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Influence of Blood Proteins on Biomedical Analysis. I. Interaction of Xanthurenic Acid with Bovine Serum Albumin[1]

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In order to characterize the amplifying effect of bovine serum albumin (BSA) on the optical density in the color reaction for the determination of xanthurenic acid (XA) using 4-aminoantipyrine (4-AAP), the interactions of XA with native and modified BSA were examined by equilibrium dialysis. BSA was modified with hydrogen peroxide (H_2O_2), *p*-chloromercuribenzoic acid (PCMB) and 2-hydroxy-5-nitrobenzyl bromide (HNBB). Binding of XA with BSA was confirmed by gel filtration of a mixture containing BSA and XA on a Sephadex G-150 column. The amount of bound XA increased dose-dependently in proportion to the BSA concentration in the mixture. Scatchard plots for the interaction of XA with BSA at pH 4.0, 5.7, 7.4 and 10.0 were obtained. The plots indicated two classes of XA-binding sites on BSA at pH 5.7 and 7.4, but gave a simple straight line with disappearance of the primary binding site at pH 4.0 and 10.0. The binding capacity (n_2K_2) in the secondary binding site decreased linearly with increasing pH of the solution from 4.0 to 10.0, suggesting that the binding force was electrostatic. The standard free energy change (ΔG_2°), standard enthalpy change (ΔH_2°) and entropy change (ΔS_2°) were -5.3 kcal/mol, -4.2 kcal/mol and 3.6 entropy units at pH 7.4, respectively. The temperature dependence of the secondary binding affinity (K_2) was large, suggesting that the association was not ionic. Consequently, these results indicate that the secondary association may involve both electrostatic and hydrophobic interactions. Any modifications of cysteine, methionine and tryptophan residues on the BSA molecule destroyed the primary binding site, and the secondary binding site was also slightly affected; thus, it is speculated that both binding sites may be associated with regions around these three amino acid residues on the BSA molecule.

In conclusion, although the relationship between the coloration and the interaction of XA with BSA must be examined more closely, the present results suggest that the binding of XA to BSA contributes substantially to the amplification of the optical density in the color reaction of XA using 4-AAP.

Keywords——bovine serum albumin; xanthurenic acid; 4-aminoantipyrine; interaction of xanthurenic acid with bovine serum albumin; equilibrium dialysis; Scatchard plot; electrostatic interaction; hydrophobic interaction

Xanthurenic acid (XA, 4,8-dihydroxyquinoline-2-carboxylic acid), which is a metabolite of tryptophan, appears in urine and blood at high levels in rats maintained with tryptophan-loaded or vitamin B_6-lacking feed.[3] Recently, it was suggested that serum albumin inhibits the diabetogenic action of XA, which arise as a result of complex formation with blood insulin; the inhibitory effect of albumin may be due to a competitive binding between XA and insulin, resulting in a low level of free XA.[4]

Previously, we reported that the addition of bovine serum albumin (BSA) to the reaction mixture depressed the decrease of optical density, and even enhanced the optical density in the color reaction for the determination of XA using 4-aminoantipyrine (4-AAP). We at-

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2) Location: *1-1, Mukogawa-cho, Nishinomiya 663, Japan.*
3) K. Statoh and J.M. Plice, *J. Biol. Chem.*, **230**, 781 (1958).
4) Y. Kotake, T. Sotokawa, E. Murakami, A. Hisatake, M. Abe, and Y. Ikeda, *J. Biochem.*, **63**, 578 (1968).

tributed this phenomenon to the interaction of XA with BSA.[5]

In the present report, we confirmed the binding of XA to BSA, and the interaction of XA with BSA was examined using native and modified BSA in order to characterize the amplifying effect of BSA on the optical density in the color reaction for the determination of XA.

