activity than on the microsomal glucose-6-phosphatase activity. Presumably, zinc taken up by the liver cells may affect the mitochondrial function of rat liver.

Previously it was reported that zinc ions affect the biochemical function in mitochondria isolated from liver cells of rats.[14,15] Concentrations lower than 4 μM Zn^{2+} cause a respiratory stimulation in coupled mitochondria isolated from rat liver.[14] Conversely, zinc ions are effective inhibitors of the respiratory chain of mitochondria. The inhibition site by 1—10 μM zinc ions is between cytochromes b and c_1, while ATPase is not inhibited.[15] Thus, the *in vitro* effect of zinc ions on the mitochondrial function in rat liver has not been fully resolved. Also, the *in vivo* effect of zinc on the liver mitochondria is little understood.

Succinate dehydrogenase is located in the inner membrane of mitochondria and participates in the electron transport system. The present findings that succinate dehydrogenase activity in rat liver mitochondria was markedly increased by zinc administration suggest that zinc may influence the mitochondrial function of rat liver. Zinc may stimulate the electron transport and oxidative phosphorylation systems in the mitochondria, but this remains to be elucidated.

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Effect of Modification of Albumin on Tolbutamide–Serum Albumin Binding[1a,b]

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The binding of tolbutamide, a representative sulfonylurea-related drug, to bovine serum albumin (BSA) modified completely or partially with three chemicals (*p*-chloromercuribenzoic acid, *p*-aminophenylmercuric acetate and *p*-hydroxynitrobenzyl bromide) was investigated by means of an equilibrium dialysis method. Scatchard plots of the data indicated that: (1) free sulfhydryl groups in the BSA molecule are not important binding sites for tolbutamide, (2) two tryptophan residues in the BSA molecule form a hydro-

phobic region, (3) one of the two tryptophan residues in the BSA molecule may be related to the binding of tolbutamide. These results are discussed in relation to the mechanisms responsible for the binding of sulfonylurea-related compounds to serum albumin.

Keywords——tolbutamide; bovine serum albumin (BSA); modified BSA; equilibrium dialysis; hydrophobic interaction; human serum albumin (HSA); sulfhydryl group; tryptophan residue; binding parameter

In our previous study,[2] the binding of twenty sulfonylurea-related compounds (SU), including seven commercial products, to bovine serum albumin (BSA) in phosphate buffer solution (pH 7.4 and 30 °C) was investigated by means of the equilibrium dialysis technique. Hydrophobicity at the primary binding site was found to play an important role in the binding. Electrostatic forces and hydrophobic forces at secondary binding sites on serum albumin molecules must also be considered.

The purpose of the present investigation was to further investigate the mechanisms responsible for the binding of SU to serum albumin. Tolbutamide was selected as a representative SU and binding experiments were undertaken using BSA, BSA treated with several reagents, and human serum albumin (HSA).

Experimental

Materials——Tolbutamide (TBM) was extracted from commercial tablets (Ono Pharmaceutical Co. Ltd.) and recrystallized from dilute EtOH. BSA (fraction V, Armour Pharmaceutical Co., U.S.A.) and HSA (fraction V, Sigma Chemical Co., U.S.A.) were used. Their molecular weights were assumed to be 69000. All chemicals were of reagent grade except for *p*-chloromercuribenzoic acid (PCMB), which was purified by the method of Boyer.[3]

Determination of Free Sulfhydryl Content in BSA——A desired volume (0—1.5 ml) of PCMB solution (6×10^{-4} M) was added to 1 ml of BSA aqueous solution (5×10^{-5} M), and the total volume was adjusted to 10 ml using 1/15 M phosphate buffer (pH 7.4). Each solution was allowed to stand at room temperature for 1 hour, then the absorbance was measured at 250 nm (A). The absorbance of similarly prepared solutions without BSA was measured at the same wavelength (B). The difference (A—B) was plotted against the amount (ml) of PCMB solution added (Fig. 1). The break point in Fig. 1 is considered to be equivalent to the free sulfhydryl group (SH) content.

Modification of BSA with PCMB and *p*-Aminophenylmercuric Acetate (PAPM)——BSA (5×10^{-6} mol) was dissolved in 17 ml of aqueous PCMB solution (6×10^{-4} M) prepared by Takagi's method.[4] The mixture was stirred for 1 hour at room temperature and dialyzed against 1.5 l of 1/15 M phosphate buffer solution (pH 7.4). The dialyzed solution was applied to a Sephadex G-25 (fine) column using the same phosphate buffer solution. After gel filtration, the total albumin concentration of the collected protein fraction was determined by Lowry's method. This fraction (PCMB–BSA) was then diluted to 5×10^{-5} M based on the above analytical value for albumin. Modification of BSA with PAPM (PAPM–BSA) was carried out in the same manner as with PCMB.

