Interaction Mode of Dicumarol and Its Derivatives with Human Serum Albumin, α_1 -Acid Glycoprotein and Asialo α_1 -Acid Glycoprotein

Mohammed Habibur RAHMAN,* Toshimi MIYOSHI,* Katsuaki SUKIMOTO,* Akira TAKADATE,** and Masaki OTAGIRI*,***

Faculty of Pharmaceutical Science, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862, Japan and Daiichi Pharmaceutical College,** 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815, Japan*

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The interaction of dicumarol and its seven other derivatives with human serum albumin (HSA), α_1 -acid glycoprotein (AGP) and desialylated AGP (asialoAGP) has been investigated by circular dichroism (CD) and fluorescence. The binding parameters of dicumarol and its derivatives obtained from fluorescence almost agreed with those obtained from CD. The binding data indicated that the total binding affinities (nK) to HSA were higher than the binding affinities to AGP and asialoAGP. Hydrophobic interaction was the driving force for the binding to all the three proteins and nK values in the binding process were found to be increased with the increase of hydrophobicity of the compound. This was evidenced by the attempts taken to correlate binding affinities with partition coefficients. Both electrostatic and van der Waals interactions were not found to play any significant role in the binding of these compounds to any of these three proteins. However, in case of the AGP and asialoAGP binding, apart from the hydrophobic interaction, some other forces may be involved as evidenced from the experimental data.

Binding was exothermic, entropy driven and spontaneous. The change of enthalpy (ΔH°) was compensated for by the change in entropy (ΔS°). Relative contribution of hydrophobic interactions in the binding of these compounds to HSA was higher than to AGP or asialoAGP. Sialic acid was not found to impart any significant role in the binding of these compounds to AGP.

Keywords — dicumarol; protein binding; hydrophobic interaction; fluorescence; thermodynamic analysis

Introduction

The significance of plasma protein binding of coumarin anticoagulants for distribution, elimination, anticoagulant effect, and pharmacokinetic drug-interactions is well known.¹⁻⁴ Dicumarol is known to bind strongly to its site on human serum albumin (HSA)⁵ and on α_1 -acid glycoprotein (AGP).^{6,7} The binding site of dicumarol was identified with site I on human serum albumin (HSA).^{8,9} Coumarin compounds such as dicumarol, coumetarol, ethylbiscoumacetate also bind to a common binding site on α_1 -acid glycoprotein (AGP) with the same affinity as that for binding to HSA.⁷ In both cases, the driving force for binding appears to be hydrophobic interactions. In this study, dicumarol and its seven other derivatives all contain two hydroxycoumarin rings. Although the plasma protein binding of dicumarol has been extensively investigated by various methods,

there has been no comparative study among the binding characteristics of the compounds used in this study to HSA, AGP and asialoAGP.

With respect to the binding mechanism of drugs to HSA, quantitative structure activity relationship (QSAR), spectroscopic and thermodynamic analysis have been frequently studied. In this paper, however, some approaches have been made in order to elucidate the interaction mode of dicumarol and its derivatives with HSA, AGP and asialoAGP on the basis of different physicochemical and thermodynamic parameters. In addition, the role of sialic acid in the binding of dicumarol and its derivatives to AGP has also been studied.

Materials and Methods

Materials — Human serum albumin (HSA; fraction V, lot no.36F-9333) and human α_1 -acid glycoprotein (AGP; lot no.44F-9345) were ob-

*** To whom correspondence should be addressed.

tained from Sigma (St. Louis, MO). The mol wt of HSA and AGP were assumed to be 66500 and 44100, respectively. Both HSA and AGP gave only one band in SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Dicumarol was purchased from Tokyo Kasei, and was used without further purification. Coumetarol and ethylbiscoumacetate were generous gifts from professor Janssen, State University of Utrecht. Other compounds listed in Table I (compounds IV—VIII) were synthesized according to the method of Sullivan *et al.*¹⁰ These compounds were identified and confirmed by melting point measurement, elemental analysis and proton nuclear magnetic resonance spectroscopic study. 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 0.067M phosphate buffer, pH 7.4.

