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Characterization of the Benzodiazepine Binding Site (Diazepam Site) on Human Serum Albumin

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The benzodiazepine-binding site (diazepam site) was investigated by studying the interaction between benzodiazepine and bilirubin in binding to human serum albumin (HSA). This paper reports some experimental observations supporting the assumption that the diazepam site on HSA is better described as the diazepam site–Site II overlapping area, consisting of the diazepam site and Site II. The fact that the binding of benzodiazepines to HSA was inhibited by Site II drugs but was not affected by bilirubin suggests that the diazepam site is the same as or very close to Site II, but is independent of the bilirubin site. When 1 or 2 mol of benzodiazepines per mol of HSA was added to bilirubin–HSA complex (0.6 : 1), the amount of bound bilirubin increased and the extrinsic Cotton effect of bilirubin–HSA complex was also strengthened. On the other hand, 1 or 2 mol of Site II drugs did not affect bilirubin–HSA complex. These apparently conflicting observations can be explained by assuming that the diazepam site and Site II overlap each other and the diazepam site acts cooperatively with the bilirubin site through an allosteric effect. The binding of benzodiazepines to the diazepam site of HSA may cause limited conformational changes around the bilirubin site.

Keywords——protein binding; binding site; human serum albumin; benzodiazepine binding site; diazepam site; bilirubin; bilirubin site; horseradish peroxidase; circular dichroism spectra

Albumin can bind, and thereby transport, various compounds such as fatty acids, bilirubin, tryptophan, steroids and a large number of other drugs. Drug interactions at the protein binding level will in most cases significantly affect the apparent distribution volume of the drugs and also affect the rate of elimination of the drugs.[1-4] The binding of a drug can be modified by the presence of other drugs or endogenous substances, which can compete for the same of the adjacent binding site(s). Therefore, exact knowledge of the location, size, and composition of the drug binding sites on the albumin molecule is important for effective therapy.

There have been many reports on drug-binding sites on human serum albumin (HSA).[5-11] Although the binding to HSA is generally assumed to be rather nonspecific, very specific binding sites have been demonstrated for some substances.

We have shown in previous studies that competitive displacement experiments with bilirubin and various drugs are very useful for the characterization and localization of the drug-binding site and the classification of drugs on the basis of binding site.[12,13] The results indicated that there are three different categories in the displacement of bilirubin by drugs, and the drugs could be classified into three groups, groups I, II and III on the basis of binding site. The primary binding sites of groups I and II drugs are called Sites I and II, which were characterized by Sudlow *et al.*[6] using the fluorescent probe 5-dimethylaminonaphthalene-1-sulfonamide and dansylsarcosine, respectively.

It is known that diazepam is bound primary to only one binding site on HSA, and its high-affinity site (diazepam site) is probably the same as Site II.[8,14] However, we were dubious about the identity of the diazepam site and Site II, because the previous study had

shown that there are considerable differences in displacing effect of bilirubin between diazepam and Site II drugs (flufenamic acid and mefenamic acid).[12] However, the previous study could not characterize the high-affinity binding site, since the experiments were carried out at a high molar ratio of drug to albumin.

In the present work, we attempted to characterize the high-affinity site of benzodiazepine, called the diazepam site, by examining the effects of benzodiazepines on the binding of bilirubin to HSA. These studies were carried out by using the equilibrium dialysis method, the peroxidase method and circular dichroism (CD) measurement.

Experimental

Materials——Bilirubin, horseradish peroxidase (type I), phenylbutazone and warfarin were purchased from Sigma Chem. Co. The benzodiazepine derivatives and other drugs were kindly provided by the manufacturers and were of JP grade: diazepam and chlordiazepoxide from Yamanouchi Seiyaku; nitrazepam, cloxazolam, mefenamic acid and flufenamic acid from Sankyo; medazepam from Shionogi; flunitrazepam from Nippon Roche. [*N*-Methyl-^{3}H]diazepam and [*N*-methyl-^{3}H]flunitrazepam were purchased from Amersham International plc. All other reagents were commercial products of special grade.

Human Serum Albumin——Crystalline HSA, essentially free of fatty acids, was obtained from Sigma Chem. Co. It contained a small amount of dimeric forms of HSA ($<1\%$) as estimated by polyacrylamide-gel electrophoresis. The molecular weight of HSA was assumed to be 66250 and the concentration was determined by using an extinction coefficient $E^{0.1\%}_{1.0\,cm}$ of 0.531 at 279 nm.[15]

Preparation of Bilirubin–HSA Solution (0.6 : 1)——HSA (198.75 mg) was dissolved in 45 ml of distilled water in a 50 ml volumetric flask, and the pH was adjusted to about 9.0 with 0.5 N NaOH. Bilirubin solution (1.8×10^{-3} M) was freshly prepared by dissolving bilirubin (5.3 mg) in 150 μl of 0.5 N NaOH and diluting the solution with 4.85 ml of distilled water. The bilirubin solution, 2.0 ml, was added to the albumin solution and mixed well, and the pH was adjusted to 7.4 with 1.0 N HCl. The stock bilirubin–HSA solution (3.6×10^{-5} M, 6.0×10^{-5} M, respectively) was prepared by making up the above mixed solution to 50 ml with distilled water. The molar ratio of bilirubin to albumin is 0.6. This solution was kept in the dark and stored in a refrigerator. Work with bilirubin-containing solutions was done in dim light, to avoid photodecomposition.

