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Optical Studies of Drug-Protein Complexes

IV. The Interaction of Warfarin and Dicoumarol with Human Serum Albumin

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SUMMARY

Human serum albumin (HSA) has three strong affinity sites for dicoumarol ($K = 7.7 \times 10^5$ M^{-1}) as well as an indeterminate number of much weaker sites. The binding of dicoumarol to HSA generates a large negative ellipticity band at 305 mμ in the normal circular dichroic spectrum of the protein. This extrinsic Cotton effect results from the perturbation of $\pi \rightarrow \pi^*$ transitions in the drug molecule by an asymmetrical center at or near the binding site for HSA. These results suggest that dicoumarol takes up a preferred orientation with respect to its HSA-binding site. The dicoumarol-HSA complex must also be rigid enough to preserve such a spatial relationship. In contrast, the binding of warfarin, acenocoumarin, ethyl biscoumacetate, and 4-hydroxycoumarin to HSA does not generate any extrinsic Cotton effects. The unique binding of dicoumarol to HSA would appear to involve the hydrophobic and electrostatic interactions of both coumarin rings.

The binding of either dicoumarol or warfarin to HSA quenches the native tryptophan fluorescence of the protein. The binding of warfarin to HSA is also accompanied by a 7-fold increase in the fluorescent yield of the drug and a shift of its fluorescence emission maximum from 400 mμ to 390 mμ. This suggests that warfarin is bound to a hydrophobic area of the HSA molecule.

Light absorbed by the HSA tryptophan groups and nonradiatively transferred to bound warfarin is re-emitted by the drug as fluorescence. This made it possible to estimate that the mean effective transfer distance between the single HSA tryptophan group and the bound warfarin molecule was 34.5 Å. Similar experiments gave a value of 23.7 Å for the mean effective transfer distance between dansylglycine and the same tryptophan group. While these distances can be considered only approximate, it is obvious that if the HSA molecule has a diameter of 56 Å (calculated assuming an anhydrous sphere of mol wt 69,000), dansylglycine and warfarin must be bound to different sites. This was confirmed by showing that warfarin does not displace dansylglycine from its binding site on HSA.

INTRODUCTION

It has recently been shown that techniques such as circular dichroism, fluorescence spectroscopy, and ultraviolet absorption spectroscopy can provide valuable information on the nature of drug-protein interactions (1–4). These studies have revealed, for example, that both hydrophobic and electrostatic interactions are important factors in the binding of anionic drugs such as phenylbutazone and flufenamic acid to serum albumin. The modifying effect of steric

parameters on binding has also been investigated (4).

In the present study, the techniques of circular dichroism and fluorescence spectroscopy have been used to examine the binding of the coumarin anticoagulant drugs warfarin and dicoumarol to serum albumin. The results clearly show that although these drugs are structurally very similar, there are fundamental differences in the way in which they interact with human serum albumin.

MATERIALS AND METHODS

Materials. Crystalline human and bovine serum albumins were purchased from Mann Research Laboratories. Each batch of albumin was dialyzed at 4° against distilled water before use. The concentration of serum albumin was determined by measuring the optical density of solutions at 280 mμ, using $E^{1\%}_{1\,cm}$ values of 5.3 and 6.6 for the human and bovine preparations, respectively. Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] was a gift from Dr. Collin H. Schroeder of the Wisconsin Alumni Research Foundation; acenocoumarin [3-(α-acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin] and ethyl biscoumacetate [ethyl bis (4-hydroxycoumarinyl) acetate] were kindly donated by Dr. Frank H. Clarke of Geigy Pharmaceuticals; dicoumarol [Dicumarol, 3,3′-methylenebis-(4-hydroxycoumarin)] was obtained from Abbott Laboratories. Dansylglycine (*N*,*N*-dimethylamino-1-naphthalene-5-sulfonyl-*N*′-glycine) was purchased from Mann Research Laboratories. All other chemicals were of reagent grade.