Experimental

Materials

All reagents used were of reagent grade. Xanthurenic acid, hydrogen peroxide (H_2O_2, 30%) and *p*-chloromercuribenzoic acid (PCMB) were products of Wako Pure Chemical Industries Ltd., Japan. 2-Hydroxy-5-nitrobenzyl bromide (HNBB, Koshland's reagent) was obtained from Nakarai Chemical Ltd., Japan. Bovine serum albumin (fraction V) was purchased from The Armour Laboratories Co., U.S.A., and cellophane tubing (20/32 inch inflated diameter, Visking Co., U.S.A.) was used as a dialyzing membrane. Other chemicals were products of Wako Pure Chemical Industries Ltd., Japan. Reagent-grade NaH_2PO_4 and Na_2HPO_4 were used to prepare 1/15 M phosphate buffer (pH 7.4). Buffer solutions of pH 10.0, 5.7 and 4.0 were prepared by addition of 5 N NaOH or 5 N HCl to 1/15 M phosphate buffer solution (pH 7.4).

Preparation of Modified BSA

Oxidation of BSA with H_2O_2[6]——Ten ml of 1/15 M phosphate buffer (pH 7.4) containing BSA (2.72 g, 40 μmol) was maintained at 0°, and one ml of 30% H_2O_2 was added to the solution. After keeping the solution at 0° for 2 hr, the reaction mixture was dialyzed overnight against 1000 ml of 1/15 M phosphate buffer (pH 7.4). The dialysate was used for experiments on the binding of XA without further purification. The content of modified protein in the dialysate was determined by an autoanalyzer (BCG method, Greiner Electron Selective Analyzer II, Greiner Electronic Ltd., Switzerland). The modification of cysteine residues (-SH) on BSA was confirmed by means of the Grote reaction.[7]

Modification of BSA with PCMB[8]——0.05 N NaOH solution (25 ml) containing 72 mg (200 μmol) of PCMB was added to 1/15 M phosphate buffer (pH 7.4) (200 ml) containing 4 g (58.8 μmol) of BSA and left for up to 2 hr. The reaction mixture was then dialyzed against 5 l of distilled water for 48 hr at 4°. Lyophilization of the dialysate gave 3.5 g of a light-yellow powder. The modification of cysteine residues (-SH) on BSA was confirmed by means of the Grote reaction.[7]

Modification of BSA with HNBB[9]——A mixture of 0.1 M citrate buffer (pH 3.0) (0.5 ml) and dimethylsulfoxide (0.5 ml) containing 18.4 mg (80 μmol) of HNBB was dropped into 0.1 M citrate buffer (pH 3.0) (10 ml) containing 2.72 g (40 μmol) of BSA and left for up to 30 min, then the reaction mixture was applied to a Sephadex G-150 column (30×2.5 cm) and eluted with 1/15 M phosphate buffer (pH 7.4). The effluent corresponding to the peak of BSA was collected and dialyzed against flowing tap water overnight. Lyophilization of the dialysate gave a sulfur-yellow powder in a yield of 2.4 g. The modification rate of BSA was determined as 2.2 HNBB/BSA (μmol/μmol)[10] from the extinction of a solution containing the modified protein at 410 nm.

Equilibrium Dialysis Method

Equilibrium dialysis as described by Hughes *et al.* was used with some modifications.[11] The dialysis studies were done with 1×10^{-3} M protein. The basal solution containing 1000 μg/ml of XA was diluted to an adequate concentration with 1/15 M phosphate buffer (pH 7.4) for each experiment. One ml of the solution containing XA and either native or modified BSA was taken into a dialysis bag which was placed in a glass vial (inner volume 20 ml) containing 9 ml of XA solution at the same concentration as that inside the bag. After continuous shaking at 120 strokes per min for 3.5 hr at 37° in a water-bath, the concentration of XA outside the bag was estimated from an optical density at the maximum absorption wavelength (340 nm) of XA at pH 7.4. A value of 6.8×10^4 was used for the molecular weight of BSA, and the molecular extinction coefficient of XA in the phosphate buffer (pH 7.4) was taken to be 6.5×10^3.

