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Effects of Fatty Acid on the Specific Drug-Binding Sites of Human Serum Albumin

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The effects of various fatty acids on the ligand-binding sites of human serum albumin (HSA) were investigated. The present results provide evidence for the independence of the four classified binding sites and for the existence of distinct high-affinity sites for long-chain (C_{12}—C_{18}) and for medium-chain (C_6—C_{10}) fatty acids. The binding of long-chain fatty acids to HSA at low molar ratios (0.5—2.0) of fatty acid to HSA caused marked conformational changes. Site I and the bilirubin-site were sensitive to these conformational changes: Site I became asymmetrical and the bilirubin-site acquired a cooperative nature. On the other hand, the diazepam-site and Site II were little affected. The binding of medium-chain fatty acids to HSA at low molar ratios (*vide supra*) caused competitive ligand displacement. These acids did not change the conformation of HSA, but they perturbed Site II to a limited extent, making Site II more asymmetric. These effects enhanced the circular dichroism spectrum of flufenamic acid–HSA complex. These results confirmed that the diazepam-site and Site II are distinct sites.

At a high molar excess (4.0—8.0) of fatty acid to HSA, the binding of ligands to HSA was inhibited in a complicated manner that appeared to reflect a combination of competitive effects and conformational changes.

Keywords——protein binding; albumin; human serum albumin; binding site; fatty acid; CD spectrum

Albumin is the physiological carrier of fatty acids, lysolecithin, and bilirubin and it also carries many hydrophobic drugs.[1] Previous reports in this series have suggested the existence of three specific binding sites for drugs on human serum albumin (HSA).[2-4] The three sites have been identified by estimating the interaction of bilirubin and various drugs by means of the peroxidase method and circular dichroism (CD) measurement. The primary binding site of bulky heterocyclic molecules such as warfarin, phenylbutazone and glibenclamide is called Site I, and that of aromatic carboxylate anions such as flufenamic acid, ibuprofen and ethacrynic acid is called Site II. Further, the benzodiazepine-binding site is distinct from the above two sites, and is called the diazepam-site.[4] However, present knowledge does not allow a complete assignment of the high-affinity binding regions for different drugs on albumin.

In vivo, the main fatty acids bound to albumin are long-chain fatty acids such as oleate, palmitate, linoleate and stearate.[5,6] However, the protein also binds with relatively high affinity fatty acids having shorter chain lengths.[7,8] It is known that the binding of various drugs to HSA is altered in the presence of fatty acid.[9-11]

The aim of the present study was to obtain information about the drug-binding sites, through the estimation of the influence of various fatty acids on drug binding to HSA. The following five ligands were used as representative ligands for the presumed four independent binding sites: warfarin and phenylbutazone for Site I; flufenamic acid for Site II; diazepam for

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the diazepam-site; bilirubin for the bilirubin-site. Saturated fatty acids with chain lengths of C_6 (caproic acid) to C_{18} (stearic acid) were used. The binding studies were carried out by the equilibrium dialysis method and CD measurement. Fluorescence measurement was also used for warfarin. In the case of bilirubin, the peroxidase method was used instead of the equilibrium dialysis method.

As the ligands used in this study have no intrinsic ellipticity, the induced CD spectra can be ascribed wholly to the extrinsic Cotton effects arising when these ligands are bound to HSA. The CD spectrum of ligand–HSA complex is generated in the wavelength region where the ligand absorbs light. Namely, the extrinsic Cotton effects appear because of the binding of the chromophore of the ligand in an asymmetrical environment on HSA.[12] Further, the CD spectrum of HSA itself in this region is probably not affected by the binding of ligands. Thus, the changes of the Cotton effects should be proportional to the concentration of the ligand–HSA complex, and should reflect the conformational changes of ligand-binding sites. Consequently, the extrinsic Cotton effects of ligand–HSA complexes can provide much valuable information on the binding of various small chromophores to the optically active macromolecule, since the CD spectra are very sensitive to the environment at the binding site.

Experimental

Materials——Bilirubin, horseradish peroxidase (type I), warfarin and phenylbutazone were purchased from Sigma Chem. Co. Flufenamic acid and diazepam were kindly provided by Sankyo and Yamanouchi Seiyaku, respectively. The following saturated fatty acids were all from P–L Biochemicals Inc.: caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid and stearic acid. [*N*-Methyl-^{3}H] diazepam was purchased from Amersham International Plc. All other reagents were commercial products of special grade.