Equilibrium Dialysis Method——The binding of diazepam and flunitrazepam to HSA was determined by the equilibrium dialysis method using a 4 ml dialysis cell (Sanko Plastic Co.) and cellophane dialysis membranes (Union Carbide). The displacing effect of bilirubin, Site I and Site II drugs on the binding of two benzodiazepines to HSA was also estimated by the equilibrium dialysis method.

Radioactive drug dissolved in absolute ethanol was mixed with unlabeled drug to give suitable concentrations. HSA solution (3.0×10^{-5} M) was prepared in the same manner as bilirubin–HSA solution by the addition of distilled water instead of the bilirubin solution. Bilirubin–HSA solution (1.8×10^{-5} M, 3.0×10^{-5} M) was obtained by diluting the stock solution two-fold with 133 mM phosphate buffer (pH 7.4). This solution is stable for at least 32 h at room temperature. Site I drug– or Site II drug–HSA solution (3.0×10^{-5} M, 3.0×10^{-5} M) was prepared by using the above HSA solution.

In the apparatus, the chambers are divided by a dialysis membrane. Two mililiters of protein solution was poured into one compartment and 2 ml of benzodiazepine solution containing radioactive drug into the other. The chamber was gently shaken for 16 h at room temperaure, and aliquots of both sides were removed for radioactivity counting. Radioactivity was determined by liquid scintillation spectrophotometery.

The binding data were studied by Scatchard analysis.[16] The following equation for a single class of n equivalent binding sites was used:

$$r/D_f = nK - rK$$

where r is the number of mol of bound drug per mol of HSA, and K and D_f are the association constant for drug–HSA complex and the concentration of free drug, respectively.

Peroxidase Method——The experimental method, based on that of Jacobsen[17] and Brodersen,[18] was described in the previous report.[12,13]

Bilirubin is bound almost exclusively to one high-affinity site on the HSA molecule with a binding constant of about 10^8 M^{-1},[19] and the Michaelis constant is high compared with the free bilirubin concentration in the system. The determination of free bilirubin concentration was done by a kinetic technique, based upon oxidation of free bilirubin with hydrogen peroxide and horseradish peroxidase. Bilirubin is thereby converted to a nearly colorless substance. Bilirubin bound to albumin is not oxidized. Changes of the equilibrium concentration of unbound bilirubin on addition of various drugs were measured throughout the binding process and the rate of decrease of light absorption of bilirubin–HSA complex at 455 nm, which is close to the spectral maximum of bilirubin–HSA complex, was

measured. The velocity, v, of oxidation was determined with and without a test drug. The ratio of these velocities was equated with the ratio of free bilirubin concentration, b. The time, $t_{0.2}$, required for completion of the fraction 0.2 of the total process was measured in each experiment. The value of b/b_0 was plotted on the ordinate against the drug concentration, D, on the abscissa.

The procedure was as follows. The drug to be tested was prepared in ethanol. The stock solution of bilirubin–HSA complex was diluted two-fold with 133 mM phosphate buffer (pH 7.4). Two mililiters of bilirubin–HSA solution was placed in a 1.0 cm cell in a spectrophotometer with a thermostatic cell holder set at 37 °C, and then as little as 5.0 μl of ethanolic drug solution was added to minimize the effects of organic solvent on the binding of bilirubin and drug to albumin and on the enzyme reaction. Then 5.0 μl of 44 mM hydrogen peroxide was added to this mixture, and the absorbance was recorded at 455 nm. After 3 min, an aliquot of 25 μM peroxidase was added and the progress of the reaction was immediately monitored by recording the absorbance change. For this, a Hitachi 557 type spectrophotometer provided with a Haake circulator (model F2) was used.

Control experiments without albumin were performed at a bilirubin concentration of 1.2 μM (peroxidase 24 pM, 5 cm optical cell) to test the effect of the drug on enzyme activity, the formation of bilirubin–drug complex or the oxidation of the drug by hydrogen peroxide and peroxidase. The effects of the organic solvent were also checked, but were undetectable.

Circular Dichroism Measurement——CD measurements were made with an automatic spectropolarimeter (JASCO J-20) equipped with a model J-DPY data processor at room temperature. This instrument was calibrated with D-10-camphorsulfonic acid. The bilirubin–HSA solution for CD measurements was prepared by diluting the stock solution four times with 133 and 66.7 mM phosphate buffer (pH 7.4). Three mililiters of bilirubin–HSA solution was placed in a 1.0 cm optical cell and an aliquot of 15 mM ethanolic drug solution was added. The maximum concentration of organic solvent was 0.8%. The effects of organic solvent on the CD measurement were undetectable. The CD spectra were obtained by smoothing of 4 runs with the data processor.