Calculation

The binding data obtained by equilibrium dialysis were analyzed according to Scatchard *et al.*[12] The following equation for a single class of n equivalent binding sites was used,

$$r/[\text{free XA}] = nK - rK \qquad (1)$$

5) E. Kaneko, K. Kobayashi, S. Kagawa, and A. Matsuoka, *Jap. J. Clin. Path.*, **26**, 726 (1978).
6) W.S. Lin, D.A. Armstrong, and G.M. Gaucher, *Can. J. Biochem.*, **53**, 298 (1975).
7) I.W. Grote, *J. Biol. Chem.*, **93**, 25 (1931).
8) P.D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).
9) A. Reis and A. Lukton, *Bioorg. Chem.*, **4**, 1 (1975).
10) HNB: 2-hydroxy-5-nitrobenzyl, $\varepsilon=18450\,\text{M}^{-1}/\text{cm}$ (410 nm, pH 10).
11) I.E. Hughes, K.F. Ilett, and L.B. Jellett, *Brit. J. Clin. Pharmacol.*, **2**, 521 (1975).
12) G. Scatchard, I.H. Scheinberg, and S.H. Armstrong, *J. Am. Chem. Soc.*, **72**, 540 (1952).

where r is the number of moles of bound XA per mol of protein, and K and [free XA] are the association constant for XA-protein complex and the concentration of free XA, respectively. If the Scatchard plot is not linear, the existence of multiple classes of site on the protein molecule can be assumed, and we can generalize Eq. 1 to Eq. 2.[13]

$$r/[\text{free XA}] = \sum n_i K_i - \sum r K_i \quad (2)$$

Here, the subscript i denotes i-th class of binding sites. Extrapolation of the limiting straight line drawn by the least-squares method for the linear portion of the Scatchard plot to the y axis gives $\sum n_i K_i$ (intercept on the y axis, total binding capacity). Similarly, extrapolation of the limiting straight line to the x axis gives $\sum n_i$ (intercept on the x axis, total number of binding sites), and the apparent K_i (slope of the straight line) can be calculated. It was reported that binding sites with a low association constant can be neglected in the study of drug-protein interaction,[14] so the present data were analyzed based on the assumption of 2 classes of sites on the BSA molecule. Values of $n_1 K_1$ and n_2 are obtained from the differences between $\sum n_i K_i$ and $n_2 K_2$, and $\sum n_i$ and n_1, respectively. Several thermodynamic parameters including standard free energy change ($\Delta G°$), standard enthalpy change ($\Delta H°$) and entropy change ($\Delta S°$) were calculated by the use of Eq. 3—5.[15]

$$\Delta G° = -RT \ln K \quad (3)$$

$$\ln(K_I/K_{II}) = \Delta H°/R\,[(T_{II}-T_I)/(T_I T_{II})] \quad (4)$$

$$\Delta G° = \Delta H° - T\Delta S° \quad (5)$$

R is the gas constant (1.987 cal/mol), and T and K are the absolute temperature and the association constant of the XA-protein complex.

Results and Discussion

Effect of BSA on the Color Reaction

The quinone dye (RQ, red quinone) formed in the color reaction for the determination of XA has a maximum absorption at 510 nm in 0.1 M carbonate buffer (pH 10.0).[4] Fig. 1A shows time course profiles of the color reaction at various concentrations of BSA. In the absence of BSA, the optical density of the reaction mixture was transiently increased within 5 min but very soon fell. However, the optical density at 30 min after the initiation of the color reaction increased dose-dependently with BSA concentration up to 0.125 mM (Fig. 1B).

When BSA was added at the last time tested in the present procedure, the time course was similar to that in the absence of BSA. However, the optical density was intermediate between those in the presence and absence of BSA, and gradually fell. Therefore, it is suggested that the potentiating and stabilizing effects of BSA on the color reaction were due to acceleration of the oxidative coupling reaction of XA with 4-AAP, with simultaneous inhibition of the decomposition of the color substance (red quinone). However, it is not clear whether these effects arise from the binding of XA with BSA or from other mechanisms.