Preparation of AsialoAGP — AGP was enzymatically desialylated as outlined by Primozic, McNamara,¹¹ using an acylneuramininyl hydrolase enzyme obtained from *Clostridium perfringens*. AGP in phosphate buffer (1.5 mg/ml) was added to the enzyme (0.357 units suspended in 5 ml of buffer). This solution was incubated at 37 °C in a water bath, while being gently rotated at 30 r.p.m. for 2h. The protein solution was filtered over an 8- μ m filter to remove the enzyme. The concentration of sialic acid in the filtrate was determined by the thio-barbituric acid method.¹² The product was dialysed against deionized water and the dialysate lyophilized. Approximately 90% of the sialic acid was removed, leaving an average of one sialic acid residue per protein molecule. The mol wt of asialoAGP was therefore 40000.

Physicochemical Properties of Dicumarol and Its Derivatives — The partition coefficient (PC) values were determined by the HPLC method. The HPLC system consisted of a Hitachi 655A-11 pump and Hitachi variable wavelength UV monitor. A column of LiChrosorb RP-18 (7 μ m, Merck, Darmstadt, Germany) was used as the stationary phase. The detection wavelength was 315 nm and the mobile phase consisted of methanol-phosphate buffer, pH 7.4 (5:5, v/v). Calculations were based on retention time meas-

urements. A calibration curve was first made by plotting the known PC values of dicumarol, coumetarol and ethylbiscoumacetate vs. their respective retention times obtained by the HPLC method. From the calibration curve the PC values of the other compounds used in this study were calculated. The experiments were carried out in triplicate. Van der Waals volume (V_w) was calculated according to the method of Moriguchi *et al.*¹³ Unfortunately, except for dicumarol, coumetarol and ethylbiscoumacetate, the pK_a values of other compounds listed in Table I could not be calculated because of their very poor solubility at lower pH (acidic pH) and higher unstability at higher pH (above pH 7.5).

Circular Dichroism (CD) — CD measurements were made on a Jasco J-50A recording spectropolarimeter (Tokyo, Japan) using a 5 mm cell at 25 °C. All solutions were scanned from wavelengths at which no induced optical activity was observed. The induced ellipticity is defined as the ellipticity of the drug-protein mixture minus the ellipticity of the protein alone at the same wavelength and is expressed in degrees. Bound drug concentration (D_b) and free drug concentration (D_f) were calculated according to the method of Rosen.¹⁴

Fluorescence — Fluorescence measurements were made using a Jasco FP-770 fluorescence spectrophotometer (Tokyo, Japan) and carried out at three different temperatures, 10°, 25° and 40 °C. Thermodynamic examinations were also carried out at the same temperatures. The fluorometric titrations were as follows: Protein solution (2 μ M, 3 ml) was titrated by the successive addition of drugs (to give a final concentration of 0.1—9 μ M), and the fluorescence intensity of protein was measured (excitation 290 nm and emission 340 nm). The total volume was less than 3 ml + 20 μ l; corrections of protein concentrations to the total volume were not made as they were insignificant. At the selected wavelength the drugs did not contribute to the fluorescence.

The fraction of drug bound, X , was determined according to Weber, Young;¹⁵

$$X = \frac{F_o - F_b}{f_a \times F_o} \quad (1)$$

where F_b and F_o are the fluorescence intensities of protein in a solution with given concentration of drug and without drug, respectively, and f_a is the maximum fraction of quenchable fluorescence. The value of f_a in case of the HSA binding was between 0.88—0.93 and in case of AGP and asialoAGP it was between 0.63—0.70 and 0.60—0.69, respectively. F_b was determined for a wide range of concentrations.

Data Analysis — All the results obtained by the above described two methods were analyzed in the form of Scatchard plots;¹⁶⁾

$$r/D_f = nK - rK \quad (2)$$

where r is the number of mol of bound drug per mol of protein, n is the number of binding sites, K is the binding constant and D_f is the free drug concentration.