Methods. Circular dichroism measurements were made at 27° with a Cary 6001 attachment to the Cary model 60 spectropolarimeter, using the following concentrations: dicoumarol, 5×10^{-5} M (4.30×10^{-5} M); warfarin, 5×10^{-5} M (2.39×10^{-5} M); 4-hydroxycoumarin, 1×10^{-4} M (2.64×10^{-5} M); acenocoumarin, 5×10^{-5} M (2.83×10^{-5} M); ethyl biscoumacetate, 5×10^{-5} M (4.41×10^{-5} M); HSA,[1] 1.45×10^{-5} M; sodium phosphate buffer (pH 7.4), 0.1 M. The figures in parentheses are the concentrations of drug bound in the presence of 1.45×10^{-5} M HSA. Results are expressed as molar ellipticities $[\theta]$ (deg·cm² dmole⁻¹), which were calculated from the formula

$$[\theta] = \frac{100\,\theta_{obs}}{lc}$$

where θ_{obs} = observed ellipticity, l = length (centimeters), and c = molar concentration. Molar ellipticities were calculated either in terms of the concentration of bound drug or in terms of the concentration of HSA, assuming a molecular weight of 69,000. The concentration of bound drugs was estimated from a Scatchard plot of equilibrium dialysis experiments (e.g., Fig. 1) or was measured directly by ultrafiltration, using an Amicon ultrafiltration cell equipped with a PM-10 filter.

Fluorescence intensities and spectra were obtained with an Aminco-Bowman spectrophotofluorometer. Cells of 1-cm path length were used for the fluorometric titrations, while a microcell having internal cross-sectional dimensions of 0.29×0.29 cm was used for all other fluorescence measurements. All readings were corrected for self-absorption. Calibration of the spectrophotofluorometer and correction of the excitation and emission spectra were carried out by the method of Chen and Hayes (5, 6). Fluorescence emission spectra (e.g., Fig. 6) were displayed in terms of relative quanta by multiplying by λ^2 at each wavelength (7). Fluorescence quantum yields were obtained by comparing the area under the corrected emission spectrum with that of a quinine standard (dissolved in 0.1 N H_2SO_4). Quinine was assumed to have a quantum yield of 0.54 when activated at 348 mμ (8). In order to check this technique, the quantum yield of tryptophan in aqueous solution and in bovine serum albumin was determined using an activation wavelength of 290 mμ. The former was found to be 0.12 (cf. 0.13 in ref. 9), while the latter was found to be 0.18 (cf. 0.18 in ref. 10). The method used to

[1] The abbreviation used is: HSA, human serum albumin.

calculate the quantum yields of the warfarin-HSA and dansylglycine-HSA complexes was essentially the same as that described by Chen and Kernohan for the dansylsulfonamide–carbonic anhydrase system (8).

A solution (2 ml) of HSA (1×10^{-5} M) containing 0.1 M sodium phosphate buffer (pH 7.4) was used for all fluorescence titrations. The drug solution (1×10^{-3} M) containing HSA (1×10^{-5} M) was added in small increments from a microsyringe. For the protein fluorescence-quenching titrations, the activation and emission wavelengths were 290 mμ and 330 mμ, respectively, while the increase in intrinsic warfarin fluorescence was observed at 400 mμ, using an activating wavelength of 320 mμ. All results were corrected for dilution and self-absorption.

The displacement of dansylglycine or warfarin from HSA by dicoumarol was measured by the fluorescence titration method previously described (4). Initial solutions (2 ml) contained HSA (1×10^{-5} M), sodium phosphate buffer (pH 7.4) (0.1 M), and either dansylglycine (2×10^{-4} M) or warfarin (1×10^{-4} M). No increase in fluorescence was observed when the concentration of warfarin or dansylglycine was doubled, indicating that saturation of the binding sites was complete. The fluorescence of the above solutions was monitored during titration with a dicoumarol solution (10^{-3} M) containing HSA (1×10^{-5} M). For dansylglycine the activation and emission wavelengths were 350 mμ and 480 mμ, respectively, while for warfarin they were 320 mμ and 400 mμ. All readings were corrected for dilution and self-absorption. Results were calculated by the method of Flanagan and Ainsworth (11).