Binding of XA to BSA

The elution pattern on gel filtration (Sephadex G-150) of XA which had been incubated in 1/15 M phosphate buffer (pH 7.4) containing 1 mM BSA at 37° for 1 hr is shown in Fig. 2. BSA-bound XA was eluted with the peak of BSA in 47% yield, and this was followed by the peak of free XA. The overall recovery of XA on gel filtration was 93%. Furthermore, on ultrafiltration of a mixture containing XA and BSA using a collodion bag (Sartorius membrane filter, SM-13200), the concentration of XA in the filtrate decreased dose-dependently with increasing BSA concentration in the mixture.[16] These results confirm that BSA has binding ability with XA.

13) I.M. Klotz and D.L. Hunston, *Biochemistry*, **10**, 3065 (1971).
14) F. Karush, *J. Am. Chem. Soc.*, **72**, 2705 (1950).
15) I.M. Klotz, "The Proteins," Vol. 1, part B, H. Neurath and K. Bailey, Eds., Academic Press Inc., New York, 1953, p. 111; A.N. Martin, "Physical Pharmacy," 2nd Ed., Lea and Febigger, Philadelphia, 1969, p. 343; M.J. Cho, A.G. Michell, and M. Pernarowski, *J. Pharm. Sci.*, **60**, 196 (1971).
16) K. Kobayashi, T. Sakoguchi, Y. Togawa, M. Kimura, and A. Matsuoka, unpublished.

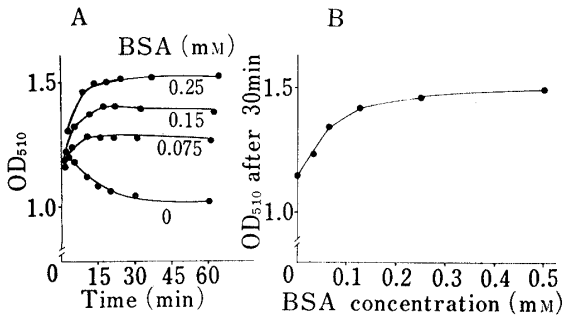

Fig. 1. Effects of BSA on the Time Course and Dose–Response Curve of the Color Reaction

Fig. 1A and 1B show the results obtained in separate experiments. Each point in Fig. 1B represents the optical density at 30 min after the initiation of the color reaction. The color reactions were performed as follows: 0.8 ml of 0.1 M carbonate buffer, 0.1 ml of 0.1 M carbonate buffer solution containing various concentrations of BSA, and 0.5 ml of XA solution (500 μg/ml) were mixed, then 0.5 ml each of 2.4% 4-AAP solution and 2.4% potassium ferricyanide were added in this order. Each reference solution was prepared by replacing XA solution with an equivalent volume of 0.1 M carbonate buffer. Each point represents the mean value of two experiments.

