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FURTHER CHARACTERIZATION OF REVERSAL OF SIGNS OF INDUCED COTTON EFFECTS OF DICUMAROL DERIVATIVES–α_1-ACID GLYCOPROTEIN SYSTEMS BY PROTRIPTYLINE

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Abstract—The interaction of dicumarol derivatives and protriptyline with respect to the binding to α_1-acid glycoprotein (AGP) has been investigated by circular dichroism (CD), equilibrium dialysis and ultrafiltration. Investigation of the induced CD spectra of dicumarol derivatives bound to AGP indicated that the conformations of these compounds were different when bound to AGP. Though all the dicumarol derivatives, protriptyline and AGP formed a ternary complex, interaction modes were different, depending upon the substituent groups at position 3 of the dicumarol molecule. On the basis of the protriptyline effect on the CD spectra of all dicumarol derivatives bound to AGP, the compounds were classified in the following way: (1) Dicumarol, ethylidenebis 4-hydroxycoumarin and propylidenebis 4-hydroxycoumarin caused reversal of the sign of ellipticity. This interaction was explained by cooperative binding. (2) Butylidenebis 4-hydroxycoumarin and pentylidenebis 4-hydroxycoumarin generated new band and disappeared ellipticity of the original Cotton effect. This interaction was also explained by the cooperative binding mode. (3) Ethylbiscoumacetate which generated the CD band similar to that of dicumarol in the absence of protriptyline, reversed the sign of the CD spectrum only at 325 nm. The interaction was anticooperative in nature. (4) Benzylidenebis 4-hydroxycoumarin represented type four which had no change in the CD spectrum by the addition of protriptyline. This interaction was explained by the two-state model accompanying the conformational change of AGP. These results suggested that all compounds, except for benzylidenebis 4-hydroxycoumarin, induced negative Cotton effects at 325 nm by taking the same asymmetrical perturbation by the addition of protriptyline and the interaction was carried out according to model 2. An attempt to study the interaction mechanism of two or more drugs with regard to the binding to protein using these models is thought to help in understanding drug–protein interactions.

Human albumin and α_1-acid glycoprotein (AGP‡) are the important drug binding proteins in plasma [1, 2]. However, the binding characteristics of ligands to the two proteins seem to be qualitatively and quantitatively different from each other. For example, human albumin strongly binds weakly acidic drugs rather than basic drugs, whereas AGP is the main plasma protein for binding basic drugs. Moreover, AGP has been generally considered to have only one common drug binding site [3] in contrast to human albumin which has at least three drug binding sites, called the warfarin site, diazepam site and digoxin site [4].

Recently, we reported that AGP may have a wide and flexible drug binding area [5]. This hypothesis was further supported by the data obtained at our laboratory that tricyclic basic drugs such as chlorpromazine and protriptyline caused a reversal of the signs of the induced Cotton effects of the dicumarol–AGP system through ternary complex formation [6]. In addition, we found that the two hydroxycoumarin rings were structurally important for the inversion of the signs of the Cotton effects [6].

Thus, the present work was undertaken to further characterize the reversal of the signs of the Cotton effects of the dicumarol–AGP complex by protriptyline. For this purpose, we synthesized 3-substituted dicumarol derivatives and examined the effects of protriptyline on the induced CD spectra of dicumarol derivatives bound to AGP. Moreover, to clarify the interaction mode between dicumarol derivatives and protriptyline when bound to AGP, mutual displacements using equilibrium dialysis or ultrafiltration techniques were carried out.

MATERIALS AND METHODS

Materials

Human AGP was donated by the Chemo-Sera-Therapeutic Research Institutes (Kumamoto, Japan). The molecular mass of AGP was assumed to be 44,100 Da. AGP gave only one band in SDS–PAGE. Protriptyline was supplied by the Yoshitomi Pharmaceutical Co. (Fukuoka, Japan). Dicumarol

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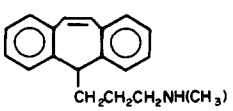
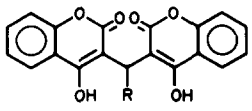
‡ Abbreviations: AGP, α-acid glycoprotein; CD, circular dichroism; compound **I**, dicumarol; compound **II**, ethylidenebis 4-hydroxycoumarin; compound **III**, propylidenebis 4-hydroxycoumarin; compound **IV**, butylidenebis 4-hydroxycoumarin; compound **V**, pentylidenebis 4-hydroxycoumarin; compound **VI**, ethylbiscoumacetate; compound **VII**, benzylidenebis 4-hydroxycoumarin.

