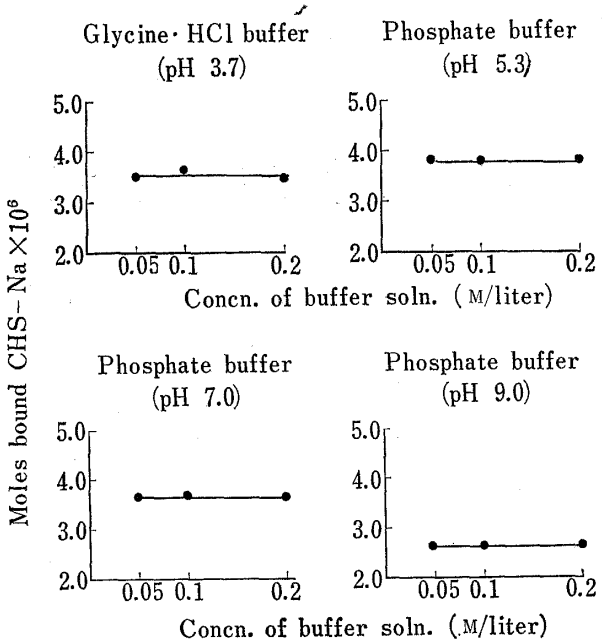

Fig. 1. The Effect of Buffer Concentration on the Binding of CHS–Na with BSA
initial concn. of CHS–Na: 2.0×10^{-3}M

The first step of studying the mode of binding between CHS–Na and BSA is to elucidate whether the binding is of reversible nature. In order to solve this problem, an equilibrium dialysis experiment was carried out according to the method described in the preceding section (Table I). The dialysis bag was removed from the external solution in which equilibrium had been completed between BSA and CHS–Na. The bag, containing an average number of 8.90 bound CHS–Na per molecule of BSA, was then immersed in a new buffer solu-

9) M. Tabachnick and H. Sobotka, *J. Biol. Chem.*, **235**, 1051 (1960).
10) S. Kojima and H. Ichibagase, *Yakugaku Zasshi*, **83**, 1108 (1963).
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tion until the second equilibrium was completed. The average number of bound CHS–Na decreased to 1.26 under a new experimental condition. Therefore, the binding of CHS–Na with BSA seemed to be a reversible process.

Table I. Reversibility of Binding

	First equilibrium	Second equilibrium
Total bound CHS–Na (mole)	7.84×10^{-6}	1.11×10^{-6}
Number of bound CHS–Na per molecule of BSA	8.90	1.26

initial concentration of CHS–Na: 8.0×10^{-3} M
0.1 M phosphate buffer at pH 5.3

When the interaction of a lower molecular compound or an ion with the proteins is a reversible one, the relation between the quantity of a bound compound (or an ion) and the concentration of a free compound (or an ion) is usually expressed by the Langmuir-type equation (1),[12]

$$\frac{1}{r}=\frac{1}{nK}\cdot\frac{1}{[c]}+\frac{1}{n} \qquad (1)$$

where r is the average number of molecules of bound CHS–Na per molecule of BSA, n is the maximum one of bound CHS–Na per molecule of BSA, K is the binding constant for the interaction, and $[c]$ is the concentration of free CHS–Na in solution when equilibrium between bound and free forms is completed.

The binding of CHS–Na with BSA was studied using 0.1M glycine–hydrochloric acid buffer of a pH of 3.7 and 0.1M phosphate buffers of pH of 4.6 to 9.0. In these experiments, the relation between $1/r$ and $1/[c]$ was expressed as a straight line (see Fig. 2). From the results shown in Fig. 2, it was obvious that the relation of binding between CHS–Na and BSA was in good accordance with the above equation (1) at any of pH values used, though the average number of bound CHS–Na molecules per BSA molecule varied depending on the pH values of buffer solutions used. Therefore, the values of n were able to be calculated from $1/n$ values which were shown as the intercepts on the ordinate axis in Fig. 2. The value of n obtained was about 9.1 in each case without reference to changes in pH value (Fig. 3). The values of K were also calculated from $1/nK$ values which were shown as the slopes of straight lines de-