The binding of dicoumarol to HSA was determined by the equilibrium dialysis method of Klotz *et al.* (12), and the results were plotted according to the method of Scatchard (13), using the relationship

$$\frac{n}{A} = KN + Kn$$

where n = number of moles of ligand bound per mole of protein, A = molar concentration of free ligand, K = association constant (M^{-1}), and N = number of ligand-binding sites per molecule of protein. Drug concentrations were measured with a Beckman DU spectrophotometer, using the following extinction coefficients: warfarin, 1.39×10^4 M^{-1} cm^{-1} (308 mμ); dicoumarol, 1.76×10^4 M^{-1} cm^{-1} (308 mμ); ethyl biscoumacetate, 2.02×10^4 M^{-1} cm^{-1} (305 mμ); acenocoumarin, 1.91×10^4 M^{-1} cm^{-1} (310 mμ); 4-hydroxycoumarin, 1.40×10^4 M^{-1} cm^{-1} (287 mμ).

Absorption spectra were recorded on a Shimadzu MPS-50L spectrophotometer, using 1-cm quartz cells.

RESULTS

Binding studies. The binding of dicoumarol to HSA was studied by equilibrium dialysis, and the results were plotted in the form of a Scatchard curve. It can readily be seen (Fig. 1) that HSA has three binding sites with high affinity for the drug, as well as an indeterminate number of binding sites with

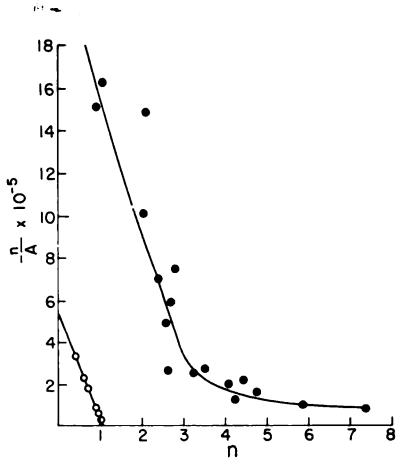

FIG. 1. *Scatchard plot of the binding of dicoumarol to HSA*

Results were obtained by equilibrium dialysis (●——●) or fluorescence quenching titration (○——○). The points for the latter curve were calculated (14) from the data presented in Fig. 4. All measurements were made in the presence of 0.1 M sodium phosphate buffer (pH 7.4). n = number of moles of dicoumarol bound per mole of HSA; A = molar concentration of free dicoumarol.

much lower affinities. The low-affinity sites could not be saturated by the addition of a large excess of dicoumarol. If it is assumed that the three strong binding sites are homogeneous and that there is no electrostatic interaction between them, an average association constant of 7.7×10^5 M^{-1} can be calculated (15).

The binding of warfarin to HSA has been studied extensively by O'Reilly *et al.* (16–18), who found that HSA has one strong binding site for the drug, with an association constant of 2.6×10^5 M^{-1}. Also present on the protein were an indeterminate number of very weak binding sites, which could not be saturated by the addition of a large excess of warfarin (16). The present equilibrium dialysis studies have essentially confirmed their results.

Circular dichroism studies. The circular dichroic spectrum of HSA exhibits two negative maxima at 209 mμ and 222 mμ, with a large positive maximum occurring at 190 mμ (19). These features are considered characteristic of the α-helical portions of a protein molecule (19). At wavelengths above 250 mμ the aromatic residues of HSA generate intrinsic Cotton effects that extend to about 310 mμ (Fig. 2) (19). Although

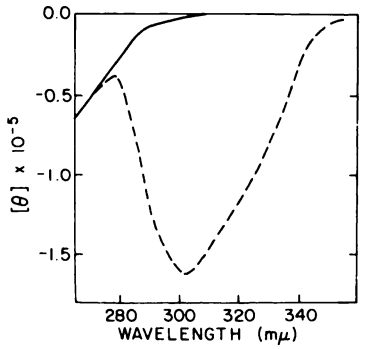

FIG. 2. *Circular dichroic spectrum of dicoumarol bound to HSA*

——, HSA (1.45×10^{-5} M); – – –, dicoumarol (5×10^{-5} M) + HSA (1.45×10^{-5} M). Molar ellipticity [θ] has been expressed in terms of the HSA concentration. All measurements were made in the presence of 0.1 M sodium phosphate (pH 7.4).

