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Influence of Blood Proteins on Biomedical Analysis. II.[1] Interaction of Gliclazide with Bovine Serum Albumin

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The interactions of gliclazide, a potential hypoglycemic drug, with native and chemically modified bovine serum albumin (BSA) were studied by means of an equilibrium dialysis technique. The Scatchard plot for the interaction of gliclazide with native BSA was a hyperbola, suggesting the existence of two (or more) classes of gliclazide-binding sites on the BSA molecule ($n_1=0.5$, $K_1=160\times10^3\,\text{M}^{-1}$; $n_2=4.5$, $K_2=4.4\times10^3\,\text{M}^{-1}$). Total binding capacity ($\sum n_iK_i$) for gliclazide-BSA binding was lower ($99.8\times10^3\,\text{M}^{-1}$) than that ($190.7\times10^3\,\text{M}^{-1}$) for tolbutamide. Modification of BSA with hydrogen peroxide, iodoacetic acid, iodoacetamide or 2-hydroxy-5-nitrobenzyl bromide lowered the binding affinity in the primary binding site or destroyed the binding site, and decreased the total binding capacity of gliclazide-BSA binding. Since the binding capacities of the primary and secondary binding sites varied upon modification of cysteine, methionine, histidine and tryptophan residues of the BSA molecule, it is possible that the binding sites are both closely associated with loops 1—4 of the BSA molecule.

Keywords——hypoglycemic drug; gliclazide; bovine serum albumin (BSA); equilibrium dialysis; Scatchard plot; binding capacity for gliclazide-BSA binding; gliclazide-BSA binding; BSA binding parameter; chemically modified BSA

It has been established that the binding of sulfonylureas to circulating proteins, such as albumin or globulin, in the living body can influence their therapeutic activities.[2] In particular, the serum albumin can interact strongly with many sulfonylureas[3] and other drugs.[4] It has been confirmed to be one of the main carrier proteins for drugs.[4] It has been accepted that the pharmacological activity of sulfonylureas is potentiated by competitive displacement by other drugs, such as salicylic acid[5] or sulfonamides,[6a] from the binding sites on serum albumin. The characterization of the drug-binding sites on the serum protein, including estimation of the binding affinities, is pharmacologically important. Moreover, knowledge of the intrinsic protein-binding properties in the blood of drugs having a narrow

range of therapeutic doses should be useful from a clinical point of view.

Several binding parameters, *e.g.*, affinity constant, number of binding sites on the protein molecule or binding ratio in the interaction of serum proteins with sulfonylureas, have been studied.[6] However, it is not well understood how functional groups on the protein molecule are involved in the interaction with the drug.

In the present paper, we describe the interactions of native and chemically modified bovine serum albumin (BSA) with gliclazide, N-(4-methylphenylsulfonyl)-N′-(3-azabicyclo[3,3,0]-octyl)urea, which is a sulfonylurea compound recently evaluated as a potential hypoglycemic drug.

Experimental

Materials——A pure reference sample of gliclazide was purchased from Dainippon Pharmaceutical Industries Co., Ltd., Japan. Bovine serum albumin (fraction V) was obtained from Armour Laboratories, U.S.A. and cellophane tubing (Visking, Co., 20/32 inch inflated diameter) was used as a dialysis membrane. Hydrogen peroxide (H_2O_2, 30%), iodoacetic acid (IAA) and iodoacetamide [IA(NH_2)] were obtained from Wako Pure Chemical Industries Ltd., Japan. 2-Hydroxy-5-nitrobenzyl bromide (HNBB, Koshland's reagent) was obtained from Nakarai Chemical Ltd., Japan. All other chemicals were products (analytical reagent grade) of Wako Pure Chemical Industries Ltd., Japan.

Preparation of Modified BSA——Iodoacetic Acid(IAA)-treated BSA:[7] Ten ml of 1/15 M phosphate buffer (pH 7.4) containing BSA (2.72 g, 40 μmol) and IAA (0.3 g, 1.6 mmol) was stirred for 3 hr at room temperature; the pH was maintained at 9.5—10.0 by the addition of 6 N KOH. The reaction mixture was dialyzed overnight against 1000 ml of 1/15 M phosphate buffer (pH 7.4). The dialysate was used for experiments without further purification. The modification of cysteine residues (-SH) in the BSA molecule was confirmed by means of the Grote reaction.[8]

Iodoacetamide[IA(NH_2)]-treated BSA: Carboxymethylation of BSA with IA(NH_2) was performed by the same procedure as in the case of IAA-treated BSA.