Table 1. Chemical structure of protriptyline and dicumarol derivatives



Protriptyline



Dicumarol

Compound	Substituent (R)
I. Dicumarol	H
II. Ethylidenebis 4-hydroxycoumarin	CH_3
III. Propylidenebis 4-hydroxycoumarin	CH_2CH_3
IV. Butylidenebis 4-hydroxycoumarin	$(CH_2)_2CH_3$
V. Pentylidenebis 4-hydroxycoumarin	$(CH_2)_3CH_3$
VI. Ethylbiscoumacetate	$COOCH_2CH_3$
VII. Benzylidenebis 4-hydroxycoumarin	C_6H_5

was purchased from Tokyo Kasei. Ethylbiscoumacetate was a gift from Prof. L. H. M. Janssen of Utrecht University. Other compounds listed in table 1 (compounds **II–V**, **VII**) were synthesized according to the method of Sullivan *et al.* [7], and the compounds were identified and confirmed by melting point measurement, elemental analysis and proton nuclear magnetic resonance spectroscopic study. The purity of the synthesized compounds was above 99%. All other materials were of reagent grade and all solutions were prepared in deionized and distilled water. All protein and drug solutions were prepared in 0.067 M phosphate buffer, pH 7.4.

Methods

Circular dichroism (CD). CD measurements were made on a Jasco J-600 spectropolarimeter (Tokyo, Japan) using 10 mm cells and at 25°. The induced CD is defined as the CD of the drug–AGP mixture minus the CD of AGP alone at the same wavelength and is expressed in degrees. AGP, dicumarol derivative (compounds **I–V** and **VII**) and protriptyline solutions all of 10 μM were used in the CD experiments. In the case of compound **VI**, the concentrations used for AGP, compound **VI** and protriptyline were 50, 50 and 100 μM, respectively.

Equilibrium dialysis and ultrafiltration. Equilibrium dialysis experiments were performed using a Sanko plastic dialysis cell (Fukuoka, Japan). The two cell compartments were separated by Visking cellulose membranes. An AGP solution (10 μM, 1.5 mL) was poured into one compartment and 1.5 mL of drug solution (0.5–30 μM) was poured into the opposite compartment. After 6 hr dialysis at 25°, the drug concentration in each compartment was assayed by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi 655A variable wavelength UV monitor. Columns of LiChrosorb RP-18 (Cica Merk, Tokyo, Japan) for dicumarol derivatives and LiChrosorb CN (E. Merck, Darmstadt, Germany) for protriptyline were used as stationary phases. The detector was set at 315 nm with the sensitivity of 0.005 A.U.F.s for dicumarol derivatives and at 250 nm with the same sensitivity of 0.005 A.U.F.s for protriptyline. The mobile phases consisted of 1.5% acetic acid solution–acetonitrile (6:4 v/v) and 1.5% acetic acid solution–methanol (3:7 v/v) for dicumarol derivatives and protriptyline, respectively. To determine adsorption on the membrane, concentrations in both compartments were measured. Bound drug concentrations and bound fractions were calculated as follows:

Bound concentrations (D_b) = drug concentration in protein compartment (D_{b+f}) − drug concentration in buffer compartment (D_f).

Bound fraction (B_f) = D_b/D_{b+f}.