Thermodynamic Analysis — Thermodynamic analysis was carried out according to the method of Pedersen *et al.*¹⁷⁾ From the temperature dependence of binding constants it is possible to calculate values for the thermodynamic functions involved in the binding process. According to the general physico-chemical theory

the change of equilibrium constant with temperature is related to changes in enthalpy and entropy as follows:

$$\frac{d(\ln k)}{dT} = \frac{1}{R} \times \frac{d}{dT} \left(\frac{\Delta H^\circ}{T} - \Delta S^\circ \right) \quad (3)$$

If ΔH° and ΔS° are both independent of temperature, Eqn. (3) can be integrated to yield the van't Hoff equation,

$$\ln K = -\frac{\Delta H^\circ}{RT} + \text{constant} \quad (4)$$

Binding constants were plotted according to Eqn. (4) and ΔH° was obtained from the slope of the regression line. The standard free energy, ΔG° , for complex formation is estimated from

$$\Delta G^\circ = -RT \ln nK \quad (5)$$

and the entropy change, ΔS° is obtained from

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (6)$$

Multiple Regression Analysis — The charac-

TABLE I. Chemical Structure, Partition Coefficient (PC) and van der Waals Volume (V_w) of the Compounds

Compound	Substituent (R)	PC	$V_w (\times 10^2 \text{ \AA}^3)$
I. Dicumarol	H	68.0	2.63
II. Coumetarol	CH_2OCH_3	24.0	2.95
III. Ethylbiscoumacetate	$\text{COOCH}_2\text{CH}_3$	21.0	3.21
IV. Ethylidenebis 4-hydroxycoumarin	CH_3	2.7	2.80
V. Propylidenebis 4-hydroxycoumarin	CH_2CH_3	105.8	2.90
VI. Butylidenebis 4-hydroxycoumarin	$(\text{CH}_2)_2\text{CH}_3$	204.9	3.10
VII. Pentylidenebis 4-hydroxycoumarin	$(\text{CH}_2)_3\text{CH}_3$	487.5	3.20
VIII. Benzylidenebis 4-hydroxycoumarin	C_6H_5	89.1	3.30

Fig. 1. Fluorescence Quenching of HSA(A), AGP(B) and AsialoAGP(C) by Dicumarol in 0.067 M Phosphate Buffer (pH 7.4) at 25 °C

[HSA] = [AGP] = [asialoAGP] = 2×10^{-6} M, dicumarol = $0.1 - 9 \times 10^{-6}$ M.

teristics of binding constants were quantitatively examined by the use of multiple regression analysis. The variations in values of log PC parameters were sufficient to attempt correlation analysis. Correlation and regression equations were calculated on a PC-9801-vm (NEC) personal computer. F-test was used to judge the reliability of regression analysis.

Results

Table I illustrates the chemical structures and different physicochemical parameters of

dicumarol and its derivatives used in this study. Binding of dicumarol and its derivatives to HSA generated monophasic extrinsic Cotton effects, but produced polyphasic extrinsic Cotton effects when bound to AGP and asialoAGP. The UV spectrum of dicumarol was characterized by two peaks at 275 nm and 315 nm at pH 7.4. These two peaks were split in the CD spectrum of dicumarol bound to AGP and asialoAGP and thus gave a polyphasic extrinsic Cotton effect. But those two peaks as were not split, generated monophasic extrinsic Cotton effect in the CD spectrum of dicumarol bound to HSA, suggest-

Fig. 2. Scatchard Plots of Dicumarol-HSA(A), -AGP(B) and -AsialoAGP(C) Interactions as Measured from Fluorescence Quenching

TABLE II. Binding Parameters of the Dicumarol-Serum Protein Systems by CD and Fluorescence at pH 7.4 and 25 °C