Results

Quantitative Characterization of the Binding of Diazepam and Flunitrazepam to HSA

As a preliminary study of the benzodiazepine binding site on the HSA molecule, the bindings of diazepam and flunitrazepam to HSA were determined by the equilibrium dialysis method. Because of the relatively low solubility of these two benzodiazepines in aqueous media the binding study was performed at relatively low molar ratios of drug to albumin. Figure 1 shows the Scatchard plots of the binding of the two benzodiazepines to HSA. The binding constants (K) for diazepam and flunitrazepam at the primary binding site ($n=1$) are $2.5\times10^5\,\mathrm{M}^{-1}$ and $0.9\times10^5\,\mathrm{M}^{-1}$, respectively. These values for diazepam are close to those reported by Kober *et al.*,[20] who used a microparticle method and equilibrium dialysis: $n=1$ and $K=1.8\times10^5\,\mathrm{M}^{-1}$. Kragh-Hansen,[21] using ultrafiltration, found $n_1=1$ and $K_1=4.7\times10^5\,\mathrm{M}^{-1}$, and commented on the existence of secondary binding sites. These differences in binding constant may be due to the experimental conditions; however, there is only one high affinity site on HSA.

Figure 1 also includes the Scatchard plots for the two benzodiazepines in the presence of bilirubin. As can be seen bilirubin does not interfere with the binding of the benzodiazepines.

As shown in Fig. 2, the binding of diazepam and flunitrazepam to HSA generated extrinsic Cotton effects, which arise from the chromophore of the drug bound in an asymmetric environment on HSA. This band is located very close to the ultraviolet (UV) absorption maximum of the two benzodiazepines. The difference CD spectra of benzodiazepine–HSA complex at 1 : 1 molar ratio were not altered in the presence of bilirubin. These findings together suggest that the diazepam site is independent of the bilirubin site.

Effects of Sites I and II Drugs on the Binding of Diazepam and Flunitrazepam to HSA

In order to determine whether Sites I and II drugs affect the binding of benzodiazepines to HSA, competitive binding studies by the equilibrium dialysis method were performed in the presence of Sites I and II drugs. Figure 3 shows Scatchard plots which clarify the nature of the displacement of diazepam and flunitrazepam from albumin by Sites I and II drugs.

Bindings of diazepam and flunitrazepam to albumin in the presence of Site II drugs were

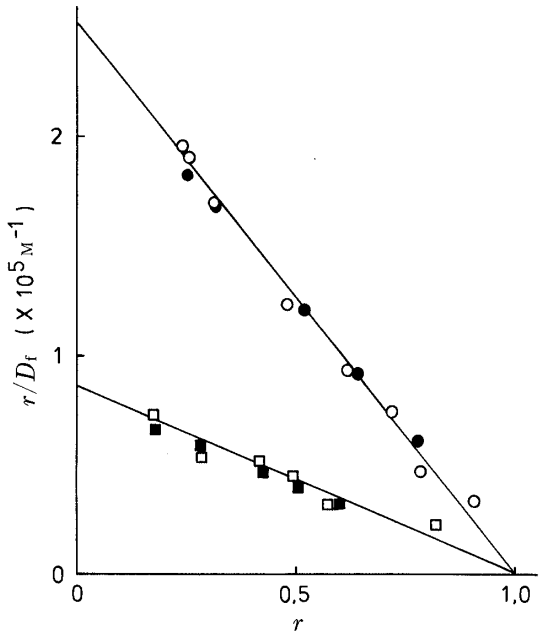

Fig. 1. Scatchard Plots of the Binding of Diazepam and Flunitrazepam to HSA (○, □) and to Bilirubin–HSA Complex (●, ■)

HSA concentration was 3.0×10^{-5} M. Bilirubin concentration was 1.8×10^{-5} M.

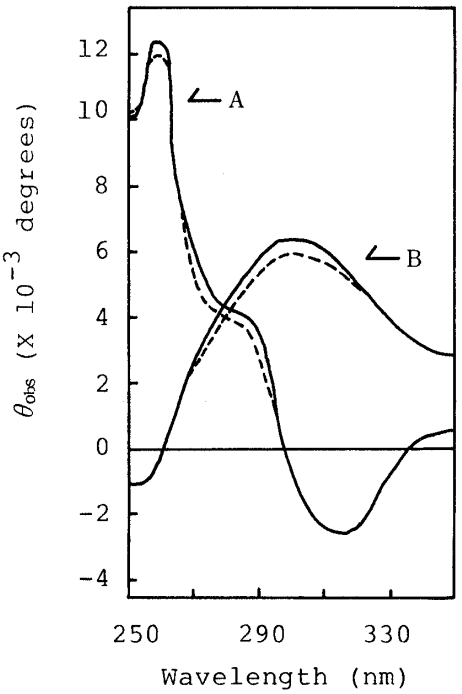

Fig. 2. Difference CD Spectra of Diazepam (A) and Flunitrazepam (B) Bound to HSA, in the Absence (——) and in the Presence (– – – –) of Bilirubin

The difference CD spectra were obtained after subtraction of the contribution from HSA itself. HSA concentration was 1.5×10^{-5} M. The molar ratios of benzodiazepine and bilirubin to HSA were 1.0 and 0.6, respectively.