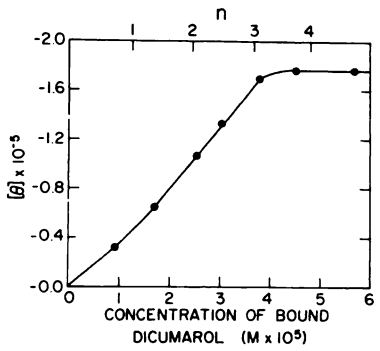

FIG. 3. *Relationship between molar ellipticity, measured at 305 mμ, and the concentration of bound dicoumarol*

Molar ellipticity [θ] was calculated with reference to the concentration of HSA, which was 1.45×10^{-5} M. All measurements were made in the presence of sodium phosphate buffer (pH 7.4). n = number of moles of drug bound per mole of protein.

dicoumarol itself is not optically active, when the drug was added to a solution of HSA a large negative Cotton effect appeared at 305 mμ (Fig. 2). Since dicoumarol has a broad ultraviolet absorption maximum between 305 and 315 mμ, there can be little doubt that this Cotton effect is extrinsic in origin. When a fixed concentration of HSA was titrated with increments of dicoumarol, the extrinsic ellipticity at 305 mμ increased sharply (Fig. 3). However, after 3 molecules of drug had been bound per molecule of protein, no further increase in ellipticity was observed (Fig. 3).

In contrast to dicoumarol, the binding of ethyl biscoumacetate, warfarin, acenocoumarin, and 4-hydroxycoumarin to HSA did not generate any extrinsic Cotton effects. Thus, although these drug molecules are also 4-hydroxycoumarin derivatives, it would appear that their interaction with HSA is fundamentally different from that of dicoumarol.

Fluorescence studies. The binding of dicoumarol to HSA quenched the tryptophan fluorescence of the protein (Fig. 4). The fluorescence titration curve (Fig. 4) suggested that quenching resulted from the

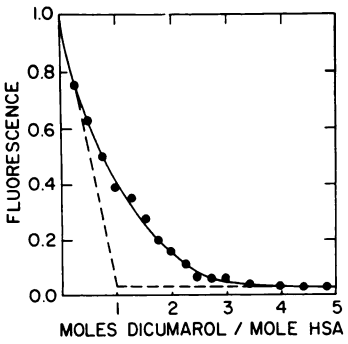

FIG. 4. *Fluorometric titration of HSA by dicoumarol*

The intensity of the tryptophan fluorescence in HSA was monitored at 335 mμ while exciting at 290 mμ. Bandwidths of excitation and emission were 12 mμ.

binding of dicoumarol to a single site on HSA. An association constant of 5.2×10^5 M^{-1} was calculated by the method of Attalah and Lata (14) (Fig. 1). The binding of warfarin to HSA also quenched the native fluorescence of the protein (Fig. 5). The fluorescence titration curve showed that quenching was associated mainly with binding to a single HSA site (Fig. 5). However, the slight downward slope of the titration curve (Fig. 5) indicated that binding of warfarin to other sites on HSA also caused some fluorescence quenching.

The quenching of protein fluorescence by the binding of warfarin was accompanied by an increase in the intrinsic fluorescence of the drug (Fig. 5). The shape and stoichiometry of the titration curve obtained by monitoring the increase in warfarin fluorescence were similar to those obtained by measuring the quenching of the native HSA fluorescence (Fig. 5). This suggested that both phenomena resulted from the same drug-protein interaction. In contrast to warfarin, the intrinsic fluorescence of dicoumarol was unchanged on binding to HSA.

The quenching of HSA fluorescence by either dicoumarol or warfarin was probably due to energy transfer from the excited state of protein tryptophan residues to the bound drug. Förster has shown (20) that in order for such a transfer to take place the emission band of the donor must overlap the absorption band of the acceptor. It can be seen clearly that this prerequisite is met by warfarin and HSA (Fig. 6). The probability of energy transfer between two molecules can be expressed in terms of the critical transfer distance, R_0, for which resonance transfer is 50% complete, by means of the equation (21, 22)

$$R_0 = \left(\frac{1.66 \times 10^{-33} \times \tau J_{\bar{\nu}}}{n^2 \times \bar{\nu}_0^2} \right)^{1/6} \quad (1)$$

where τ is the donor fluorescence decay time, $\bar{\nu}_0$ is the mean of the peak positions (in wave numbers) of the donor emission and lowest energy absorption bands, $J_{\bar{\nu}}$ is the overlap integral, and n is the refractive index.