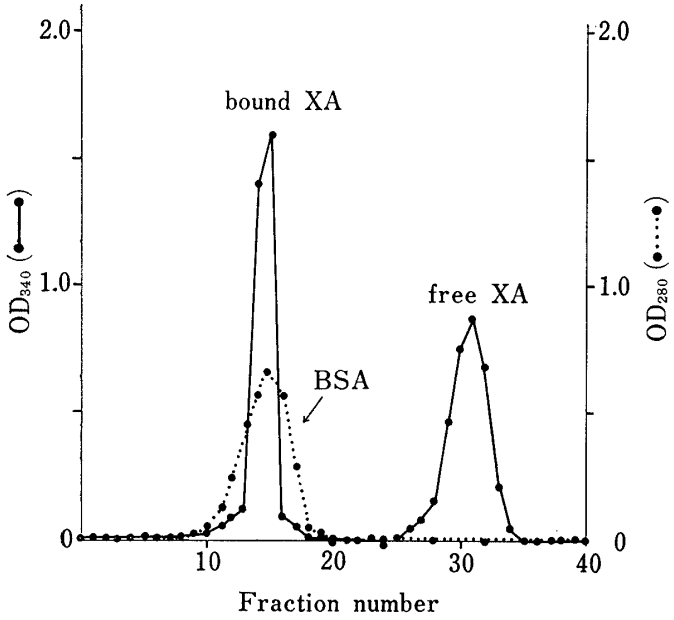

Fig. 2. Gel Filtration of BSA-bound XA from Sephadex G-150

A mixture (5 ml) of 1/15 M phosphate buffer (pH 7.4) containing 5 μmol of BSA and 31.5 μmol of XA was incubated for 1 hr at 37°, then 1 ml of the reaction mixture was applied to a Sephadex G-150 column (1.5×90 cm). The column was eluted with 1/15 M phosphate buffer (pH 7.4) at a flow rate of 0.4 ml/min, and fractions of 4 ml were collected (10 min per fraction) using a fraction collector. The optical density of each fraction was measured at 280 and 340 nm.

The interaction of XA with BSA was examined by the equilibrium dialysis method using Visking tubing. The equilibration of XA with the dialysis membrane required 60—90 min under the present experimental conditions, but the amount of XA bound to BSA inside the bag reached a maximum in 3—4 hr on shaking at 37°. A partial dissociation of free XA from the XA-bound protein was observed on shaking for more than 6 hr (Fig. 3A), so the following equilibrium dialyses were carried out for 3 hr at 37°. The amount of BSA-bound XA increased linearly in proportion to the BSA concentration in the mixture, as shown in Fig. 3B.

Analysis of the Interaction of XA with BSA

Fig. 4A shows Scatchard plots for the interaction of XA with BSA at various pH values 4.0, 5.7, 7.4 and 10.0). The plots at pH 4.0 and 10.0 were simple straight lines (pH 4.0: $n_2=$

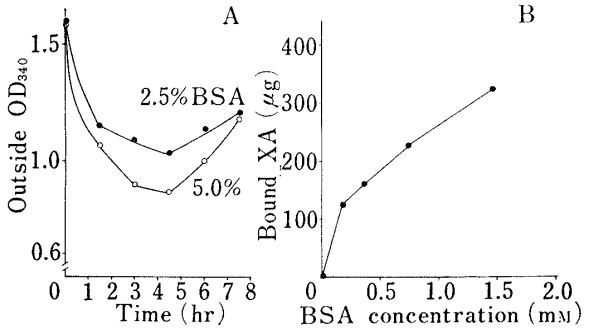

Fig. 3. Time Course and Extent of XA-binding with BSA

The amount of XA bound was calculated from the decrease of XA concentration outside the dialysis bag after 3.5 hr.

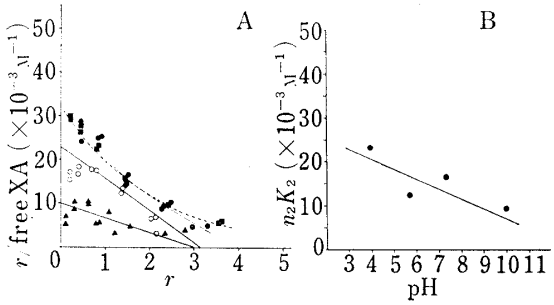

Fig. 4. Scatchard Plots for the Interaction of XA with BSA at Various pH Values and the Relationship between pH and nK Value

—○—, pH 4.0, -- ■---, 5.7, —●—, 7.4, —▲—, 10.0.

3.2, pH 10.0: n_2=3.0) indicating disappearance of the primary binding sites. However, the plots at pH 5.7 and 7.4 were hyperbolic curves, indicating multiple classes of XA-binding sites on the BSA molecule.