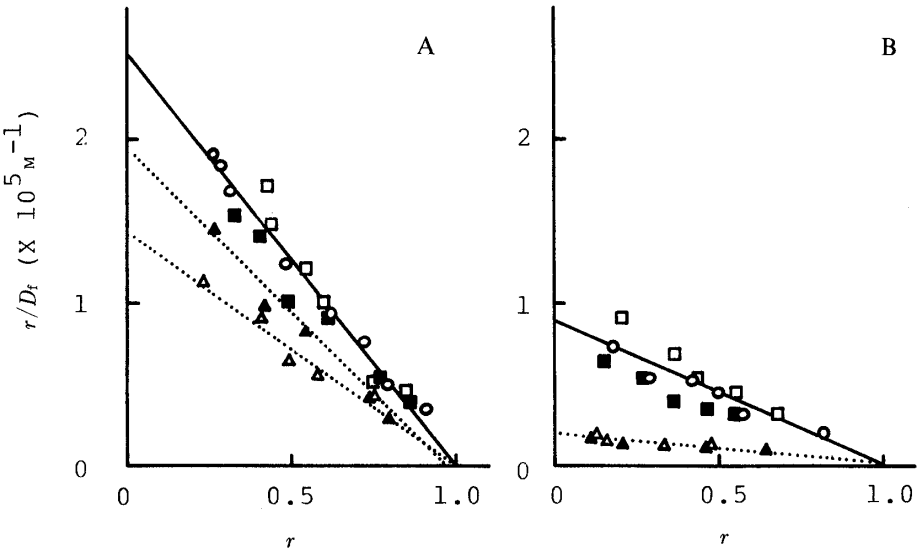

Fig. 3. Scatchard Plots of the Binding of Diazepam (A) and Flunitrazepam (B) to HSA in the Presence of Sites I and II Drugs

HSA concentration was 3.0×10^{-5} M. The molar ratios of Sites I and II drugs to HSA were 1.0.

○, alone; □, warfarin; ■, phenylbutazone; △, flufenamic acid; ▲, mefenamic acid.

The dotted lines show the binding of diazepam (A) and flunitrazepam (B) in the presence of Site II drugs. The dotted lines for binding of diazepam were constructed by using $n = 1.0$ and $K = 1.43 \times 10^5$ M^{-1} in the presence of flufenamic acid, and $n = 0.93$, $K = 1.95 \times 10^5$ M^{-1} in the presence of mefenamic acid. The dotted line for binding of flunitrazepam was constructed by using $n = 1.0$ and $K = 2.0 \times 10^4$ M^{-1} in the presence of Site II drugs.

diminished competitively. Clearly flufenamic acid displaced diazepam more effectively than mefenamic acid. In contrast, the bindings were not displaced by Site I drugs. These findings indicate that benzodizaepins and Site II drugs compete for a common high-affinity binding site on albumin.

Effects of Benzodiazepines on the Binding of Bilirubin to HSA

(1) By the Peroxidase Method——The effects of six benzodiazepines, Sites I and II drugs on bilirubin–HSA complex (0.6 : 1) were examined by the peroxidase method. The relative changes of the free bilirubin concentration caused by drugs are depicted in Figs. 4 and 5.

Addition of Site I drugs such as phenylbutazone and oxyphenbutazone resulted in an increase of the b/b_0 value with increasing concentration of these drugs (Fig. 4). In contrast, the effects of Site II drugs such as flufenamic acid and mefenamic acid were different. The b/b_0 values were little changed at low drug concentrations but were significantly changed at about 2-fold molar excess of drug over albumin. These results for Sites I and II drugs were in good agreement with those of the previous report.[13] On the other hand, as shown in Fig. 5, addition of benzodiazepines to the bilirubin–HSA complex resulted in a decrease of the b/b_0 value, that is, in a decrease of free bilirubin concentration. It should be noted that the concentration of free bilirubin was reduced in the presence of a sufficient amount of benzodiazepine to saturate almost completely the primary benzodiazepine-binding site (diazepam site). Maximal cooperativity of all six benzodiazepines was reached at the molar ratio of 4 for ligand to HSA. Medazepam was a potent cooperator for the binding of bilirubin to HSA.

Thus, addition of benzodiazepines produced remarkable relative changes of the free bilirubin concentration. The effects were clearly different from those of Sites I and II drugs.