Before the value of R_0 for the warfarin-HSA system could be calculated, the fluorescence emission spectrum of the protein (Fig. 6) had first to be corrected by multiplying by λ^2 at each wavelength (23) and then to be replotted on the wave number scale. From this curve it was possible to calculate (8) that $\bar{\nu}_0 = 32.7 \times 10^3$ cm^{-1} and $J_{\bar{\nu}} = 1.19 \times 10^{11}$ cm^3 mM^{-2}. By assuming that $n = 1.6$ and by using $\tau = 4.5$ nsec (24),

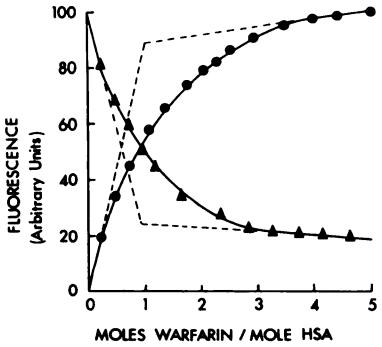

FIG. 5. *Fluorometric titration of HSA by warfarin*

The intensity of the tryptophan fluorescence (▲——▲) in HSA was monitored at 320 mμ while exciting at 290 mμ. The excitation and emission wavelengths for warfarin fluorescence (●——●) were 320 mμ and 400 mμ, respectively. Bandwidths of excitation and emission were 12 mμ.

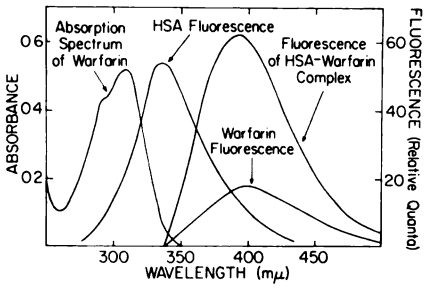

FIG. 6. *Spectra of warfarin and HSA*

The absorption spectrum of warfarin was measured in a 1-cm quartz cell, using a drug concentration of 4×10^{-4} M in the presence of 0.1 M sodium phosphate (pH 7.4). Fluorescence emission spectra were recorded using microcells (0.29×0.29 cm) and are displayed in terms of relative quanta. The concentrations were: HSA, 1.55×10^{-5} M; warfarin alone, 4×10^{-5} M; warfarin, 4×10^{-5} M, + HSA, 1×10^{-5} M. The HSA solution was activated at 290 mμ, while those containing warfarin were activated at 320 mμ. Bandwidths of excitation and emission were 12 mμ.

R_0 was calculated to be 26.2 Å. This value permitted the estimation of the mean effective transfer distance, R, between donor (tryptophan) and acceptor (warfarin) from the following formula (21).

$$1 - x = \frac{1}{(R_0/R)^6 + 1} \qquad (2)$$

where x is the fraction of photons transferred.

The quantum yield of tryptophan fluorescence in HSA was found to be 0.063 (Table 1), which is less than half that reported (10) for bovine serum albumin. Thus about 94% of the photons of light absorbed by the tryptophan moiety of HSA are lost by radiationless transitions. The quenching data (Figs. 4 and 5) are therefore only concerned with the 6% of the photons emitted as fluorescence. It is obvious, therefore, that in order to estimate the efficiency of energy transfer between tryptophan and warfarin, an alternative approach must be employed.

The fluorescence excitation spectrum of warfarin (Fig. 7) showed a close correspondence to the absorption spectrum (Fig. 6), with a maximum occurring at 310 mμ. However, in the presence of HSA the fluorescence excitation spectrum of warfarin also had a second maximum at 280 mμ (Fig. 7). While warfarin itself has little absorption at 280 mμ (Fig. 6), the tryptophan residues of HSA absorb strongly at this wavelength. This can be seen clearly in the absorption spectrum of the warfarin-HSA complex (Fig. 7). Thus photons absorbed by the protein tryptophan residues must have been nonradiatively transferred to the bound warfarin molecules, which then emitted them as fluorescence. To calculate the efficiency of this transfer, the quantum yield of fluorescence of the warfarin-HSA complex was measured, using activation wavelengths of 290 mμ and 320 mμ. The 290 mμ wavelength was chosen to avoid any contributions from protein tyrosine groups (8).