Serum protein	Fluorescence		CD	
	<i>n</i>	<i>K</i> ($\times 10^6 \text{ M}^{-1}$)	<i>n</i>	<i>K</i> ($\times 10^6 \text{ M}^{-1}$)
HSA	1.1 $\pm$ 0.02	1.8 $\pm$ 0.10	1.4 $\pm$ 0.04	2.1 $\pm$ 0.22
AGP	0.9 $\pm$ 0.01	1.4 $\pm$ 0.08	1.2 $\pm$ 0.09	1.7 $\pm$ 0.21
AsialoAGP	0.8 $\pm$ 0.03	1.5 $\pm$ 0.19	1.0 $\pm$ 0.07	1.8 $\pm$ 0.25

All values are mean of three determinations $\pm$ S.D. *n* = number of binding sites, *K* = binding constant, as determined from Scatchard plots.

TABLE III. Binding Parameter of Dicumarol and Its Derivatives to HSA, AGP and AsialoAGP as Measured by Fluorescence Quenching at pH 7.4 and 25 °C

Compound	HSA		AGP		AsialoAGP	
	<i>n</i> ^{a)}	<i>K</i> ($\times 10^6 \text{ M}^{-1}$)	<i>n</i> ^{a)}	<i>K</i> ($\times 10^6 \text{ M}^{-1}$)	<i>n</i> ^{a)}	<i>K</i> ($\times 10^6 \text{ M}^{-1}$)
I	1.1	1.8 $\pm$ 0.10	0.9	1.4 $\pm$ 0.08	0.8	1.5 $\pm$ 0.19
II	0.9	1.8 $\pm$ 0.09	0.9	1.3 $\pm$ 0.07	0.8	1.3 $\pm$ 0.16
III	0.7	2.4 $\pm$ 0.12	1.1	1.1 $\pm$ 0.09	0.8	1.2 $\pm$ 0.14
IV	1.3	1.0 $\pm$ 0.13	0.7	1.2 $\pm$ 0.14	0.7	1.0 $\pm$ 0.12
V	1.2	1.8 $\pm$ 0.21	0.9	1.5 $\pm$ 0.18	0.8	1.6 $\pm$ 0.04
VI	0.9	2.5 $\pm$ 0.23	0.8	2.4 $\pm$ 0.21	0.8	2.1 $\pm$ 0.08
VII	0.9	2.8 $\pm$ 0.19	0.8	2.3 $\pm$ 0.22	0.8	2.4 $\pm$ 0.07
VIII	0.8	2.5 $\pm$ 0.07	1.5	0.7 $\pm$ 0.21	1.3	0.7 $\pm$ 0.09

All values are mean of three determinations $\pm$ S.D. *n* = number of binding sites, *K* = binding constant, as measured from Scatchard plots.

a) S.D. of all *n* values were below 0.03.

ing that the geometry of the binding sites of dicumarol and its derivatives on HSA is not the same as that on AGP or asialoAGP. As a representative example, the fluorescence quenchings of HSA, AGP and asialoAGP by dicumarol are shown in Fig. 1. The Scatchard plots obtained from Fig. 1 are shown in Fig. 2. Table II lists the binding parameters of dicumarol bound to HSA, AGP and asialoAGP by fluorescence and CD. Binding parameters of dicumarol and its derivatives to the three proteins by the fluorescence technique at 25 °C are summarized in Table III. Job plots were constructed for dicumarol and its derivatives bound to HSA, AGP and asialoAGP by maintaining the total concentration of drug and protein at a constant value of $5 \times 10^{-6} \text{ M}$.¹⁸⁾ As a representative example, the Job plots for the dicumarol-HSA, -AGP, and -asialoAGP systems are shown in Fig. 3. The inflection points for these plots were near 0.5. It can readily be seen (Fig. 4) that there was a good

Fig. 3. Job Plots of Relative Fluorescence Intensities as a Function of Mole Fraction of Dicumarol

Total concentration of [serum protein] + [dicumarol] was kept constant at $5 \times 10^{-6} \text{ M}$.

(●) dicumarol-HSA system, (▲) dicumarol-AGP system and (○) dicumarol-asialoAGP system.

F_0 , fluorescence intensity of dicumarol + protein; F_p , fluorescence intensity of protein alone.