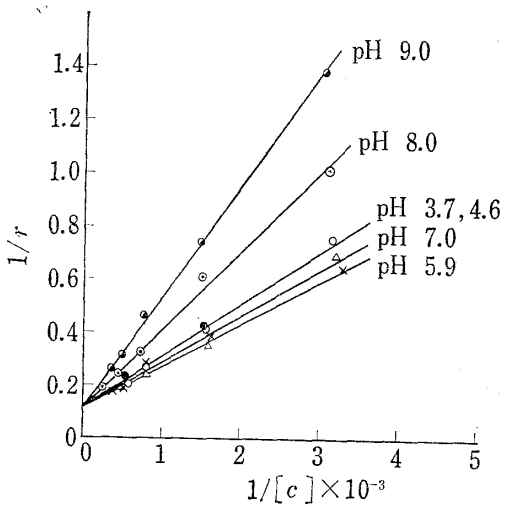

Fig. 2. Binding of CHS–Na with BSA in Various pH Values at 6°

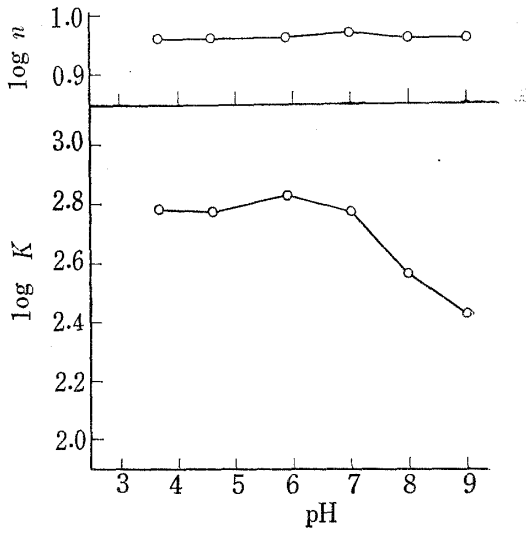

Fig. 3. Variaion with pH of n and K Values of CHS–Na Bound with BSA Molecule

12) I.M. Klotz, F.M. Walker, and R.B. Pivan, *J. Am. Chem. Soc.*, **68**, 1486 (1946).

scribed above. As shown in Fig. 3, the values of K were much the same within the range of pH of 3.7 to 7.0, however, they decreased gradually in accordance with an increase in pH value.

In order to investigate further the binding properties of CHS–Na with BSA, a test was carried out using albumin in which the amino groups had been selectively acetylated. The pattern of binding of CHS–Na with such an acetyl–amino BSA (91% of whole amino groups were acetylated) is illustrated in Fig. 4, together with some results obtained from unacetylated BSA under the same experimental conditions. The extent of binding between CHS–Na and the acetyl–amino BSA distinctly decreased as compared with that observed between CHS–Na and BSA. Thus it may be considered that the amino groups in BSA participate in CHS–Na binding. Then, Klotz[13] has demonstrated that the binding of an anion with a protein is generally based on electrostatic force, by which an anion is bound with the positively- charged amino acid residues in a protein molecule. On the other hand, CHS–Na seems to exist mostly as an ionized form in various buffer solutions used in this experiment, as reported previously.[14] Accordingly, it is presumed that an anionic form of CHS–Na is bound with cationic nitrogen groups in the BSA molecule by means of electrostatic force. Consequently, it may be presumed further that the positive charge in a BSA molecule decreases concomitantly with the increase in pH value, so that the progressive decrease in value of K, in other words, the decrease in the strength of binding of CHS–Na with BSA, may occur above pH 7.0.

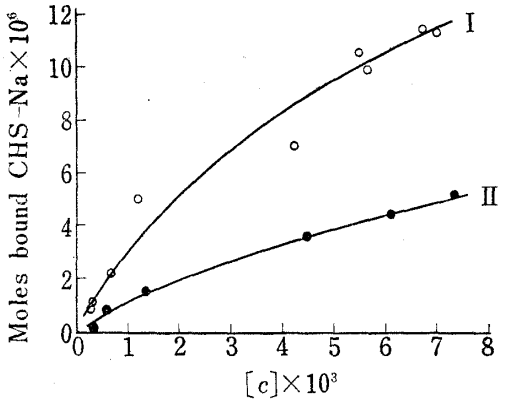

Fig. 4. Comparison of the Extent of Binding of Acetyl–amino BSA (II), with that of the Parent Albumin (I)

0.1 M phosphate buffer at pH 5.3

Furthermore, the relation among the binding constant, K, the binding affinity, $\Delta\mu^\circ$, and the surface potential of BSA, ϕ, is expressed by the following equation (2),[15] where R

$$RT \ln K = -\Delta\mu^\circ + \phi F \qquad (2)$$

is the gas constant, T is the absolute temperature, and F is the Faraday constant. The value of the binding affinity of CHS–Na toward BSA, $\Delta\mu^\circ$, calculated from the equation (2), was −3.58 Kcal/mole. Therefore, it was found that this binding affinity of CHS–Na was smaller than those of some sulfonamides, which were about −6 Kcal/mole as reported by Nakagaki, *et al.*[15]

Results of further studies on the interaction of CHS–Na with proteins will be reported afterwards.

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