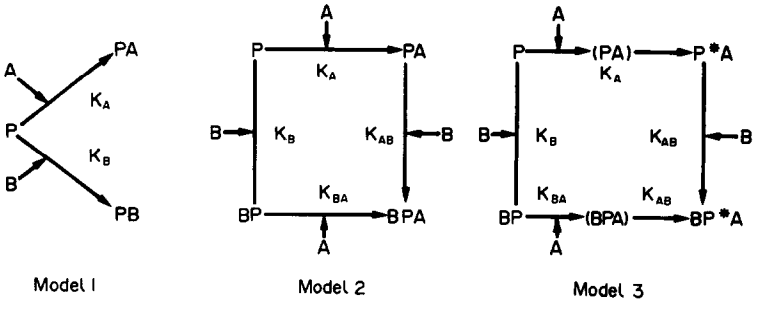
As some of the dicumarol derivatives (compounds **II–V**) were highly unstable in phosphate buffer, pH 7.4, ultrafiltration technique was chosen for those compounds instead of equilibrium dialysis. Ultrafiltration experiments were performed using a micropartition system MPS-3 (Amicon Corp., Danvers, MA, U.S.A.). The bound fraction was corrected for adsorption of drugs on the membrane by studying the system without AGP. The drug concentrations were determined by HPLC as described in equilibrium dialysis. Bound drug concentrations and bound fractions were calculated as follows:

Bound concentration (D_b) = total drug concentration (before filtration) (D_t) − filtered drug concentration/f_a.

Bound fraction (B_f) = D_b/D_t

where f_a is the ratio of the concentration after ultrafiltration without AGP with respect to the total concentration. The f_a of protriptyline was 0.94.

Data analysis. The binding of two ligands to the protein was complicated and could not be explained by a simple competition interaction alone. For example, dicumarol and protriptyline increase in binding to AGP mutually. Therefore, in the present study, on the basis of 1:1 complex formation by all compounds bound to AGP [6, 8], we treated the



Scheme 1. Interaction models.

data according to the assumption that interaction of two ligands bound to AGP simultaneously had taken place by interaction models illustrated in Scheme 1 [9, 10].

Model 1, a competitive model in general, was analysed by using the following equations:

$$\frac{[PA]}{[P_t]} = \frac{K_A[A_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (1)$$

$$\frac{[PB]}{[P_t]} = \frac{K_A[B_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (2)$$

where $[P_t]$ is the total AGP concentration, $[A_f]$ and $[B_f]$ are the concentrations of the free ligands.

Model 2, adopted by Kragh-Hansen [9], represented the case of two drugs binding to AGP together. In this model (Scheme 1), $K_{BA} = x \cdot K_A$ and $K_{AB} = x \cdot K_B$, where factor x is defined as the coupling constant. If A and B bind independently to AGP, then $x = 1$. In other cases, $x > 1$ and $x < 1$ indicated cooperative binding and anticooperative binding, respectively. The x value was calculated as follows [9]:

$$[P_t] = [P] + [PA] + [PB] + [BPA] \quad (3)$$

where $[P_t]$ is the total concentration of AGP and $[P]$ is the free concentration of AGP.

This relationship is readily transformed to

$$[P_t] = [P] + K_A \cdot [P][A_f] + K_B[P][B_f] + x \cdot K_A \cdot K_B \cdot [A_f][B_f][P]. \quad (4)$$

The concentration of bound A $[A_b]$ is given by:

$$[A_b] = [A_t] - [A_f] = K_A \cdot [P][A_f] + x \cdot K_A \cdot K_B \cdot [A_f][B_f][P] \quad (5)$$

where $[A_t]$ represents the concentration of total A. Subtracting equation (5) from equation (4) gives:

$$[P_t] - [A_b] = [P] + K_B \cdot [B_f][P]. \quad (6)$$

Since $[P_t]$, $[A_t]$, $[A_f]$, K_B and $[B_f]$ are known, it is possible to calculate $[P]$, and the known values for K_A and $[A_f]$, in equation (4) give x.

Model 3, a two-state model, named MWC (Monod–Wyman–Changeux) [10] or allosteric model was adopted on the basis of the N–B transition of human serum albumin (Janssen *et al.* [11]). The following equations are used to explain this model.

$$\frac{[PB]}{[P_t]} = f \cdot \frac{K_{AB}[B_f]}{1 + K_{AB}[A_f]} + (1 - f) \cdot \frac{K_B[B_f]}{1 + K_B[B_f]} \quad (7)$$

$$f = \frac{[PA]}{[P_t]} = \frac{K_A[A_f]}{1 + K_A[A_f]} \quad (8)$$

where f is the ratio of the concentration of the allosteric transition state (P^*A) of AGP generated by the binding of A to the total concentration of AGP(P_t). According to this model, (PA) and (BPA) are intermediate states, K_{BA} is equal to K_A and K_{AB} is the binding constant of B associated with allosteric transition of AGP. The N–B transition is a conformational change at pH 6–9 where the binding of the drug is affected by the binding of protons. This model accommodates the variation in bound B resulting from the conformational change in AGP generated by the binding of A to AGP.