HSA——HSA (essentially fatty acid-free, less than 0.005%; prepared from Fraction V human albumin) was obtained from Sigma Chem. Co. It contained a small amount of dimeric forms of HSA (less than 0.5%) as estimated by polyacrylamide-gel electrophoresis. The molecular weight was taken as 66500.[13] The concentration of albumin solutions was checked spectrophotometrically at 279 nm using an extinction coefficient of $E_{1\,\mathrm{cm}}^{0.1\%}$ of 0.531.[14]

Solutions——All binding experiments were conducted with media containing 0.1 M sodium phosphate buffer, pH 7.4. The concentration of HSA was 3×10^{-5} M. The molar ratio of each drug to albumin was kept constant at 1.0, while in the case of bilirubin, the ratio was kept at 0.6. Radioactive diazepam dissolved in ethanol was mixed with unlabeled diazepam to give suitable concentrations. Under the present conditions, the observed effects are mainly attributable to the interaction with the high-affinity binding site for the ligand. Fatty acids with different carbon lengths (C_6—C_{18}) were dissolved in absolute ethanol. The amount of ethanolic fatty acid solution added to ligand–HSA solution was always such that the final ethanol concentration was less than 0.5%, in order to minimize the effect of organic solvent on the binding of bilirubin and the drugs to albumin.

Binding Studies——The binding of drug to HSA in the presence and absence of fatty acid was determined by the equilibrium dialysis method as described previously.[4] The concentration of free drug was determined spectrophotometrically, except for that of radio-labelled diazepam, which was determined by liquid scintillation spectrophotometry with a toluene-Triton X-100 scintillator solution. The binding of bilirubin to HSA in the presence and absence of fatty acid was estimated by the peroxidase method. As described in the previous report,[3] the determination of free bilirubin concentration was done by a kinetic technique, based upon the oxidation of free bilirubin with hydrogen peroxide and horseradish peroxidase. Control experiments were carried out with addition of the same amount of ethanol only. Each measurement was performed in triplicate. The free fraction of ligands in the presence of fatty acids is presented as the ratio (f/f_0) with respect to that in the absence of fatty acid. Here f denotes the concentration of free ligand and subscript 0 indicates the absence of fatty acid.

CD Measurements——CD measurements were made with an automatic spectropolarimeter (JASCO J-20) equipped with a model J-DPY data processor (calibrated with *d*-10-camphorsulfonic acid) at room temperature. A CD spectrum was obtained by smoothing of 4 to 8 runs with the data processor. The effects of fatty acids on the induced CD spectra of ligand–HSA complexes were examined by measuring the difference CD spectra. Each difference CD spectrum was obtained by subtracting the CD spectrum of HSA alone from that of the ligand–HSA complex.

Fluorescence Measurements——The fluorescence of warfarin bound to HSA (3×10^{-6} M) was determined at excitation and emission wavelengths of 315 and 388 nm, respectively. The experiments were performed with a Hitachi 650-10S fluorescence spectrophotometer at room temperature.

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Results

Effects of Fatty Acid on Drug Binding to HSA

Bilirubin——The relative changes of the free bilirubin concentration induced by fatty acids are depicted in Fig. 1. In the concentration range of 0.5—2.0 mol of caprylic acid or capric acid per mol of albumin, a weak displacement of bilirubin was observed. Further addition of these acids caused a decrease in bilirubin binding, to give $f/f_0=1.58$ for caprylic acid or $f/f_0=2.48$ for capric acid in the presence of 8.0 mol of fatty acid per mol of protein. In contrast, the addition of 0.5—2.0 mol of lauric acid, myristic acid, palmitic acid or stearic acid per mol of albumin resulted in a pronounced increase of bilirubin binding. Higher concentrations of these acids markedly displaced bilirubin.

The relation between the carbon chain length of the added fatty acid and the binding of bilirubin to HSA is shown in Fig. 2; there are clear differences between the effects of medium-chain and long-chain fatty acids on the binding of bilirubin to HSA. These findings suggest that long-chain fatty acids have a cooperative effect. As shown by the value of $f/f_0=0.7$, lauric acid seems to have the strongest cooperative effect. The medium-chain fatty acids decreased the binding of bilirubin.