Fig. 4. Relationships between Binding Affinities and Partition Coefficient Values of Dicumarol and Its Derivatives (A) HSA system, (B) AGP system and (D) asialoAGP system. 1:I, 2:II, 3:III, 4:IV, 5:V, 6:VI, 7:VII, 8:VIII. (see Table I).

correlation between the partition coefficients and the binding affinities (nK) of dicumarol and its derivatives.

Thermodynamic parameters were calculated by the usual procedure and are summarized in Table IV. Compensation plots of entropy change, ΔS° , *versus* overall enthalpy change, ΔH° , for the binding of dicumarol and its derivatives to HSA, AGP and asialoAGP are illustrated in Fig. 5.

The influence of oleic acid and sodium chloride on the binding of dicumarol and its derivatives to the three proteins was investigated and the results with dicumarol are illustrated in Fig. 6.

Discussion

Comparison of Methods

For quantitative correlation studies, all the binding constants must be measured by the same method with a high degree accuracy and precision. Initially, we attempted to estimate the binding parameters of dicumarol bound to HSA, AGP and asialoAGP using the fluorescence and the CD technique, and the results were given in the form of a Scatchard plot. The intrinsic fluorescence arising from the tryptophan residue of HSA, AGP and asialoAGP was significantly quenched, since the binding sites of these compounds are believed to be located near the tryptophan residues of these proteins.^{7,19} The amount of quenching directly reflected the amount of the drug bound to protein.

TABLE IV. Thermodynamic Parameters of Dicumarol and Its Derivatives

Compound	HSA			AGP			AsialoAGP		
	$\Delta G^{oa)}$	$\Delta H^{ob)}$	$\Delta S^{oc)}$	$\Delta G^{oa)}$	$\Delta H^{ob)}$	$\Delta S^{oc)}$	$\Delta G^{oa)}$	$\Delta H^{ob)}$	$\Delta S^{oc)}$
I	-35.1	-6.5	95.9	-34.4	-4.3	101.2	-34.5	-4.5	100.5
II	-34.8	-6.7	94.5	-34.1	-6.1	93.9	-34.1	-7.6	89.0
III	-34.2	-6.6	94.8	-33.7	-5.9	93.3	-34.0	-7.6	88.6
IV	-34.2	-8.0	87.4	-33.6	-9.7	80.5	-33.9	-11.2	76.1
V	-33.3	-6.2	97.5	-34.5	-3.7	103.3	-34.6	-3.5	104.5
VI	-35.5	-5.0	102.4	-35.6	-2.3	111.9	-35.3	-2.9	108.8
VII	-35.7	-3.9	106.3	-35.6	-0.7	117.1	-35.7	-1.0	116.1
VIII	-35.2	-6.4	96.7	-33.5	-1.4	107.9	-33.0	-1.2	106.7

a) kJ/mol, b) kJ/mol, c) J/K° mol.

Fig. 5. Compensation Plots of Entropy, ΔS° , versus Overall Enthalpy, ΔH° , for the Binding of Dicumarol and Its Derivatives to HSA(A), AGP(B) and AsialoAGP(C)

Numbers used indicate the same compounds as those used in Fig. 4.

Binding constants obtained by both methods were found in close agreement with each other. The fluorescence quenching method was used for further studies because of its simplicity and high sensitivity.

Comparison of Binding to HSA, AGP and AsialoAGP

The linearity obtained in the Scatchard plot obviously indicated the presence of only one class of binding sites for these compounds on HSA, AGP and asialoAGP. Construction of Job plots also indicated the formation of a 1:1 complex, as shown in Fig. 3 for dicumarol, as a represen-

tative example, bound to HSA, AGP and asialoAGP. In order to minimize the variations among the binding constants, the total binding affinity (nK) instead of binding constant (K) was considered for following analysis, based upon 1:1 complex formation. As shown in Table III, it is clear that the binding affinity of dicumarol and its derivatives bound to HSA was greater than AGP or asialoAGP.