Acetylated BSA:[9] BSA (2.72 g, 40 μmol) was dissolved in 10 ml of 1/15 M phosphate buffer (pH 7.4), and the reaction mixture was kept at 0° with constant stirring. Acetic anhydride (1 ml) was added, and the pH of the reaction mixture was maintained at 7.4—8.5 by the addition of 6 N KOH. The reaction was carried out for 3 hr, and then the solution was dialyzed overnight against 1000 ml of 1/15 M phosphate buffer (pH 7.4). The dialysate was used for experiments without further purification. H_2O_2-oxidized and HNBB-modified BSA were prepared according to the methods described in our previous report.[1]

Equilibrium Dialysis Method——Equilibrium dialysis as described by Hughes *et al.*[10] was used with several modifications.[1] The dialysis studies were done with 1×10^{-3} M protein. The basal solution, containing 400 μg/ml of gliclazide, was diluted to an adequate concentration with 1/15 M phosphate buffer (pH 7.4) for each experiment. One ml of the solution containing gliclazide 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 gliclazide solution at the same concentration as that inside the bag. After continuous shaking of the vial at 120 strokes per min for 3 hr at 37° in a water-bath, the concentration of gliclazide outside the bag was estimated from the optical density at the maximum absorption wavelength (225 nm) of gliclazide 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 gliclazide in the phosphate buffer (pH 7.4) was taken to be 12.5×10^3.

Calculation——The binding data obtained by equilibrium dialysis were analyzed according to Scatchard *et al.*[11] The following equation for a single class of n equivalent binding sites was used:

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

where r is the number of moles of bound drug per mole of protein, and K and [free drug] are the association constant for the drug-protein complex and the concentration of free drug (gliclazide), 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].[12]

$$r/[\text{free drug}] = \sum n_i K_i - \sum r K_i \qquad [2]$$

Here, the subscript i denotes the 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. The 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.

Results and Discussion

Conventional methods for equilibrium dialysis, including dynamic dialysis, require a long time (8—24 hr) for equilibration for the drug across the dialysis membrane.[3,6] However, under the present experimental conditions, the equilibration of gliclazide required only 2—3 hr, as shown in Fig. 1. Therefore, the following equilibrium dialyses were carried out for 3 hr.

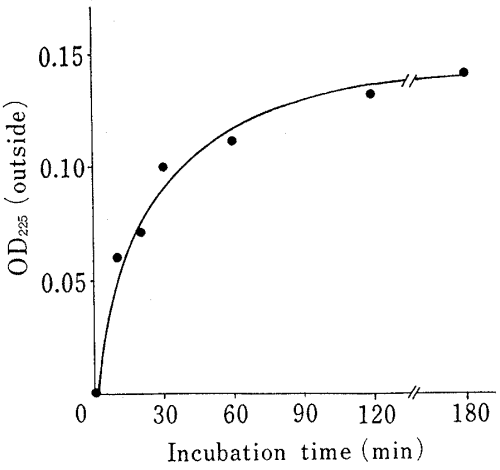

Fig. 1. Equilibration of Gliclazide with a Dialysis Membrane

The solution (1 ml), containing 20 μg/ml of gliclazide and 1 mM BSA, was taken into the dialysis bag. The bag was placed in a glass vial containing 9 ml of gliclazide solution at the same concentration at that inside the bag. The vial was shaken at 37°. Each point represents the mean value of two experiments.