The bilirubin-HSA complex exhibited a CD spectrum with a negative band at 404 nm and a positive band at 458 nm, arising from the orientation of two dipyrromethene moieties of the bilirubin molecule.[15] Figure 3 illustrates the effects of the fatty acids on the extrinsic Cotton effect of bilirubin–HSA complex. Caprylic acid reduced the intensity of the CD spectrum of bilirubin–HSA complex without shifting the peak positions. However, the other fatty acids with longer carbon chains both decreased the intensity and induced shifts of the peak positions. These spectral changes of bilirubin–HSA complex are probably the result of conformational changes of the protein caused by binding of fatty acid.

These results suggest that the conformational changes of albumin caused by addition of 1.0—2.0 mol of lauric acid, palmitic acid or stearic acid per mol of albumin induce cooperativity in the binding of bilirubin to HSA, and/or an alteration in the direction of the

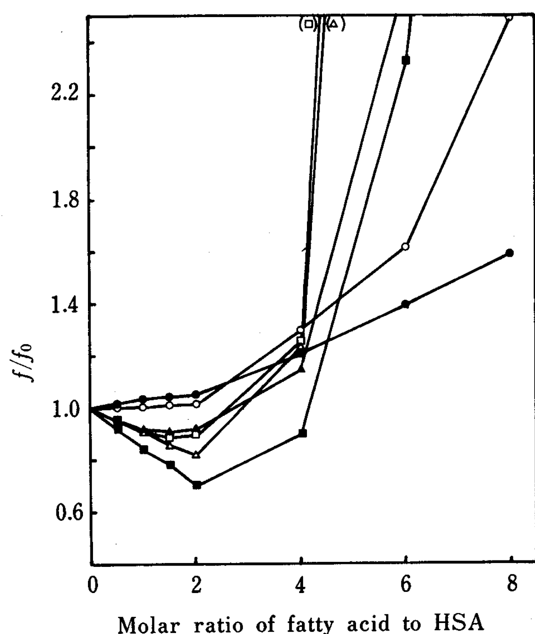

Fig. 1. Effect of Increasing Concentrations of Fatty Acids on HSA Binding of Bilirubin

Fatty acids used were caprylic acid (●), capric acid (○), lauric acid (■), myristic acid (△), palmitic acid (□) and stearic acid (▲).

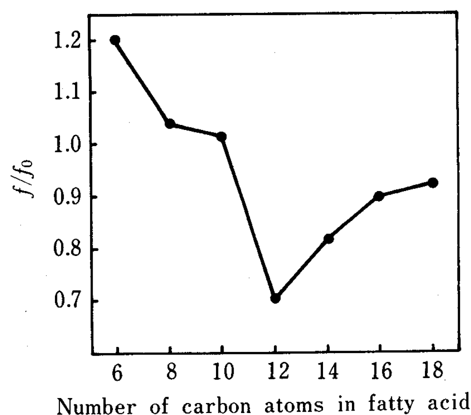

Fig. 2. Effect of Fatty Acid Chain Length on HSA Binding of Bilirubin with a Molar Ratio of Fatty Acid to Protein of 2 : 1

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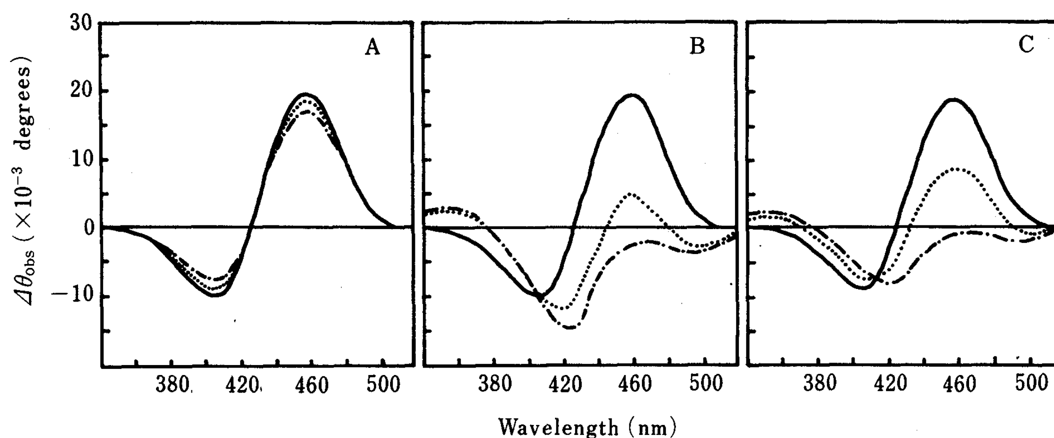

Fig. 3. Effect of Fatty Acids on the CD Spectra of Bilirubin–HSA Complex

Fatty acids used were caprylic acid (A), lauric acid (B) and stearic acid (C) with a molar ratio of fatty acid to albumin of 2:1 (------) and 6:1 (−·−·−).
The solid line is the result in the absence of fatty acid.

transition moment between the two interacting chromophores. On the other hand, it is suggested that caprylic acid and capric acid with shorter carbon chains have a competitive effect on the binding of bilirubin to HSA.