Modification of BSA with *p*-Hydroxynitrobenzyl Bromide (HNB)——Twenty-five ml of BSA solution (4×10^{-4} M) was acidified to pH 3 by addition of 1 N HCl, and 1 ml of acetone solution of HNB (10—250 μmol/ml) was added with stirring at 30°C. The pH was maintained at 3 by addition of small increments of 1 N NaOH using a pH-stat (Hiranuma K.K., PS-11) during the reaction. The reaction mixture was dialyzed for 24 hours against 2 l of 1 mM HCl at 4°C and then passed through a Sephadex G-25 (fine) column using the same HCl solution. The protein fraction was frozen and dried, and BSA treated with HNB (HNB–BSA) was obtained. The fluorescence intensities of the solutions of BSA and HNB–BSA (1×10^{-5} M) were measured at an excitation wavelength of 275 nm and emission wavelength of 340 nm (Hitachi spectrofluorometer, model MPF-4). The amount of tryptophan residues remaining in HNB–BSA could be calculated from the results of spectrofluorometric measurement.

Equilibrium Dialysis——The general procedure and treatment of data were the same as described previously.[5] The concentration of TBM was determined by using a Hitachi spectrophotometer, model 181.

Results and Discussion

Free SH Content in BSA——A typical titration curve of BSA with PCMB, a specific sulfhydryl reagent,[3] is given in Fig. 1. When the amount (ml) of PCMB was increased, the titra-

tion curve showed a distinct break at the point equivalent to free SH content. Similar titration experiments were carried out repeatedly, and the free SH content of BSA was calculated. The results are summarized in Table I. An average free SH/BSA ratio of 0.58 was obtained. Urea concentrations higher than 2 M induced alterations in the molecular structure of BSA, but the above ratio remained unchanged in 7 M urea solution. When L-cysteine was chosen as the standard for this titration, the ratio became 0.96. This value is almost identical with the theoretical ratio (=1.0).

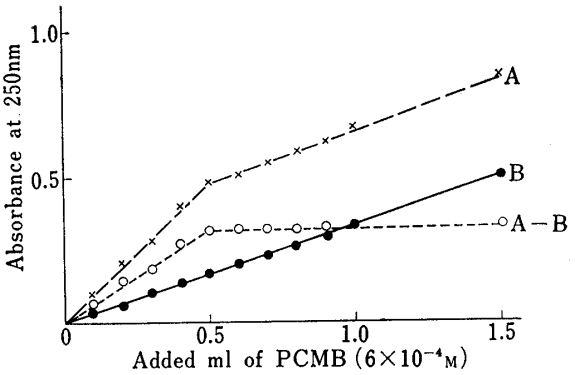

Fig. 1. Changes in Absorbance following the Reaction of PCMB with BSA

A, PCMB with BSA; B, PCMB alone.

Recently, Brown[6] and Meloun[7] have reported independently the complete amino acid sequences of BSA and HSA. According to their papers, there are 35 cysteine residues and 17 disulfide bonds are formed with 34 cysteine residues in both types of albumin. Only one cysteine residue is of the thiol type. Therefore, the theoretical free SH/BSA ratio must be one. In the present study, however, it was shown that BSA contained only 0.58 mol of SH per mol of BSA. Gabr and his associates[8] reported that HSA consists of two forms; one with one SH per molecule (two—thirds) and another with no SH. If this is also the case for BSA, the apparent free SH/BSA ratio should be modified by a factor of 0.67. The ratio obtained in these experiments (0.58 as average) is in reasonably good agreement with this value.

Table I. Determination of Free SH in the BSA Molecule[a]

BSA (1 ml)	PCMB 6×10^{-6} M (ml)	SH/BSA	
5×10^{-4} M	0.48	0.58	
	0.49	0.59	
	0.47	0.56	
	0.47	0.56	
	0.49	0.59	in 7 M Urea
	0.49	0.59	in 7 M Urea
8×10^{-4} M	0.79	0.59	
	Average	0.58	
Cystein 3×10^{-4} M	0.48	0.96	

a) Phosphate buffer (1/15 M, pH 7.4) was used as a solvent and the reaction was carried out at 30 °C for 1 h.

Binding of TBM with PCMB-BSA and PAPM-BSA——TBM binding with PCMB-BSA and PAPM-BSA was not significantly different from that with intact BSA, as shown in Figs 2 and 3.

Ohkubo[9] concluded that the reactive SH is located at the border between a polar helical segment and a hydrophobic area on the surface of the intact albumin molecule. It can be seen (Fig. 1 and Table I) that PCMB and PAPM reacted completely with the reactive SH. It was observed in separate experiments that these two reagents were not significantly displaced by TBM. These results suggest that the specific binding site for TBM on the BSA molecule is not the free sulfhydryl group.