(2) By CD Measurement——In order to further characterize the diazepam site, we used a CD spectropolarimeter and studied the spectral changes of bilirubin–HSA complex induced by benzodiazepines. When a ligand binds to a protein, new extrinsic Cotton effects are

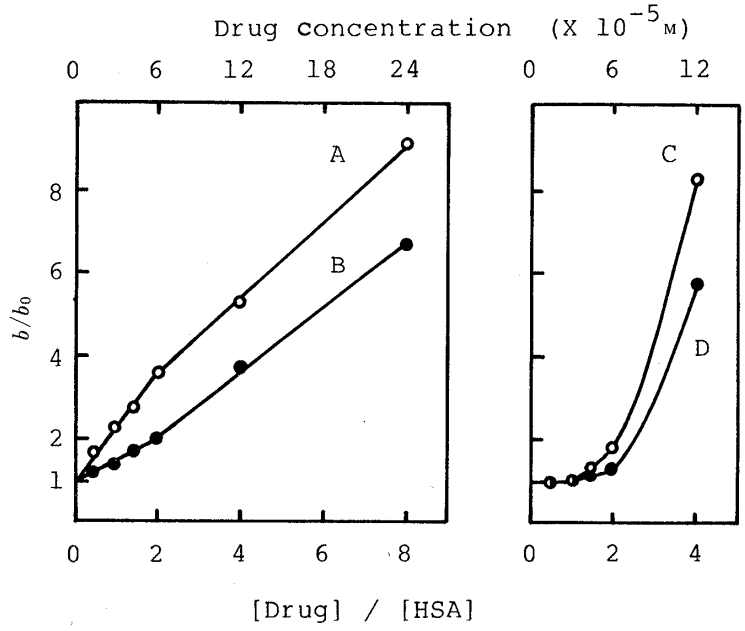

Fig. 4. Relative Concentration of Free Bilirubin in Bilirubin–HSA Complex with and without Sites I and II Drug as a Function of Drug Concentration

HSA concentration was 3.0×10^{-5} M. The molar ratio of bilirubin to HSA was 0.6. A, phenylbutazone (Site I); B, warfarin (Site I); C, flufenamic acid (Site II); D, mefenamic acid (Site II).

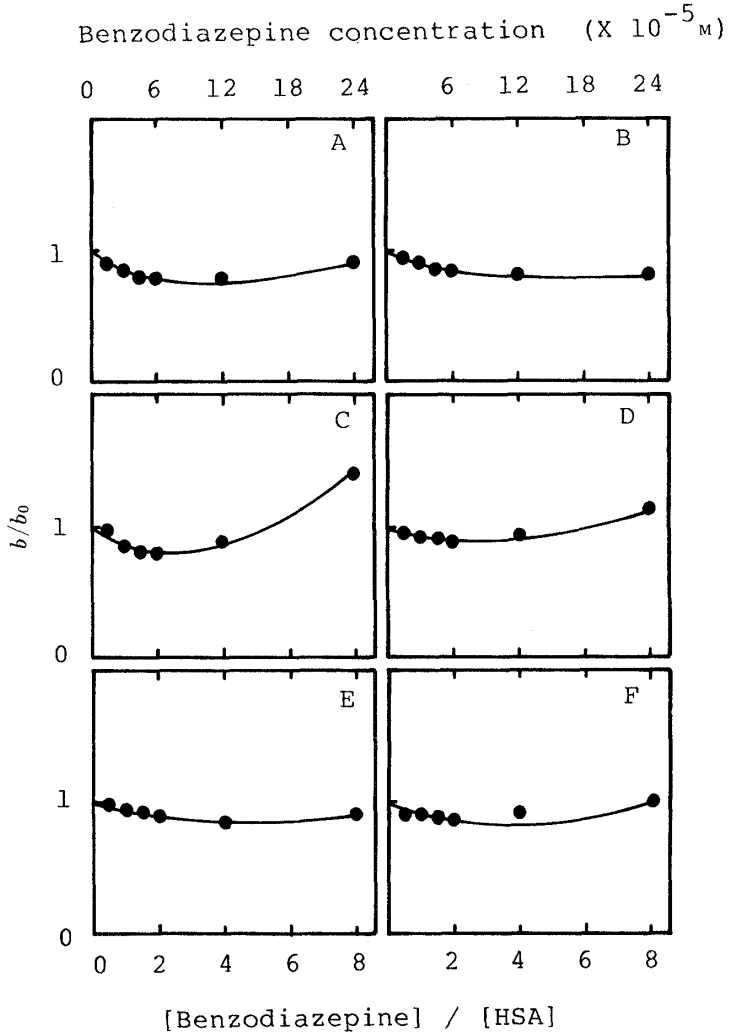

Fig. 5. Relative Concentration of Free Bilirubin in Bilirubin–HSA Complex with and without Benzodiazepine as a Function of Drug Concentration

A, diazepam; B, flunitrazepam; C, medazepam; D, chlordiazepoxide; E, nitrazepam; F, cloxazolam.

obtained at wavelengths where the ligand has absorption bands (360—510 nm). Figure 6 shows the absorption spectrum and the CD spectrum of the bilirubin–HSA complex. The bilirubin–HSA complex exhibited a CD spectrum with a negative band at 404 nm and a positive band at 458 nm. Under these conditions (low molar ratio of bilirubin to HSA), bilirubin is essentially bound only to the primary site. As bilirubin has no ellipticity of its own, the spectral changes can be fully ascribed to the extrinsic Cotton effects arising when bilirubin binds to HSA. Some of the substances used in these experiments generated their own optically active absorption bands because the chromophores were asymmetrically perturbed after binding to albumin, but these bands were in different spectral regions from the bilirubin-induced band.