Warfarin——As shown in Fig. 4, addition of caprylic acid to the warfarin–HSA complex (1:1) resulted in an increase of the concentration of free warfarin with increasing concentration of this acid. Other medium-chain fatty acids such as caproic acid and capric acid gave similar results. The fluorescence intensity of warfarin–HSA complex was also reduced in proportion to the displacement of warfarin. In contrast, addition of stearic acid up to 4-fold molar excess over albumin slightly reduced the concentration of free warfarin. Similar results were obtained with myristic acid or palmitic acid. Warfarin fluorescence was enhanced markedly by addition of up to 2.0 mol of lauric acid or stearic acid per mol of albumin. Larger amounts of fatty acids reduced the fluorescence intensity.

The relation between the carbon chain length of fatty acid and the binding of warfarin is shown in Fig. 5. Medium-chain and long-chain fatty acids showed clearly different effects on warfarin binding. The fluorescence intensity was enhanced more markedly than the amount of bound warfarin. Addition of 2.0 mol of myristic acid strongly enhanced the binding of warfarin. Addition of lauric acid up to 2.0 mol per mol of albumin had no influence on the binding of warfarin to HSA.

The effects of various fatty acids on the CD spectra of warfarin–HSA complex are illustrated in Fig. 6. Warfarin–HSA complex gave a new Cotton effect at around 315 nm, where the ligand had absorption bands. Caprylic acid slightly decreased the CD intensity. At 2.0 mol of lauric acid per mol of albumin, a positive ellipticity around 315 nm could be seen, but at higher molar excess of this acid, the ellipticity was increased. The ellipticity around 280—295 nm was also increased. Addition of stearic acid gave a more positive ellipticity around 315 nm and a more negative ellipticity around 260—295 nm.

The observations (Figs. 4—6) that the binding of long-chain fatty acid to the primary site increased both the difference CD intensity and the fluorescence of warfarin–HSA complex, even though the change in warfarin binding was smaller, indicate that the conformation around the warfarin binding site (Site I) of albumin became more asymmetrical.

Phenylbutazone——The addition of 1.0—2.0 mol of fatty acids resulted in a displacement of phenylbutazone from albumin (Fig. 7(A)). The medium-chain fatty acids displaced phenylbutazone more effectively than the long-chain fatty acids. However, at a higher molar excess of lauric acid, palmitic acid and stearic acid, the binding of phenylbutazone was

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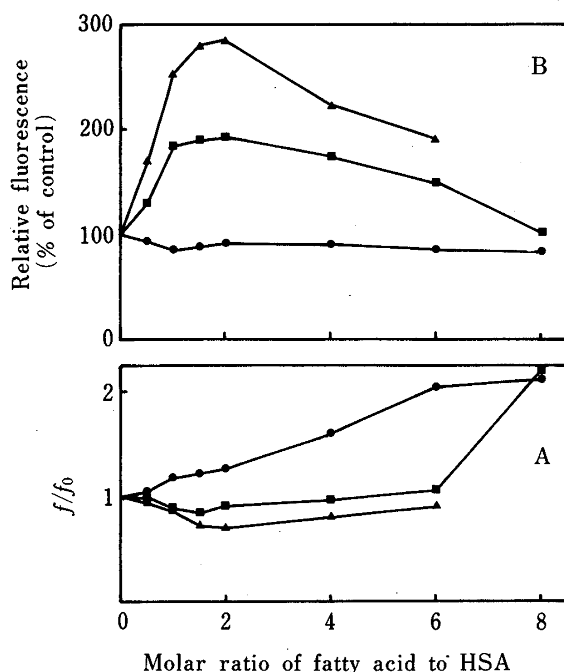

Fig. 4. Effect of Increasing Concentrations of Fatty Acids on HSA Binding of Warfarin

(A) changes of free warfarin concentration; (B) changes of fluorescence intensity of warfarin–HSA complex.

Fatty acids used were caprylic acid (●), lauric acid (■) and stearic acid (▲). The fluorescence intensity was measured at 388 nm for emission and at 315 nm for excitation.