RESULTS

The CD spectra of dicumarol derivatives bound to AGP in the presence and absence of protriptyline

Initially, the induced CD spectra of dicumarol and six derivatives bound to AGP were examined. The spectra were different in shape and magnitude depending upon the substituents present at position 3 of the dicumarol molecule (Table 2). Moreover, the binding constants of these compounds increased depending on their hydrophobicities [8].

Effects of protriptyline on the CD spectra of dicumarol derivatives bound to AGP were qualitatively and quantitatively different (Table 2). Binding of protriptyline to AGP did not generate any measurable extrinsic Cotton effects at wavelengths longer than 260 nm under the given experimental conditions. So the compounds were separated into four major groups on the basis of the effects produced by protriptyline as follows (Fig. 1):

1. Figure 1a shows the complete reversal of the signs of the CD spectra by the addition of protriptyline. This group consisted of the compounds **I**, **II** and **III**.

2. In Fig. 1b, the CD spectra of the compounds **IV** and **V** showed new induced negative Cotton effects at 325 nm with the disappearance of the negative Cotton effects at 300 nm by the addition of protriptyline.

3. Figure 1c is for the compound **VI**, where the

Table 2. CD characteristics of dicumarol and its derivatives bound to AGP in the absence and presence of protriptyline

Compound	Without protriptyline		With protriptyline	
	λ_{max} (nm)	θ_{obs} (mdeg)	λ_{max} (nm)	θ_{obs} (mdeg)
I	325	+1.5	325	−3.0
	300	−1.3	290	+1.9
	275	−3.4		
II	325	+1.8	325	−3.0
	300	−1.3	290	+1.9
	275	−4.2		
III	300	−1.7	325	−4.5
	275	−1.3	290	+2.5
IV	300	−3.0	325	−3.0
V	300	−2.1	325	−1.9
VI	325	+1.5	325	−5.0
	300	−4.5		
	275	−7.5		
VII	300	−4.5	300	−4.5

The following concentrations were used: AGP, 10 μM; compounds (**I–V, VII**), 10 μM; protriptyline, 10 μM. In case of the compound **VI**, the following concentrations were used: AGP, 50 μM; compound **VII**, 50 μM; protriptyline, 100 μM.

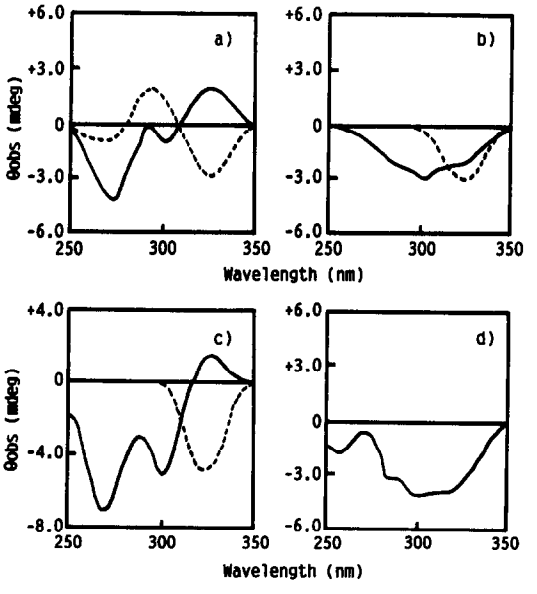


Fig. 1. Effects of protriptyline on induced CD spectra of compound **I** (dicumarol) and its derivatives bound to AGP (a) compound **I**–AGP system, (b) compound **IV**–AGP system, (c) compound **VI**–AGP system, (d) compound **VII**–AGP system. (——) Without protriptyline, (– – – –) with protriptyline. (a), (b) and (d) systems: the following concentrations were used: AGP, 10 μM; compounds **I, IV, VII** and protriptyline, 10 μM. (c) system: the following concentrations were used: AGP, 50 μM; compound **VI**, 50 μM; protriptyline, 100 μM.