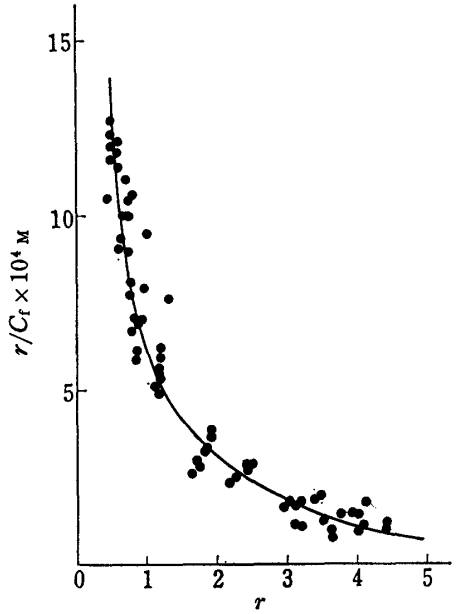

Fig. 2. Scatchard Plots for the Binding of Tolbutamide to PCMB-BSA at pH 7.4 and 30 °C

All points are experimental. The solid line was computed using the binding parameters calculated from the data for intact BSA.

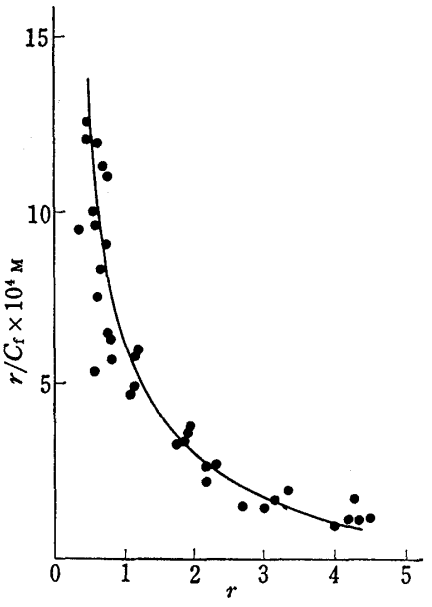

Fig. 3. Scatchard Plots for the Binding of Tolbutamide to PAPM-BSA at pH 7.4 and 30 °C

All points are experimental. The solid line was computed using the binding parameters calculated from the data for intact BSA.

Table II. Synthesis of HNB-Modified BSA (HNB–BSA)

Exp. No.	BSA: HNB	Fluorescence[a] intensity	Remaining[b] Trp
1	1 : 25	1.29	0.06
2[c]		1.34	0.06
3		2.17	0.10
4		1.97	0.09
5		3.53	0.17
6		4.22	0.20
7[c]	1 : 10	8.14	0.38
8		7.73	0.36
9[c]	1 : 8	12.92	0.61
10	1 : 6	14.32	0.67
11	1 : 5	19.26	0.91
12	1 : 3	24.47	1.15
13[c]	1 : 2.4	27.27	1.28
14	1 : 2	23.89	1.12
15		29.09	1.37
BSA		42.51	2.00

a) $F_{375\text{-}340}$, arbitrary units.
b) $2 \times \frac{\text{(HNB–BSA fluorescence intensity)}}{\text{(BSA fluorescence intensity)}}$
c) These HNB–BSA preparations were used for equilibrium dialysis experiments (Fig. 4).

Modification of BSA with HNB——Horton[10] has reported that HNB shows preferential specificity for the tryptophan residue of protein molecules in acid solution. Hydrophobic forces play a role in binding specificity, and so the tryptophan residue in the BSA molecule may participate in the binding of TBM. The effect of HNB concentration on the remaining tryptophan residue, *i.e.*, the changes in fluorescence characteristics associated with HNB

binding to BSA, is shown in Table II. It is clear that the apparent amount of remaining tryptophan residues in the BSA molecule decreases with increasing HNB concentration.

Binding of TBM with HNB–BSA——Intact BSA and HNB–BSA with various amounts of intact tryptophan residues were used for binding with TBM. The Scatchard plots for TBM are presented in Fig. 4. As shown in Fig. 4, the plots for binding of TBM gradually fall below that of intact BSA with decrease of the apparent remaining tryptophan content of the BSA molecule. When it was almost zero, that is, when the hydrophobic region on the BSA molecule disappeared, the Scatchard plot became nearly linear, as shown in Fig. 4 (△). Mathematical analysis of the binding parameters was carried out and these values, the binding constants (K) and the maximum number of binding sites (n) are summarized in Table III. It is evident from Table III, that the secondary binding parameters of intact BSA and those of HNB–BSA (Exp. No. 13, tryptophan residue content almost 0) are almost identical. Therefore no clear difference in the binding of TBM at secondary binding sites could be demonstrated between BSA and HNB–BSA, though the tryptophan residue may be involved in TBM binding at the primary binding sites.