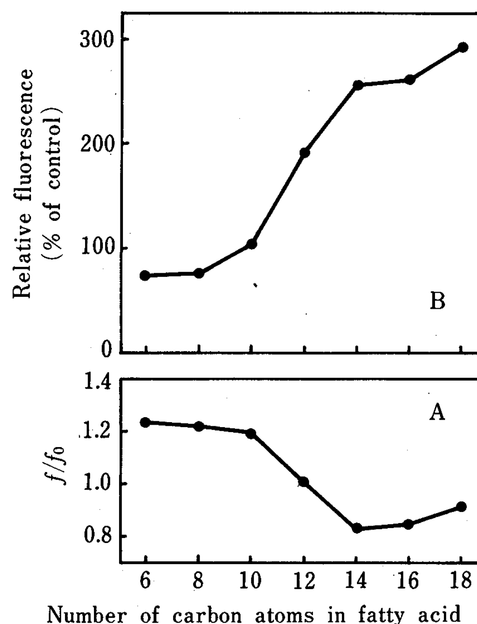

Fig. 5. Effect of Fatty Acid Chain Length on HSA Binding of Warfarin with a Molar Ratio of Fatty Acid to Protein of 2 : 1

(A) changes of free warfarin concentration; (B) changes of fluorescence intensity of warfarin–HSA complex.

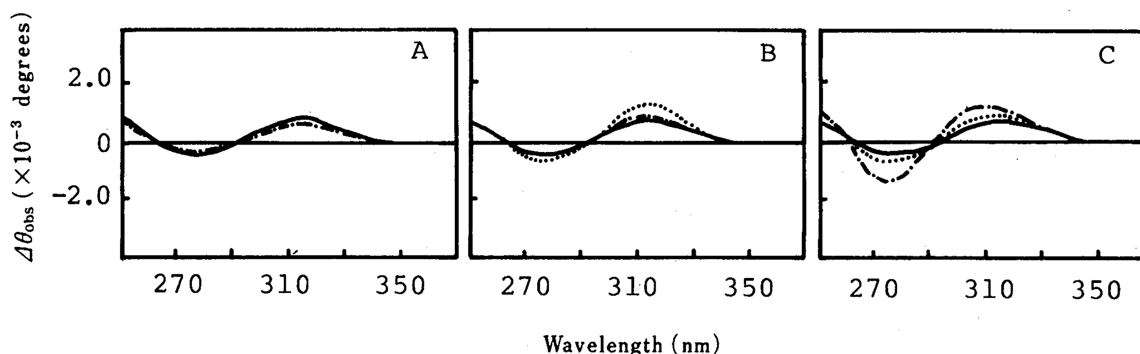

Fig. 6. Effect of Fatty Acids on CD Spectra of Warfarin–HSA Complex

Details and symbols are the same as in Fig. 3.

diminished more effectively. Stearic acid bound to the primary site had the least effect on the binding of phenylbutazone to HSA.

Phenylbutazone gave a strong extrinsic Cotton effect with a large positive ellipticity band at 288 nm when bound to HSA. As shown in Fig. 8, in the presence of caprylic acid the difference CD intensity of phenylbutazone–HSA complex (1 : 1) was markedly diminished. In contrast, addition of lauric acid and stearic acid enhanced the positive ellipticity at 288 nm of this complex. Stearic acid had stronger effects than lauric acid. These results were similar to those in the case of warfarin.

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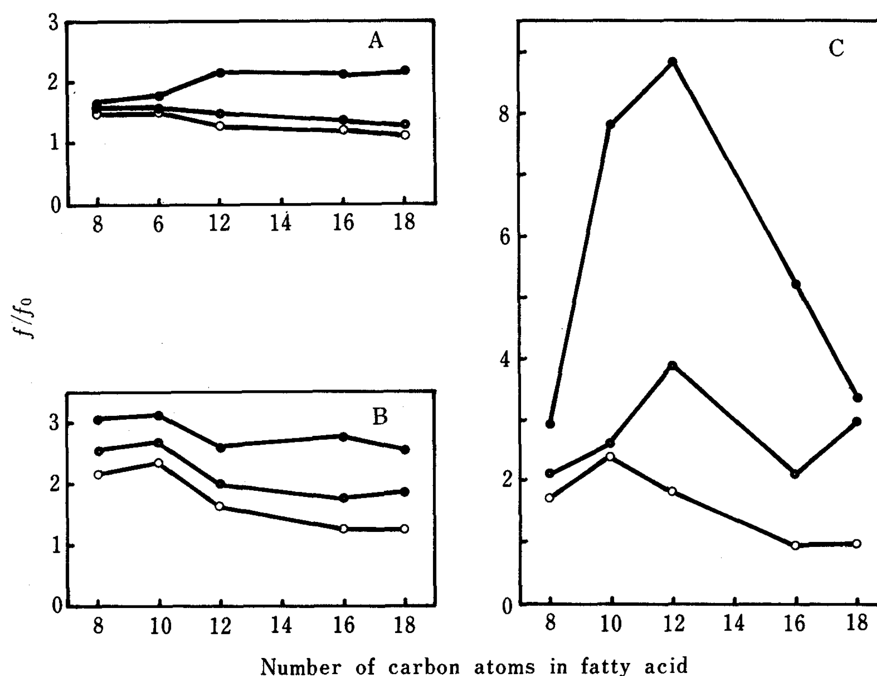