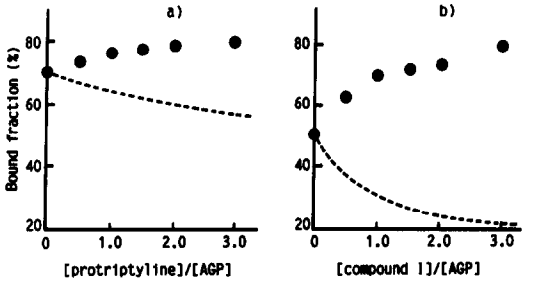


Fig. 2. Compound **I** (dicumarol) bound fraction in presence of protriptyline (a) and protriptyline bound fraction in presence of compound **I** (dicumarol) (b) at pH 7.4 and 25°. (– – – –) Theoretical curve assuming competition model (model 1). The following concentrations were used: AGP, 10 μM; compound **I** (dicumarol) (a) and protriptyline (b), 10 μM. Each value represents the average of two experiments ± SD (within the symbol).

addition of protriptyline generated a new negative Cotton effect at 325 nm in place of the original. The concentration used for compound **VI** was five times higher than that used for the other compounds because of its very small CD ellipticity.

4. The CD spectrum of compound **VII** was unaffected by the addition of protriptyline (Fig. 1d).

Effects of protriptyline on the bound fraction of dicumarol derivatives and vice versa

To elucidate the interaction between two ligands responsible for the above-mentioned four types of spectral behavior, the changes in bound fraction of

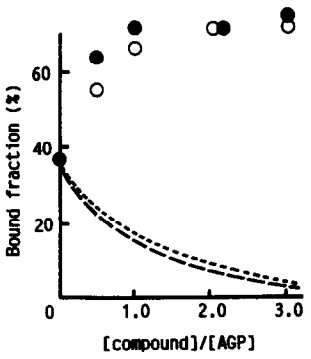


Fig. 3. Protriptyline bound fraction in presence of dicumarol derivatives by ultrafiltration at pH 7.4 and 25°. (○) Compound **II**, (●) compound **III**, (– – – –) theoretical curve (for compound **II**) assuming competition model (model 1), (——) theoretical curve (for compound **III**) assuming competition model (model 1). The following concentrations were used: AGP, 10 μM; protriptyline, 10 μM. Each value represents the average of two experiments ± SD (within the symbol).

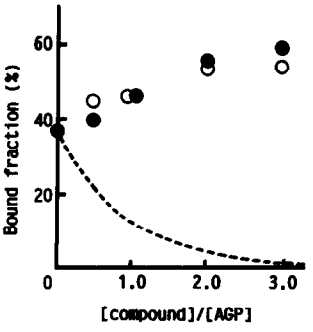


Fig. 4. Protriptyline bound fraction in presence of dicumarol derivatives by ultrafiltration at pH 7.4 and 25°. (○) Compound **IV**, (●) compound **V**, (– – – –) theoretical curve (for both compounds) assuming competition model (model 1). The following concentrations were used: AGP, 10 μM; protriptyline, 10 μM. Each value represents the average of two experiments ± SD (within the symbol).

protriptyline in the presence of dicumarol derivatives and *vice versa* were examined by equilibrium dialysis and ultrafiltration techniques.

The bound fraction of dicumarol was increased with the rise in protriptyline concentration (Fig. 2a) and *vice versa* (Fig. 2b). These data, thus, were treated according to model 2 of cooperative interaction. The coupling constant x was calculated to be 4.6 ± 1.30 (mean ± SE) (Fig. 2).

The bound fraction of protriptyline in the presence of compounds **II** and **III** was examined by ultrafiltration. The bound fraction of protriptyline increased with the increase of the concentration of these compounds (Fig. 3). However, the bound fractions of these compounds in the presence of protriptyline could not be determined because of their high instability in phosphate buffer. These interactions like those of dicumarol were also considered according to cooperative interaction (model 2).

Figure 4 shows the bound fraction of protriptyline in the presence of the compounds **IV** and **V**. These data indicate a behavior similar to dicumarol, so these interactions were also treated as cooperative (model 2).