Figure 7 illustrates the effects of diazepam and flunitrazepam on the extrinsic Cotton effect of the bilirubin–HSA complex (0.6 : 1). The spectral changes can be better seen in the difference spectrum obtained by subtracting the bilirubin–HSA complex spectrum from the spectrum induced by the coexisting drug. As benzodiazepines were added to the bilirubin–HSA complex, the difference CD spectra showed a steady increase in the intensity of a positive Cotton effect at about 458 nm and a negative Cotton effect at about 404 nm, with a

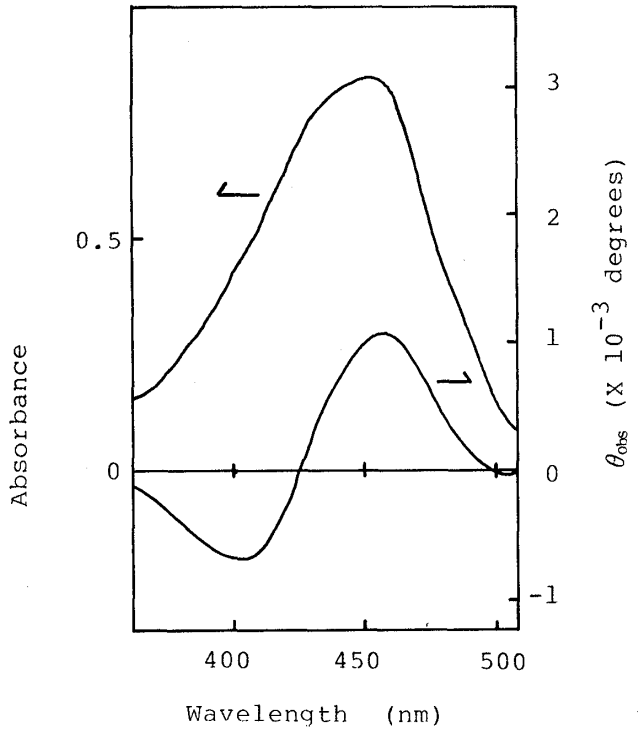

Fig. 6. Absorption Spectra and CD Spectra of Bilirubin–HSA Complex in the Visible Region

HSA concentration was 1.5×10^{-5} M. The molar ratio of bilirubin to HSA was 0.6.

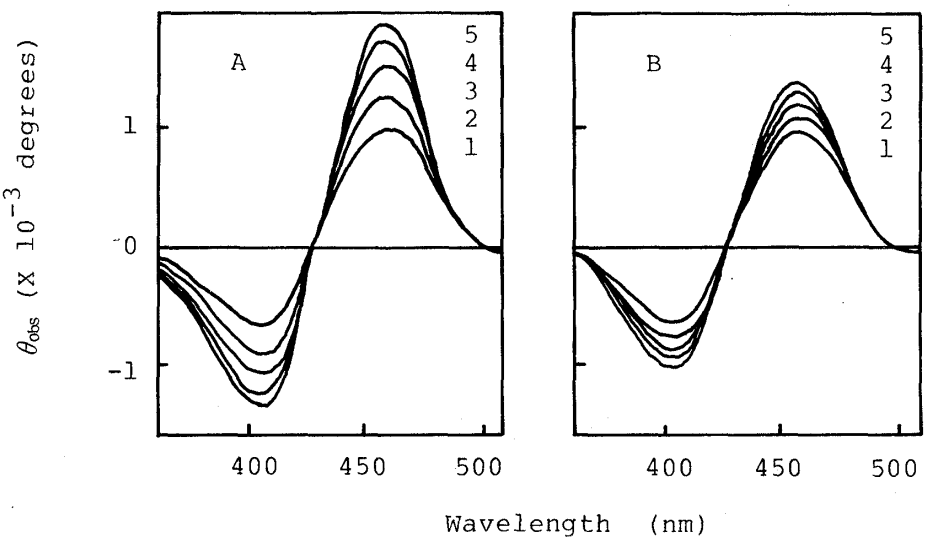

Fig. 7. Change of CD Spectra of Bilirubin–HSA Complex upon Addition of Diazepam (A) and Flunitrazepam (B)

HSA concentration was 3.0×10^{-5} M. The molar ratio of bilirubin to HSA was 0.6. The molar ratios of benzodiazepines to HSA were (1) 0, (2) 1.0, (3) 2.0, (4) 4.0 and (5) 8.0.

crossover point at 426 nm. However, the spectra did not shift at molar ratios of less than 8 of drug to HSA. These results suggest that the binding of benzodiazepines causes the asymmetrical perturbation of bound bilirubin.