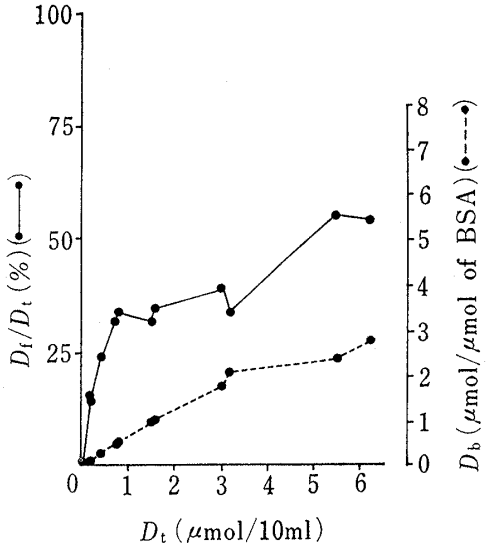

Fig. 2. Gliclazide-binding with BSA

D_t, D_b and D_f/D_t represent the total concentration of gliclazide in the medium (10 ml), the amount of gliclazide bound to BSA and the ratio (%) of free drug level to total drug level, respectively. Each point represents the mean value of two experiments.

Figure 2 shows the relationship between the ratio (D_f/D_t) of the free drug level to total drug concentration and the total drug concentration (D_t, μmol/10 ml). The binding ratio decreased as D_t increased up to 0.8 μmol/10 ml, and then kept a constant value untill about 3 μmol/10 ml. The binding ratio was 86—90% at a drug concentration (0.03—0.3 μmol/10 ml) corresponding to the blood level produced by an effective dose. This result suggests that the binding ratio of gliclazide to BSA is not constant but variable, depending on the relative amounts of drug and protein in the medium. Analogous findings have been reported in binding studies on other drugs, such as phenytoin or salicylic acid.[13]

Scatchard plots for the interaction of gliclazide with native and modified BSA are demonstrated in Fig. 3. The Scatchard plot for the interaction of gliclazide with native BSA was a hyperbola, like that of tolbutamide, suggesting the existence of two (or more) classes of gliclazide-binding sites on the BSA molecule.[6b,14] The interaction of gliclazide with H_2O_2-oxidized

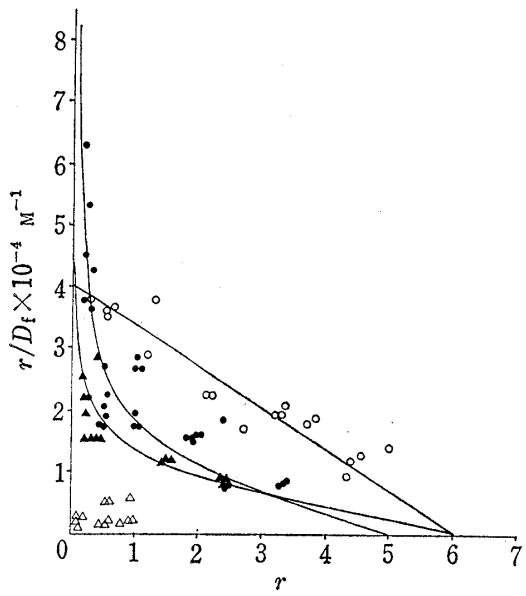

Fig. 3. Scatchard Plots for the Interactions of Gliclazide with Native and Modified BSA

●: native, ▲: H_2O_2-oxidized, ○: IAA-treated, △: acetylated.

BSA also gave a hyperbolic Scatchard plot, though its affinity constant in the primary binding site was greatly reduced. In the case of IAA-treated BSA, the plot was a simple straight line, suggesting the disappearance of the primary binding site for gliclazide. Acetylation of BSA with acetic anhydride largely removed the binding activity for the drug.

Several binding parameters for the interactions of gliclazide with native and modified BSA are summarized in Table I. H_2O_2-oxidation of cysteine and methionine residues in BSA reduced the binding capacities (nK) of the primary and secondary binding sites to $9.3\times10^3\,M^{-1}$ (11.6%) and $8.0\times10^3\,M^{-1}$ (40%) from 80×10^3 and $19.8\times10^3\,M^{-1}$ for native BSA, respectively. Modifications of cysteine, methionine and histidine residues in BSA with IAA or IA (NH_2), and of tryptophan with HNBB destroyed the primary binding site for gliclazide, whereas the binding capacity (n_2K_2) of the secondary binding site increased several-fold. Similar results were observed in the interaction between tolbutamide and IAA-treated BSA. The enhanced binding capacity of the secondary binding site may be derived from some configurational changes of the BSA molecule, which permits a facile approach of the drug to the binding site. This observation seems to be interesting in relation to the amplifying effect of IAA on glucose-induced insulin release from rat islets that is induced by modification of the sulfhydryl groups on the B cell membrane (hypothetical glucoreceptor).[15]