QSAR Analysis

Since the binding of drugs to proteins usually depends upon their physicochemical properties,^{20,21} quantitative relationships between

Fig. 6. Effects of Oleic Acid and Sodium Chloride on the Observed Ellipticities of the Dicumarol-HSA(●), -AGP(▲) -Asialo AGP(○) Complexes at pH 7.4 and 25 °C

[HSA] = [AGP] = [asialoAGP] = 1×10^{-5} M, [dicumarol] = 1×10^{-5} M

physicochemical properties and binding of drugs to proteins can be established by multiple regression analysis. Therefore, partition coefficients, (PC) as a measure of hydrophobicity were correlated with binding affinities for HSA, AGP and asialoAGP in an attempt to interpret the binding mode of these compounds to HSA, AGP and asialoAGP. Figure 4 shows relationships between PC of dicumarol and its derivatives and their binding affinities to HSA, AGP and asialoAGP. The fitted straight lines gave

$$\begin{aligned} \text{for HSA: } \log nK &= 0.126 (\pm 0.005) \log PC \\ &\quad + 6.04 (\pm 0.01), \\ n &= 8, r = 0.993, s = 0.006 \end{aligned} \quad (7)$$

$$\begin{aligned} \text{for AGP: } \log nK &= 0.130 (\pm 0.033) \log PC \\ &\quad + 5.88 (\pm 0.001), \\ n &= 8, r = 0.840, s = 0.022 \end{aligned} \quad (8)$$

and for asialoAGP:

$$\begin{aligned} \log nK &= 0.141 (\pm 0.039) \log PC \\ &\quad + 5.828 (\pm 0.072), \\ n &= 8, r = 0.828, s = 0.031 \end{aligned} \quad (9)$$

Partition coefficients, as a measure of hydrophobicity were correlated with binding affinities for HSA, AGP and asialoAGP with correlation coefficients of 0.993, 0.840 and 0.828, respectively. Several reports describe a linear relationship between the lipophilicity of drugs and their binding constants to proteins.^{22,23} It is evident (Fig. 4) that with the increase of lipophilicity, the binding affinities of these compounds to the three proteins were increased linearly, suggesting that hydrophobic interaction was the driving force in the binding of these compounds to the three proteins. The slope of each system was less than unity, which suggests that dicumarol and its derivatives were bound to hydrophobic surfaces on HSA, AGP and asialoAGP rather than to deeply buried sites in the three proteins.²⁴ The intercepts of the AGP plot was almost similar to that of the asialoAGP plot, indicating that the binding sites on the two proteins may have almost the same hydrophobicity.

Since the calculation of the pK_a values of some of the compounds used in this study (compounds IV—VIII in Table I) was not feasible (reason has already been mentioned), the correlation between the pK_a values and the binding

affinities was not studied.

No improved correlation was found when attempts were made to include the term V_w in the equations. This indicates the noninterference of the steric effect in the binding process of these compounds to the three proteins. This finding is somewhat in contrast to the previous finding reported by Maruyama *et al.*⁷ The reason for this contrast is probably due to the fact that in the previous work different coumarin compounds containing both monohydroxy and dihydroxy coumarin rings were used (V_w values varied with a spread of above 50%), which were probably sufficient to impart the steric effect in the binding of those compounds to AGP. But in this study as the coumarin compounds containing only two hydroxy coumarin rings have always been used, the variations in the van der Waals volumes were not enough (only about 12%) to show the steric effect in the binding process to any of these three proteins.