As shown in Figs. 8 and 9, difference ellipticities at 458 nm were plotted against the molar ratio of drug to HSA. Progressive addition of Site I drugs resulted in a progressive decrease in the intensity of CD bands. In the case of Site II drugs, the induced ellipticity of bilirubin–HSA complex (0.6 : 1) at 458 nm showed little change on addition of up to 2 mol of drugs per mol of HSA, although it was decreased when further drugs were added. This reduction may depend

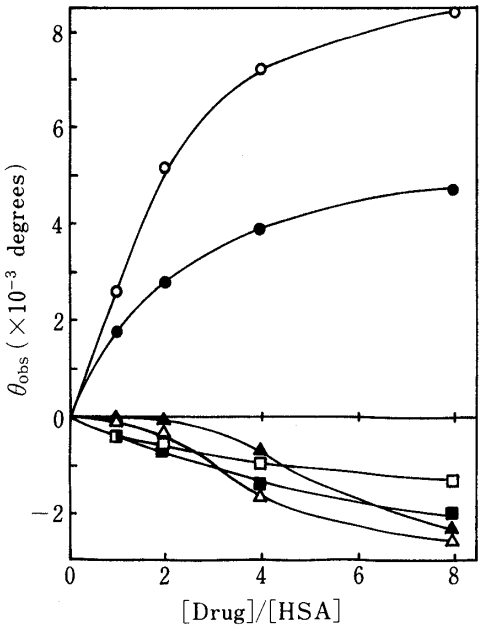

Fig. 8. Plots of Differential Ellipticity at 458 nm for Bilirubin–HSA Complex upon Addition of Site I drug, Site II drug, Diazepam and Flunitrazepam

—□—, warfarin (Site I); —■—, phenylbutazone (Site I); —△—, flufenamic acid (Site II); —▲—, mefenamic acid (Site II); —○—, diazepam; —●—, flunitrazepam.

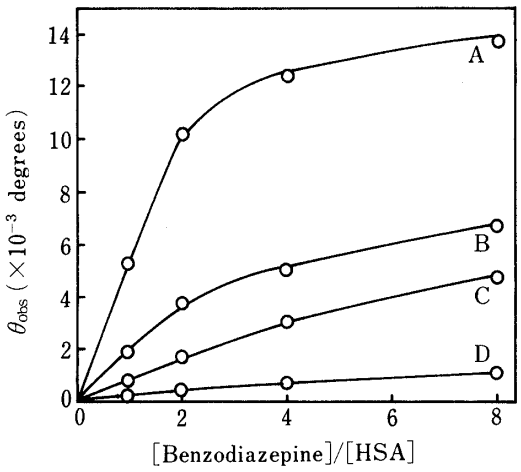

Fig. 9. Plots of Differential Ellipticity at 458 nm for Bilirubin–HSA Complex upon Addition of Benzodiazepines

A, medazepam; B, chlordiazepoxide; C, cloxazolam; D, nitrazepam.

on a diminished binding of bilirubin to albumin, as judged from the results of the peroxidase method (Fig. 4).

On the other hand, addition of benzodiazepines resulted in an increase of CD ellipticities of the bilirubin–HSA complex over the whole range. When benzodiazepines were added in 4-fold molar excess, the changes of the extrinsic CD spectra reached plateau levels. These findings, together with the results of the peroxidase method might suggest that the enhancement of ellipticity at 458 nm is due to the increasing amount of bound bilirubin. However, there is no parallel relation between the enhancement of the extrinsic Cotton effect and the increasing amount of bound bilirubin. Therefore, the increasing ellipticity may be caused by both a change of the rotational strength of bilirubin–HSA complex and an increased binding of bilirubin to albumin. The larger effects obtained with medazepam are in good agreement with the result in the peroxidase experiments.

Discussion

A number of relatively selective binding sites for various ligands have been shown to exist on HSA. These binding sites include Sites I and II for drugs, as well as a relatively specific site for bilirubin and fatty acids.[22,23] Site I on HSA showed affinity for bulky heterocyclic molecules with a negative charge and Site II for aromatic carboxylate anions. Digitoxin seems to bind to another drug binding site.[8]

Jacobsen[17,19] showed that the major bilirubin binding sites on HSA are of two classes, the first containing a single site (bilirubin site) at which bilirubin is bound with an association constant of $1.4 \times 10^8\ \text{M}^{-1}$, and the second containing two sites with corresponding constants of $5.0 \times 10^5\ \text{M}^{-1}$. It is known that the main part of the bilirubin site is located within the

second domain in loop 4, including Lys-240.[24-26] In the previous study, we showed that bilirubin was a suitable marker for characterization of the drug binding site on HSA.[12,13] It has been demonstrated that the bilirubin site is very close to Site I, but is independent of Site II.

Competitive binding studies with a benzodiazepine (diazepam or flunitrazepam) or Site II drug (flufenamic acid or mefenamic acid) revealed that the two ligands compete for a common high-affinity site (Fig. 3). Sjöholm *et al.*[8] have shown that diazepam was competitively displaced by Site II drugs, *e.g.* ethacrynic acid, ibuprofen and dansylsarcosine, using HSA immobilized in macroporous microparticles of polyacrylamide. Moreover, the literature contains supporting evidence for the competitive binding of diazepam and Site II drugs.[14] It is, therefore, suggested that the diazepam site is identical or very close to Site II. However, in the present study we observed both qualitatively and quantitatively different effects between benzodiazepine and Site II drugs for the binding of bilirubin to HSA.