Measurements of quantum yield were made with a solution containing 1×10^{-5} M HSA, 4×10^{-5} M warfarin, and 0.1 M sodium phosphate buffer (pH 7.4). Although ultrafiltration indicated that 1.7 molecules of warfarin were bound per molecule of HSA, it was assumed that energy transfer was occurring at only one of the protein-binding sites. Figure 5 shows that this site was completely occupied by warfarin molecules

TABLE 1

Quantum yields of warfarin, HSA, and the warfarin-HSA complex

The quantum yield of the warfarin-HSA complex was determined using a solution containing 1×10^{-5} M HSA, 4×10^{-5} M warfarin, and 0.1 M sodium phosphate buffer (pH 7.4). The method used for calculation, as well as the corrections employed, is described in the text.

Compound	Exciting wavelength	λ_{max} of emission	Quantum yield
	mμ	mμ	
HSA	290	335	0.063
Warfarin	320	400	0.012
Warfarin-HSA	290	390	0.015[a]
Warfarin-HSA	290	335	0.013[a]
Warfarin-HSA	320	390	0.090

[a] Quantum yield calculated on the basis of the tryptophan absorption.

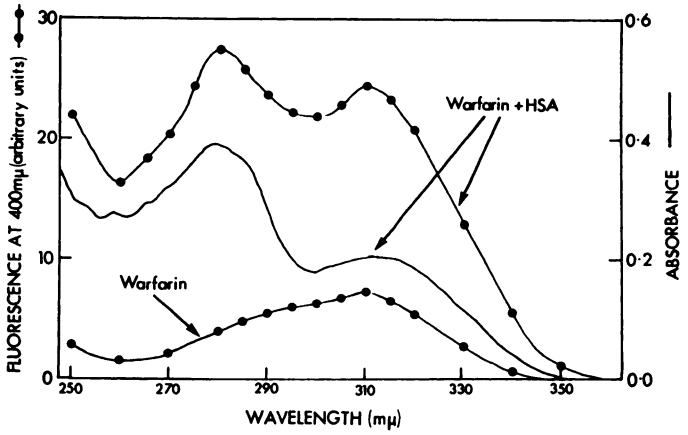

FIG. 7. *Spectra of warfarin and the warfarin-HSA complex*

The activation spectra of warfarin and the warfarin-HSA complex (●——●) have been corrected for the intensity of the activating light. Concentrations were: warfarin alone, 4×10^{-5} M; warfarin, 4×10^{-5} M, + HSA, 1×10^{-5} M. The absorption spectrum of the warfarin-HSA complex (——) was measured in a 1-cm quartz cell, using a solution containing 1×10^{-5} M HSA and 4×10^{-5} M warfarin. A blank containing 2.3×10^{-5} M warfarin was used to compensate for absorption due to the free drug. All solutions contained 0.1 M sodium phosphate buffer (pH 7.4).

under the conditions used in this experiment. The quantum yield of warfarin bound to this unique site [$Q_{(W)}$] was determined by activating the drug-protein complex at 320 mμ. Appropriate corrections were made for the fluorescence of the unbound warfarin. Corrections were also made for the fluorescence of warfarin bound to HSA at sites other than those at which fluorescence enhancement occurred, by assuming that the quantum yield was the same as that of the unbound drug. The fluorescence emission spectrum resulting from activation at 290 mμ was first corrected for the unquenched tryptophan fluorescence. Then the quantum yield of energy absorbed by the tryptophans then transferred to the bound warfarin molecules [$Q_{(H)}$] was calculated from the equation

$$A_{290(Wf)} \cdot Q_{(Wf)} + A_{290(W)} \cdot Q_{(W)} + A_{290(H)} \cdot Q_{(H)} = A_{290(M)} \cdot Q_{290(M)}$$