Table I. Several Binding Parameters for the Interactions of XA with Native and Modified BSA

Modification	Temp. (°)	Binding parameter					
		n_1	k_1 ($\times 10^{-3}$ M^{-1})	n_1K_1 ($\times 10^{-3}$ M^{-1})	n_2	k_2 ($\times 10^{-3}$ M^{-1})	n_2K_2 ($\times 10^{-3}$ M^{-1})
Native pH 10.0	37	—	—	—	3.0	3.3	9.9
7.4	37	1.1	11.8	13.0	2.9	5.5	16.0
7.4	20	2.6	6.9	17.9	1.3	8.2	10.7
5.7	37	1.6	9.1	14.6	3.4	3.5	11.9
4.0	37	—	—	—	3.2	7.2	23.0
Oxidation (H_2O_2)	37	—	—	—	2.7	9.7	26.2
Mercaptidation (PCMB)	37	—	—	—	3.6	9.4	33.8
Trp-modification (HNBB)	37	—	—	—	4.2	4.4	18.5

All binding experiments using modified BSA were carried out in 1/15 M phosphate buffer (pH 7.4).

Several binding parameters for the interaction of XA with chemically modified BSA are summarized in Table I. Since the isoelectric point of BSA is around pH 4.7, the negative charge on BSA must increase as the pH of the solution increases. At the same time, this also increases the negative charge on the XA molecule, so the elevation of pH may weaken the electrostatic interaction between XA and BSA. Analogous interactions of several sulfonamides with BSA were studied by Nakagaki *et al.*[17] and the binding modes were suggested to be electrostatic from the relationship between the amount of bound drug and the pH of the solution. In the present study, the binding capacity (n_2K_2) of the secondary binding sites on BSA linearly decreased with increase in the pH of the solution (Fig. 4B). This result suggests that the main binding force of XA with BSA in the secondary binding sites is electrostatic. However, a decrease of the temperature from 37° to 20° causes a 49% increase in the affinity constant (K_2). This is characteristic of an exothermic drug-protein interaction.[18] The large temperature dependence suggests that the secondary association is not ionic.[18]

Thermodynamic parameters for the interaction of XA and BSA are summarized in Table II. In the first class of sites ΔG_1° was −2.9 kcal/mol at 20° to −5.8 kcal/mol at 37°, and the standard enthalpy change (ΔH_1°) was positive (5.6 kcal/mol), suggesting hydrogen bonding.[19] Overall, these results indicate that the association in the primary binding sites is not ionic.

Table II. Thermodynamic Parameters for the Interaction of XA with BSA

Temperature (°)	ΔG_1° (kcal/mol)	ΔH_1° (kcal/mol)	ΔS_1° (e. u.)	ΔG_2° (kcal/mol)	ΔH_2° (kcal/mol)	ΔS_2° (e. u.)
37	−5.8			−5.3		
20	−2.9	5.6	36.8	−3.0	−4.2	3.6

The binding experiments were carried out 1/15 M phosphate buffer (pH 7.4) at 20° and 37°. Thermodynamic parameters were derived from the data in Table I.

17) M. Nakagaki, N. Koga, and H. Terada, *Yakugaku Zasshi*, **83**, 586 (1963).
18) M.J. Crook and K.F. Brown, *J. Pharm. Pharmacol.*, **26**, 304 (1974).
19) T. Kadota, "Chemistry of Proteins," Vol. 1, ed. by S. Mizushima and S. Akabori, Kyoritsu Shuppan Co., Ltd., Tokyo, 1954, p. 237.

The standard energy change (ΔG_2°) ranged from -5.0 to -5.9 kcal/mol in the interaction of XA with BSA under four conditions of pH, and these values are in the range ($\Delta G^\circ = -5$ to -7 kcal/mol) observed commonly in electrostatic interactions.[20] In the second class of sites, the contribution to ΔG_2° by ΔH_2° was very large (79.2%), while the contribution of ΔS_2° was small (3.6 entropy units). Judging from these results, the binding force of XA with BSA at the secondary sites was concluded not to be electrostatic only. The secondary association may involve both electrostatic and hydrophobic interactions.