The peroxidase method and the CD spectropolarimetric method are utilized for investigating competitive binding of bilirubin and various drugs to HSA. In studies by the peroxidase method, addition of diazepam to bilirubin–HSA complex (0.6 : 1) produced remarkable features in terms of the relation of the b/b_0 value with added concentration of drug, giving results which were clearly different from those of Sites I and II drugs (Figs. 4 and 5). At 1 mol of ligand per mol of HSA, the binding of bilirubin to HSA was increased cooperatively by all benzodiazepines, but was not affected by Site II drugs. The linear relation in the case of Site I drugs is compatible with competitive binding of Site I drugs to the bilirubin site on the HSA molecule. On the other hand, the finding that the first two molecules of Site II drugs could be bound to HSA without interfering with the binding of bilirubin, indicates that Site II is independent of the bilirubin site. These findings indicate the existence of cooperative binding between the bilirubin site and the diazepam site.

Furthermore, in studies by the CD method, benzodiazepine produced different effects on the CD spectrum of bilirubin–HSA complex as compared with Site II drugs (Figs. 8 and 9). Progressive addition of benzodiazepine to the bilirubin–HSA complex produced a steady increase in the extrinsic Cotton effects, as would be expected for the purely additive effect of saturating a single site. On the other hand, addition of 2 mol of Site II drug had little effect on the ellipticity of the complex, though further addition reduced the CD intensity. Differences in the two binding sites of Site II drugs are, therefore, easily recognized. In contrast, Site I drugs decreased the intensity of ellipticity of bilirubin–HSA complex. This may be due to displacement of bilirubin from the bilirubin site. These effects were more clearly defined in the case of the peroxidase method (Fig. 4).

Bilirubin became optically active upon binding to albumin (Fig. 6). Bilirubin in buffer solution is not optically active. These extrinsic Cotton effects were very sensitive to conformational and ionic changes around the chromophore, and so may be a sensitive probe for the region of the binding site. This method is direct, and not subject to the ambiguities and technical problems inherent in absorption spectroscopy, equilibrium dialysis using membranes, or gel filtration and related methods which measure the release of bound bilirubin or binding of competitive ligand without regard to the specific nature and site of the interaction. In addition, when CD methods are used, binding is viewed in terms of asymmetric alignments of interacting species, and thus a unique competitive mechanism may be elucidated.

Under the present experimental conditions, bilirubin binding should occur almost entirely at the primary binding site, and the simple assumption can be made that the entire Cotton effect arises from a special spatial arrangement of the bilirubin at an asymmetric locus on the binding site rather than from a perturbation of the bilirubin chromophore at the site. The strong enhancement of the CD spectrum of bilirubin–HSA complex caused by addition of benzodiazepines is probably due to both the increase of the amount of bound bilirubin and

asymmetric conformational changes in the bilirubin binding site. However, the combined results of the peroxidase method and CD measurement indicated a stronger contribution of asymmetric conformational changes.

The present results suggest that the diazepam site and Site II are the same or are very close to each other, but the diazepam site is independent of the bilirubin site. On the other hand, it was found that the binding of benzodiazepine to the diazepam site could increase the binding capacity of HSA for bilirubin, whereas binding of a Site II drug to Site II on HSA did not affect the bilirubin binding. These findings together indicate that the benzodiazepine and Site II drug binding sites may not be identical.

These observations can all be explained by assuming a diazepam site–Site II overlapping area. The apparent differences (see Figs. 4, 5, 8 and 9) seem to be due to the effects of possible conformational changes of the albumin molecule accompanying binding of benzodiazepine. Benzodiazepine may introduce different conformational changes depending on differences in the location of the binding site and in the association constant. The observed competitive interaction between diazepam and Site II drugs (Fig. 3) may be due to conformational changes induced by diazepam.

There are several reports concerning the location of the diazepam site on HSA.[5,27] Sjödin *et al.*[28] demonstrated that a part of the diazepam site must be located in a large trypsin-resistant fragment of HSA (residues 182—585) because this fragment is still able to bind diazepam, though the affinity is much weaker. Tyr-411 in HSA was concluded to be a part of the diazepam site, based on the pronounced reduction of the binding of diazepam after its selective modification with tetranitromethane.[29] Thus, the diazepam site must be formed by the HSA tertiary structure.

The apparent cooperativity of binding of benzodiazepine and bilirubin suggests the existence of site-to-site interaction (diazepam site → bilirubin site) with an accompanying local allosteric effect on the bilirubin site. The one or two molecules of benzodiazepine may have an allosteric effect on the binding of bilirubin to the bilirubin site. The data of the present study strongly suggest that the bilirubin site is independent of the diazepam site–Site II overlapping area but that the diazepam site acts cooperatively on the bilirubin site with a site-to-site effect. It is clear that detailed investigations should be made before a decrease in binding of a ligand caused by the presence of another ligand can be interpreted as binding to a common binding site.

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