Figure 5 shows the bound fraction of protriptyline in the presence of the compound **VI** (Fig. 5a) and *vice versa* (Fig. 5b). Both bound fraction profiles had a tendency to decrease, but they did not fit the theoretical curve assuming competition (model 1). So this interaction was analysed by the anticooperative interaction model (model 2), the coupling constant x was 0.6 ± 0.24 (mean ± SE, N = 8) (Fig. 5).

It is easily seen (Fig. 6a) that the bound fraction of compound **VII** was almost unchanged by the addition of protriptyline, suggesting that both compounds bound to AGP independently. The finding is also supported by CD data. However, the

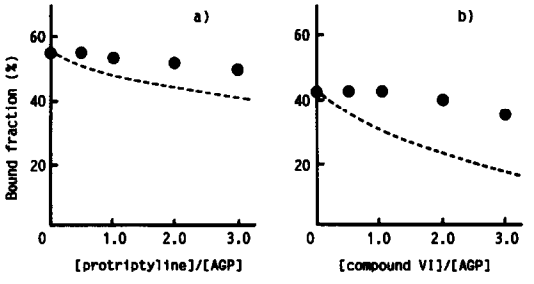


Fig. 5. Compound **VI** bound fraction in presence of protriptyline (a) and protriptyline bound fraction in presence of compound **VI** (b). (– – – –) Theoretical curve assuming competition model (model 1). The following concentrations were used: AGP, 10 μM; compound **VI** (a) and protriptyline (b), 10 μM. Each value represents the average of two experiments ± SD (within the symbol).

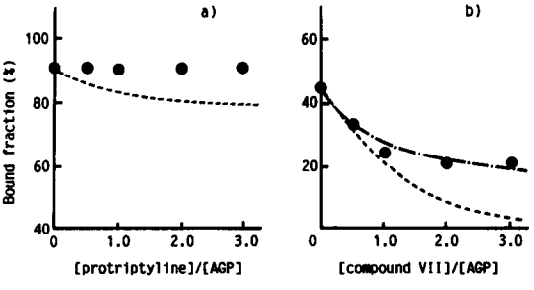


Fig. 6. Compound **VII** bound fraction in presence of protriptyline (a) and protriptyline bound fraction in presence of compound **VII** (b). (– – – –) Theoretical curve assuming competition model (model 1). (—·—) Theoretical curve assuming two-state model (model 3). The following concentrations were used: AGP, 10 μM; compound **VII** (a) and protriptyline (b), 10 μM. Each value represents the average of two experiments ± SD (within the symbol).

bound fraction of protriptyline was decreased by the addition of compound **VII** (Fig. 6b), but failed to fit the theoretical curve for competitive interaction especially at high concentration. Therefore, these data were treated according to the two-state model (model 3), where the data fitted the theoretical curve well. The binding constants of protriptyline in the absence and presence of compound **VII** were $1.1 \times 10^5\ M^{-1}$ and $2.5 \times 10^4\ M^{-1}$, respectively.

DISCUSSION

In our previous work, we found that basic tricyclic drugs such as protriptyline caused the reversal of the sign of the CD spectra of dicumarol bound to AGP with cooperative interactions [6]. There was no reversal of the CD signs of the drugs not containing two hydroxycoumarin rings by protriptyline [6]. In the present work, the interaction mode between dicumarol derivatives bound to AGP and protriptyline was studied by CD, equilibrium dialysis and ultrafiltration techniques in order to elucidate the effects of substituents in the dicumarol molecule at position 3.

The extrinsic Cotton effects depend on the spatial relationship between the asymmetric center and the perturbed chromophore of the ligand [12]. So, the variation in the induced CD spectra of dicumarol derivatives bound to AGP indicated that some compounds existed in different conformations in the binding site of the AGP molecule by steric hindrance, because all compounds showed the similar biphasic UV spectra in phosphate buffer in the UV range of 260 to 350 nm.

AsialoAGP (removal of sialic acid from AGP) behaved like AGP (results not shown). Sialic acid therefore seemed not to be involved in these interactions. Furthermore, dicumarol and protriptyline did not interact without AGP (data not shown). In addition, we have previously suggested that AGP has partially overlapping binding sites for basic drugs and acidic drugs [5]. It is not known why AGP possesses this kind of characteristic binding site. This question seems to relate the vital function of AGP. However, the data obtained in the present work can be explained as follows.