Every primary binding site for XA on BSA disappeared on modification with H_2O_2, PCMB or HNBB. Scatchard plots for the interaction of XA with modified BSA were simple straight lines which gave n_2 values ranging from 2.7 to 4.2 (Fig. 5). The affinity constant (K_2) in the interaction between XA and BSA modified with H_2O_2 or PCMB increased by about 50% (9.7×10^3 and $9.4\times10^3\,\text{M}^{-1}$, respectively) compared with that for native BSA ($6.3\times10^3\,\text{M}^{-1}$). These results suggest that the conformation around the cysteine residues of BSA changed on modification with these reagents, resulting in increased affinity for XA at the secondary binding sites. The total binding capacity ($\Sigma n_i K_i$) was reduced by about 50% ($18.5\times10^{-3}\,\text{M}^{-1}$) for tryptophan-modified BSA and about 30% ($26.2\times10^3\,\text{M}^{-1}$) for H_2O_2-oxidized BSA compared with that for native BSA ($35.1\times10^3\,\text{M}^{-1}$). However, the binding capacity in the interaction between XA and PCMB-modified BSA was slightly reduced ($33.8\times10^3\,\text{M}^{-1}$).

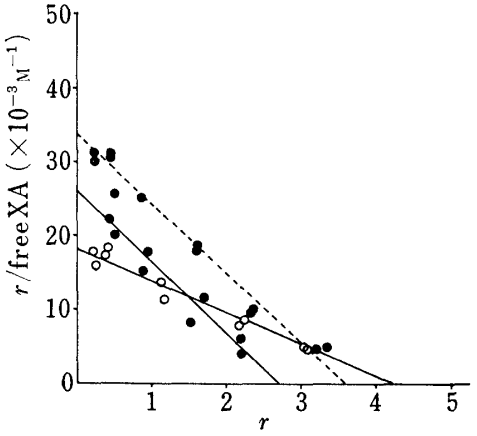

Fig. 5. Scatchard Plots for the Interaction of XA with Modified BSA

—●—, H_2O_2-oxidized BSA,
---●---, PCMB-treated BSA,
—○—, HNBB-treated BSA.

Consequently, any modification of cysteine, methionine and tryptophan residues on BSA destroyed the primary binding sites for the interaction of XA with the protein, whereas the secondary binding sites were only slightly affected by these modifications. Thus, it was suggested that the primary and secondary binding sites of BSA are associated with loops 1—4,[21] which include cysteine, methionine and tryptophan residues.

These present results are not sufficient to clarify fully the binding mode of XA to the primary binding sites on BSA, and no information is available on the interaction of BSA with XA analogs, such as quinaldic acid, kynurenic acid or other tryptophan metabolites. Goto and his co-workers reported recently that 75% of BSA binding was hydrophobic at the primary binding sites in the interaction of several sulfonylureas with BSA,[22] so the binding mode of XA to the primary binding sites on BSA may involve hydrophobic interaction, as mentioned above.

Effects of BSA on the Production of Other Red Quinones

The effects of BSA on various compounds which could or could not bind with BSA in the color reaction using 4-AAP were examined. 8-Hydroxyquinoline, which binds with BSA, showed an effect similar to that with XA on addition of BSA, resulting in a stable coloration. In the cases of phenol and dimethylaniline, with low affinity for BSA, on the contrary, the optical density decreased within 20 min on addition of BSA.

20) M. Nakagaki, N. Koga, and H. Terada, *Yakugaku Zasshi*, **84**, 516 (1964); H. Kitagawa, T. Noguchi, and R. Ito (Ed.), "Absorption, Distribution, Metabolism, Excretion of Drugs and Pharmacological Effects," Nankodo Co., Ltd., Tokyo, 1971, p. 88; J. Clausen, *J. Am. Chem. Soc.*, **70**, 943 (1948).
21) P.Q. Behrens, A.M. Spiekerman, and J.R. Brown, *Fed. Proc.*, **34**, 591 (1975).
22) S. Goto, H. Yoshitomi, and M. Nakase, *Chem. Pharm. Bull.*, **26**, 472 (1978).

In conclusion, although more compounds should be examined to characterize the effect of BSA on the coloration, the present results suggest that the interaction of XA with BSA contributes substantially to the amplification of optical density in the color reaction of XA using 4-AAP.