Fig. 7. Effect of Fatty Acid Chain Length and Concentration on HSA Binding of Drug with a Molar Ratio of Fatty Acid to Albumin of 1 : 1 (○), 2 : 1 (●) or 6 : 1 (●)

(A) phenylbutazone; (B) diazepam; (C) flufenamic acid.

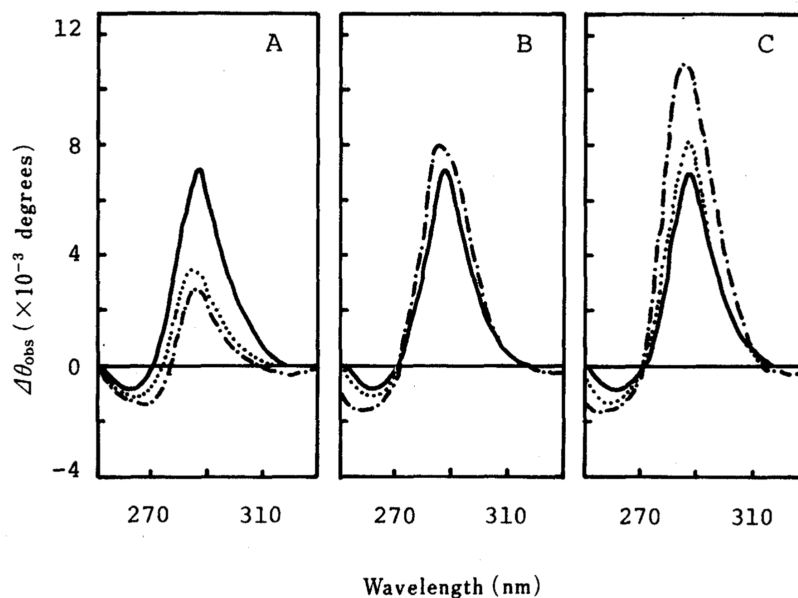

Fig. 8. Effect of Fatty Acids on CD Spectra of Phenylbutazone–HSA Complex

Details and symbols are the same as in Fig. 3.

The observation that the difference CD intensity of phenylbutazone–HSA complex increased in the presence of stearic acid although the binding amount of phenylbutazone was low indicates that the phenylbutazone binding site (Site I) is in an asymmetrical environment that is susceptible to conformational changes of the protein. Further, the results for warfarin and phenylbutazone suggest that they have a common binding site.

Diazepam——As shown in Fig. 7(B), the addition of 1.0—2.0 mol of caprylic acid or

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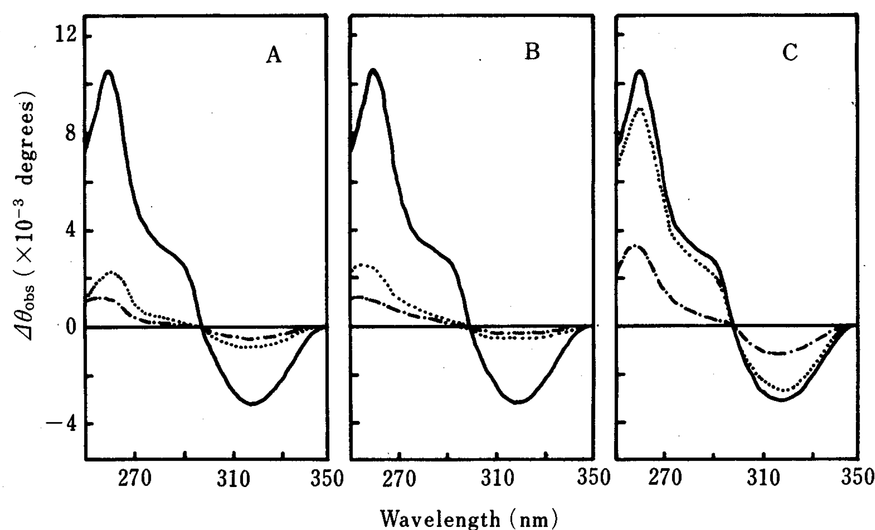

Fig. 9. Effect of Fatty Acids on CD Spectra of Diazepam–HSA Complex
Details and symbols are the same as in Fig. 3.