Compounds **I–VI** when bound to AGP in the presence of protriptyline seemed to possess the same orientation at around 325 nm, though the orientation of compounds **I–III** in the range 250–300 nm were different from those of compounds **IV–VI**. The orientation at around 325 nm might be attributed to the phenyl ring portion of compounds **I–VI** when bound to AGP as the UV absorption of the phenyl ring portion of the compounds was undoubtedly obtained within the range of 300 to 350 nm.

The interaction of compounds **I–III** with AGP in the presence of protriptyline was accompanied by the reversal of the CD signs at 325 and 300 nm as shown in Fig. 1a. As shown in Figs 2 and 3, the interaction was cooperative. The interaction of compounds **IV** and **V** in the presence of protriptyline generated a new CD band at 325 nm but the original disappeared at 300 nm (Fig. 1b). However, it is evident from Fig. 4 that this interaction was also cooperative similar to that of compounds **I–III**.

In the absence of protriptyline, compound **VI** with AGP showed a CD spectrum similar to that of dicumarol when bound to AGP in the absence of protriptyline (Fig. 1c). But with protriptyline, compound **VI** reversed the CD sign only at 325 nm. The interaction was anticooperative as shown in Fig. 5. As all the experimental conditions before and after the addition of protriptyline in the CD experiments were the same, the cause of this interaction and the different CD behavior might be attributed to the characteristic steric effect produced by the substituent at position 3. In the absence of protriptyline, the steric effect probably did not play any role in the CD spectrum, as the CD spectrum was quite similar to that of compounds **I–III**. However, with protriptyline, the steric effect had some influence in changing the orientation of compound **VI** during binding to AGP, which ultimately caused a change in the CD spectrum as did compounds **IV** and **V**.

Compound **VII** when bound to AGP in the presence of protriptyline showed no change in the CD spectrum (Fig. 1d). The interaction was neither cooperative nor anticooperative. The possibility of independent binding was also rejected because of the evidence of the decreased bound fraction of protriptyline in the presence of compound **VII**. However, the bound fraction of compound **VII** was constant in the presence of protriptyline. Thus, the overall interaction was tried to explain by two-state model accompanying the possibility of conformational change of AGP as shown in Fig. 6b. In the case of other compounds, there were changes in the CD spectra in the presence of protriptyline through ternary complex formation. In the case of compound **VII** also, the presence of ternary complexation was evident (Fig. 6) but there was no change in the CD spectrum in the presence of protriptyline. The reason for this completely different interaction with AGP and CD behavior produced by compound **VII** was not very clear. However, this unchangeable behavior of the CD spectrum of compound **VII** in the presence of protriptyline may be explained by the following reasons.

1. The presence of phenyl bulky group at position 3 of compound **VII** made the interaction of protriptyline with compound **VII** difficult, causing a very small formation of the ternary complex. Thus, it is probable that the very small concentration and molecular ellipticity of the ternary complex produced were not sufficient to change the chirality of the complex. Consequently CD behavior was unchanged.

2. Phenyl group at position 3 of compound **VII** might bind to a special pocket present in AGP. This characteristic binding possibly prevented the change of the orientation of compound **VII** during binding to AGP in the presence of protriptyline. Therefore, though the formation of the ternary complex was evident, it could not change the orientation of compound **VII**, causing no change in the CD spectrum. However, it was not clear whether any of the above reasons or both were responsible for the unchangeable nature of the CD spectrum of compound **VII** in the presence of protriptyline.

In conclusion, drug binding sites on the AGP molecule were partially overlapped by the binding

sites of acidic drugs and basic drugs. Therefore, each drug bound to these sites was influenced by another. That the effects were very variable was seen by changing the substituent groups of a drug. Taking into consideration the present data and the previous results [5, 6, 13], it can be said that the binding sites of drugs and fatty acids on the AGP molecule were very close to each other, suggesting that AGP has a wide and flexible ligand binding area. Thus, it should be noted that endogenous substances such as fatty acids and steroid hormones [3] and exogenous substances such as drugs share the same binding area on AGP in sharp contrast to human albumin.

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