Thermodynamic Analysis

Thermodynamic parameters can be interpreted in terms of forces that stabilize the protein-ligand complex.²⁵ From the thermodynamic data listed in Table IV, it is evident that binding of dicumarol and its derivatives to all these proteins is accompanied by an entropy gain and the enthalpies are of negative values. The decrease of binding strength of HSA, AGP and asialoAGP for dicumarol and its derivatives with increasing temperature is characteristic of an exothermic reaction and has been reported by many protein interactions.²⁶ The binding process was always spontaneous as evidenced by the negative sign of ΔG° values. For typical hydrophobic interactions both ΔS° and ΔH° are positive, while the sources of negative enthalpy and entropy changes arise from van der Waals interactions and hydrogen bonding formation in low dielectric media.²⁵ However, for electrostatic interaction, negative enthalpy may play a role but for actual or true electrostatic interaction ΔH° is expected to be very small or almost zero.²⁷ Therefore, there is no doubt that hydrophobic interaction played the most significant role in the binding of dicumarol and its derivatives to these plasma proteins. The overall binding was exothermic as indicated by the negative sign of en-

thalpy. In our experimental conditions, the enthalpy change (ΔH°) was compensated for with a variation of the entropy change (ΔS°) (Fig. 5). The linear relationship between ΔH° and ΔS° (Fig. 5) supported that the binding of these drugs to all the three proteins was mediated mainly by one common high affinity binding site. The possibility of unfolding of the protein molecule during the binding process because of high positive ΔS° values can be rejected in raising the evidence that unfolding of proteins presumably requires the breaking or bending of several bonds and should result in an endothermic reaction of appreciable magnitude,²⁸⁾ but sufficient evidence has already been produced in favor of an exothermic reaction.

This finding was supported by experiments with induced CD spectra of dicumarol and its derivatives to HSA, AGP and asialoAGP in the presence of fatty acid and neutral salt. Oleic acid and sodium chloride were used to confirm the binding force of dicumarol and its derivatives to the three proteins. In spite of the presence of the carboxylic group in oleic acid, the binding of oleic acid to plasma proteins is mainly hydrophobic.^{29,30)} On the other hand, the sodium chloride binding to plasma proteins represents true electrostatic interactions.³¹⁾ Figure 6, as a representative example, illustrates the effects of oleic acid and sodium chloride on the induced CD spectra of dicumarol bound to HSA, AGP and asialoAGP. The induced CD intensity of dicumarol bound to the three proteins remained unaffected by the addition of sodium chloride. On the other hand, the induced CD intensity of dicumarol bound to AGP and asialoAGP was significantly decreased by the addition of oleic acid. However, the effect of oleic acid on the induced CD intensity of dicumarol bound to HSA was qualitatively and quantitatively different from those to AGP or asialoAGP. At a lower concentration of oleic acid, the CD intensity of dicumarol bound to HSA was increased but it decreased with the increase in oleic acid concentration. This phenomenon can be explained by the relationship between the binding sites of oleic acid and dicumarol on HSA. At a lower concentration oleic acid binds to its high affinity sites on HSA causing some conformational changes

in the HSA molecule, which ultimately affects dicumarol binding indirectly (or allosterically) by increasing its molecular ellipticity with HSA. Oleic acid in higher concentration binds not only to its high affinity binding sites but also to its low affinity binding sites. Some of these low affinity binding sites are the high and low affinity binding sites of drugs. So dicumarol was probably displaced from its binding sites at the higher concentration of oleic acid, causing a fall in the molecular ellipticity of dicumarol with HSA. On the other hand, in the case of AGP binding, the high affinity binding sites of oleic acid and drugs may possibly be the same; thus dicumarol was displaced from its binding sites even at the lower concentration of oleic acid as evidenced by the decline in the molecular ellipticity produced by the very low concentration of oleic acid. These results as stated above indicate that dicumarol and its derivatives bound to these proteins are not so much affected by charges. Binding characteristics of dicumarol and its derivatives to AGP and asialoAGP were found almost identical, suggesting that sialic acid did not have significant influence on the binding of dicumarol and its derivatives to AGP.

In conclusion, the total binding of dicumarol and its derivatives to HSA is greater than to AGP or asialoAGP, and hydrophobic interaction is the driving force for binding in all the systems. However, the relative contribution of hydrophobic interaction is higher in the case of binding of dicumarol and its derivatives to HSA than to AGP or asialoAGP, where some other forces may play a role. Sialic acid does not have any significant role in the binding of dicumarol and its derivatives to AGP.

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