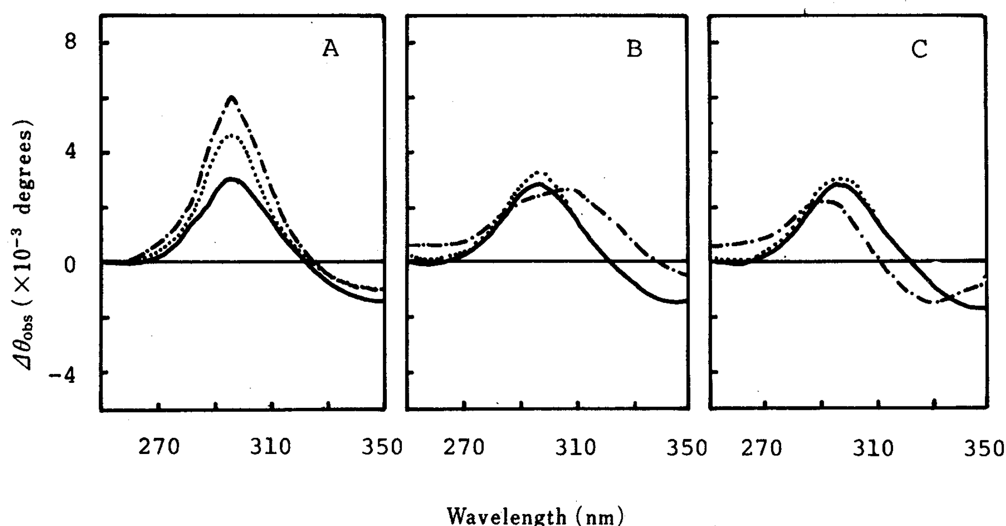

Fig. 10. Effect of Fatty Acids on CD Spectra of Flufenamic Acid–HSA Complex
Details and symbols are the same as in Fig. 3.

capric acid per mol of albumin resulted in a pronounced decrease in diazepam binding. Increasing the amount of the two fatty acids caused a small increase in the concentration of free diazepam. In contrast, addition of 1.0 mol of palmitic acid or stearic acid per mol of albumin resulted in only a small displacement of diazepam from HSA. The influence of lauric acid on the binding of diazepam is intermediate between those of the fatty acids with shorter and longer chain lengths at molar ratios less than 2.

Diazepam showed strong extrinsic Cotton effects when bound to HSA. The difference spectrum can roughly be characterized as biphasic with a positive and a negative extrinsic Cotton effect below and above 300 nm, respectively (Fig. 9). When caprylic acid or lauric acid was added at the molar ratio of two or six, the extrinsic Cotton effects were greatly diminished. These reductions may reflect decreased binding of diazepam to albumin, as judged from the equilibrium dialysis data (Fig. 7(B)). In contrast, when stearic acid was added at a molar ratio of two, the CD spectrum of the diazepam–HSA complex was little affected. However, addition of 6.0 mol of this acid per mol of albumin reduced the original extrinsic

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Cotton effects to half. These findings indicate that the diazepam-site is not affected by the conformational change induced by long-chain fatty acid, and is the same as the binding site for medium-chain fatty acids (caprylic acid and capric acid).

Flufenamic Acid——Flufenamic acid was displaced by caprylic acid, capric acid and lauric acid at low molar ratios per mol of albumin (Fig. 7(C)). However, the addition of 1.0 mol of palmitic acid or stearic acid per mol of albumin could not displace flufenamic acid, though higher concentrations of these acids had stronger displacement effects. Lauric acid markedly displaced flufenamic acid at higher molar ratios.