The binding parameters for primary sites obtained from the Scatchard plots, Fig. 5, of the binding of HSA and BSA–TBM are shown in Table III. Brown[6] demonstrated that

Table III. Binding Constants for Tolbutamide–Albumin at pH 7.4 and 30 °C

Albumin	K_1 $\times 10^5$ M^{-1}	n_1	K_2 $\times 10^3$ M^{-1}	n_2
BSA	2.49	0.75	5.65	5.14
HNB–BSA	—	—	5.45	4.65
HSA	2.06	0.90	3.56	3.45

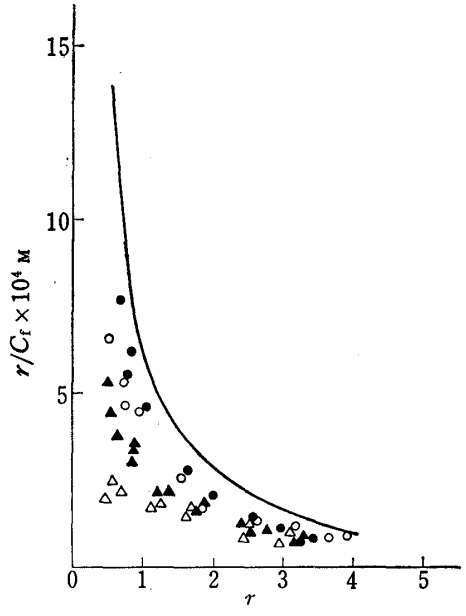

Fig. 4. Scatchard Plots for the Binding of Tolbutamide to HNB-BSA at pH 7.4 and 30 °C

The solid line was computed using the binding parameters calculated from the data for intact BSA. All points are experimental, and correspond to Exp. Nos. 2, 7, 9 and 13 in Table II. These are shown as △, ▲, ○ and ●, respectively.

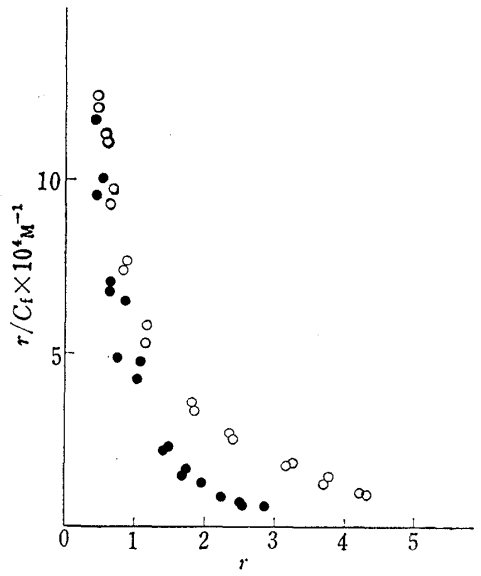

Fig. 5. Scatchard Plots for the Binding of Tolbutamide to HSA or BSA

Key: ○ BSA, ● HSA.

there are one and two tryptophan residues in HSA and BSA, respectively, and that one of the two tryptophan residues in the BSA molecule occupies the same position as the residue in the HSA molecule. The maximum binding number was approximately one in this experiment. The above results suggest that the hydrophobic region (intimately related to the binding site of TBM) on the albumin molecule is located in the same position in the HSA and BSA molecules. The possibility that conformational differences of the serum albumin molecule are induced by modification with the three chemicals must be considered. However, this was not investigated in the present study.

References and Notes

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Heterogeneity of Histamine N-Methyltransferase

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The distribution of histamine N-methyltransferase (HMT, EC 2.1.1.8) activity was studied in various tissues of the guinea-pig and rat. The properties of HMT in rat kidney were shown to differ from those of HMT in the brain and kidney of guinea-pig and the brain of rat. Quinacrine, a potent HMT inhibitor, inhibited the activity of HMT by 50% at concentrations of about 10^{-5} M in rat kidney and about 10^{-7} M in other tissues.

Keywords——histamine N-methyltransferase; guinea-pig and rat tissues; histamine methylation; quinacrine affinity; multiple form

Histamine N-methyltransferase (HMT, EC 2.1.1.8) occurs ubiquitously in the organs and tissues of animals,[2,3)] and its high degree of substrate specificity is well known.[2,4,5)] Studies on multiple forms of the enzyme and on the mechanism of histamine methylation, however, have yielded conflicting data, irrespective of species and tissue[3,6–11)] and therefore the properties of this enzyme are still uncertain. The purpose of the present work was to examine the heterogeneity of tissue HMT in common laboratory animals.