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

Modification	Binding parameter						
	n_1	K_1 ($\times10^{-3}\,M^{-1}$)	n_1K_1 ($\times10^{-3}\,M^{-1}$)	n_2	K_2 ($\times10^{-3}\,M^{-1}$)	n_2K_2 ($\times10^{-3}\,M^{-1}$)	Σn_iK_i ($\times10^{-3}\,M^{-1}$)
(Gliclazide)							
Native	0.5	160.0	80.0	4.5	4.4	19.8	99.8
H_2O_2-oxidized	0.3	31.0	9.3	5.7	1.4	8.0	17.3
IAA-treated	—	—	—	6.0	6.7	40.0	40.0
IA(NH_2)-treated	—	—	—	2.5	22.0	55.0	55.0
HNBB-treated	—	—	—	3.1	10.5	32.9	32.9
Acetylated	—	—	—	—	—	—	—
(Tolbutamide)							
Native	0.8	200.0	160.0	5.3	5.8	30.7	190.7
IAA-treated	—	—	—	4.0	15.0	60.0	60.0

IAA, iodoacetic acid; IA(NH_2), iodoacetamide; HNBB, 2-hydroxy-5-nitrobenzyl bromide.

The total binding capacity ($\sum n_iK_i$) in gliclazide-BSA binding was lower ($99.8\times10^3\,M^{-1}$) than that $190.7\times10^3\,M^{-1}$) of tolbutamide. This lower binding capacity for gliclazide may be attributed to a lower binding affinity (K_1) of the primary binding site on the BSA molecule. The total binding capacity in the interaction of gliclazide with modified proteins was lower than that with native BSA. Since the binding capacities of the primary and secondary binding sites varied upon modification of cysteine, methionine, histidine or tryptophan residues of the BSA molecule, it was speculated that both binding sites are closely ascociated with loop 1—4,[16] which include these four amino acid residues.

More recently, it was reported that tryptophan residues were associated with the binding of tolbutamide to BSA,[17] in accord with our present result. Goto and his co-workers demonstrated that 75% of BSA binding was hydrophobic at the primary binding sites in the interaction of several sulfonylureas with BSA and 90% of BSA binding could be explained by both hydrophobic and electrostatic interactions at the secondary binding sites.[18] Therefore, the binding mode of gliclazide with BSA may also involve both electrostatic and hydrophobic interactions.

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Synthesis and Evaluation *in Vitro* of 4-Acetamidophenyl Phosphate[1)]

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4-Acetamidophenyl phosphate was synthesized by a phosphorylation procedure with polyphosphoric acid to examine its properties as a prodrug of acetaminophen. Separation of the ester from the phosphorylation mixture was carried out by precipitation with organic solvents. 4-Acetamidophenyl phosphate was soluble in water, was practically tasteless, and was quickly hydrolyzed to acetaminophen by alkaline phosphatase at 37°. The ester was stable in aqueous solution at neutral and acidic pH at 37°.

Keywords——4-acetamidophenyl phosphate; phosphate ester; prodrug; synthesis; acetaminophen; polyphosphoric acid; hydrolysis; high performance liquid chromatography; gas chromatography; gas chromatography-mass spectrometry

Although acetaminophen lacks the anti-inflammatory effect of the salicylates, it is probably the analgesic-antipyretic of choice as an alternative to aspirin, particularly in patients allergic to aspirin or with a history of peptic ulcer.[2)] Although drops, elixir, and syrup are available[2)] for pediatric patients, the drug has a bitter taste.

In the present study, we synthesized a phosphate ester of acetaminophen, employing the phosphorylation procedures used for the synthesis of uridine-5′-phosphate.[3)] The ester was found to be very soluble in water, practically tasteless, and stable as a solid or in solution. Further, it was hydrolyzed quickly in the presence of alkaline phosphatase. Thus, the ester may be administered orally in the form of an aqueous solution to pediatric patients. Injections may also be prepared because of the high water-solubility and chemical stability of the ester in solution.