The flufenamic acid–HSA complex exhibited a positive CD band at 295 nm. As shown in Fig. 10, the difference CD spectrum was altered by fatty acids with different chain lengths. Increasing amounts of caprylic acid enhanced the CD intensity at 295 nm. When lauric acid or stearic acid was added at a molar ratio of two, the CD spectrum of the flufenamic acid–HSA complex was little affected. However, lauric acid and stearic acid induced complex changes at higher molar ratios. Lauric acid and stearic acid induce the conformational change of HSA in a different manner, which may reflect these differences in CD spectra. The enhancement of CD intensity of flufenamic acid–HSA complex, even though flufenamic acid was displaced, indicates that caprylic acid perturbed Site II, causing it to become more asymmetrical.

Discussion

A number of studies have shown that albumin undergoes a conformational transition (the N–B transition) in the pH range of 6—9. Wilting *et al.*[18] have shown that the N–B transition causes a change at Site I, resulting in enhanced binding and molar ellipticity of warfarin in the B-form, and Blauer *et al.*[19] have demonstrated an effect of pH on the CD spectrum of bilirubin–HSA complex. Thus, spectral analysis using warfarin and bilirubin is very useful for the estimation of the conformational alteration of HSA. However, Wanwimolruk and Birkett pointed out that the conformational change induced by addition of fatty acids was different from that induced by pH change.[20]

In previous reports, we have indicated that Site I is identical or very close to the bilirubin-site but is independent of Site II.[3] Further, it was found that the diazepam-site was different from Site II even though they may partially overlap.[4] Various reports have indicated that fatty acids with long-chain lengths (C_{12}—C_{18}) and fatty acids with medium-chain lengths (C_6—C_{10}) have different high-affinity binding sites.[21-23] Those reports also indicate that the primary binding site of long-chain fatty acids is located at loop 7 within domain 3,[21] whereas that of medium-chain fatty acids is located at loops 3—4.[24] The binding area of medium-chain fatty acid is the same as that of warfarin or bilirubin.[23,25] There are several reports concerning the location of the drug-binding site or the bilirubin-site on HSA. It is considered that the main part of Site I or the bilirubin-site is located within loop 4 in the second domain, including Lys-240, and the diazepam-site is within domain 3, containing Tyr-411.[25-29]

In the present investigation, the effects of various fatty acids on independent binding sites for ligands were studied. Although the patterns varied, the results provide good evidence for the independence of the four classified binding sites. Further, the present results strongly support the proposal of separate high-affinity binding sites for long-chain and medium-chain fatty acids.

All ligands used as representative ligands for the four independent binding sites were displaced by medium-chain fatty acids. The present results indicate that medium-chain length fatty acids compete with the binding of ligand, but have little effect on the conformation of HSA. However, our finding of enhanced CD intensity of the flufenamic acid–HSA complex (Fig. 10), in spite of large displacement (Fig. 9), suggests that medium-chain fatty acids cause local structural change at Site II. If overall conformational changes were induced, the other binding sites would also be affected. These results suggest that the diazepam-site and Site II

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are independent.

The binding of long-chain fatty acids to HSA causes marked conformational alterations. As lauric acid and stearic acid induce the conformational change of HSA in a different manner, the effects of either acid on the CD spectra of the ligand–HSA complex are apparently diverse (Figs. 3,6,8 and 10). Site I and the bilirubin-site are affected markedly by stearic acid. Since an increase of binding amount and decrease of the spectral shift of bilirubin are seen (Figs. 1 and 2), it seems likely that the bilirubin-site acquires cooperativity as a result of the conformational change induced by the binding of stearic acid. Further, the difference CD intensity of the complexes of Site I drugs with HSA was enhanced, although little change in binding amount occurred (see Figs. 4—8). Similarly, the fluorescence intensity of warfarin–HSA complex was also enhanced (Figs. 4(B) and 5(B)). These results indicate that Site I is transformed to a more asymmetrical form. On the other hand, Site II and the diazepam-site are little affected by long-chain fatty acids, except for a slight change of the CD spectrum. The present results are in accord with the observations of Wanwimolruk and Birkett, who concluded that the long-chain fatty acids do not bind to either Site I or Site II but exert different allosteric effects on the two sites.[20]

The results of this study have confirmed the independence of the four binding sites. It is clear from the present studies that Site I and the bilirubin-site are sensitive to conformational change of HSA induced by the binding of long-chain fatty acids, whereas the diazepam-site and Site II are not. Wanwimolruk and Birkett have already reported that conformational changes caused by pH or fatty acid modify Site I, but not Site II.[20] It is suggested that the binding of long-chain fatty acids to a part of domain 3, the primary binding site, induces conformational change of domain 2, including Site I and the bilirubin-site.

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