where $A_{290(Wf)}$ = optical density at 290 mμ of the unbound warfarin + optical density at 290 mμ of the warfarin bound to HSA sites at which no fluorescence enhancement occurred; $Q_{(Wf)}$ = quantum yield of unbound warfarin; $A_{290(W)}$ = optical density at 290 mμ of warfarin bound to HSA sites at which fluorescence enhancement occurred; $A_{290(H)}$ = optical density of the HSA at 290 mμ; $A_{290(M)}$ = optical density at 290 mμ of the warfarin-HSA mixture; and $Q_{290(M)}$ = quantum yield of the warfarin-HSA mixture activated at 290 mμ. The results of these experiments are given in Table 1. It can be seen, therefore, that the photons absorbed by the tryptophan are transferred to the bound warfarin molecules with a yield of 16.6% (0.015/0.090 × 100). Because the quantum yield of tryptophan in the complex was 0.015, only about 18% (16.6 + 1.5) of the photons absorbed by the complex can be accounted for. Thus about 82% of the photons are still lost in radiationless transitions in comparison to the 94% lost from the uncomplexed protein. The mean effective transfer distance was calculated from Eq. 2 to be 34.5 Å.

The binding of dansylglycine to a single site on HSA results in a large increase in the fluorescence quantum yield of the ligand, together with a marked blue shift of the fluorescence emission spectrum (3). This observation suggests that the HSA binding

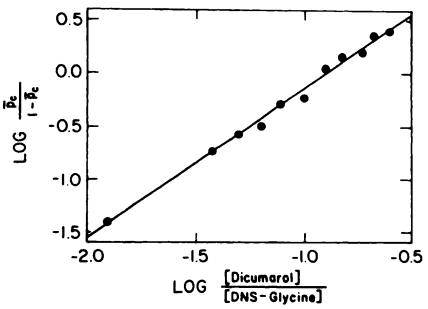

FIG. 8. *Hill plot for the replacement of dansylglycine by dicoumarol*

The concentrations were: HSA, 1×10^{-5} M; dansylglycine (DNS-glycine), 2×10^{-4} M; sodium phosphate buffer (pH 7.4), 0.1 M. Activation and emission wavelengths were 350 mμ and 480 mμ, respectively. Bandwidths for excitation and emission were 12 mμ. $\bar{p}_c$ = fractional occupation of dansylglycine-binding sites by dicoumarol.

site for dansylglycine is located in a hydrophobic region of the protein. This same site also binds phenylbutazone and flufenamic acid, since these anionic drugs have been shown (3, 4) to displace dansylglycine from HSA competitively. Although dicoumarol also displaced dansylglycine from HSA (Fig. 8), warfarin did not. The association constant of dansylglycine for HSA was found by fluorescence titration (3) to be 1.45×10^5 M^{-1}. From this value the association constant of dicoumarol for HSA was calculated (11) from the data in Fig. 8 to be 1.12×10^6 M^{-1}. However, dicoumarol did competitively displace warfarin from HSA (Fig. 9). If it is assumed that dicoumarol has an association constant of 7.7×10^5 M^{-1} (Fig. 1), the association constant for warfarin would be 2.7×10^5 M^{-1} (11). This value is similar to that obtained by O'Reilly (18) from equilibrium dialysis studies.

Since energy transfer between tryptophan and the dansyl group have already been reported (8, 25), it was of interest to estimate the transfer distance of dansylglycine in a complex with HSA for comparison with the value already calculated for warfarin. For the dansylglycine-HSA system it was calculated (8) that $J_{\bar{\nu}} = 7.3 \times 10^{10}$ cm^3 mM^{-2} and $\bar{\nu}_0 = 32.8 \times 10^3$ cm^{-1}. By assuming that $n = 1.6$ and $\tau = 4.5$ nsec, R_0 was found to be 24.2 Å. From Table 2 the efficiency of transfer between tryptophan and dansylglycine was 52.8% (0.235/0.443 × 100). R was calculated to be 23.7 Å by means of Eq. 2.

The fluorescent yield of warfarin increased when the drug was dissolved in certain solvents, such as dimethylformamide and glycerol, but was relatively unaffected by

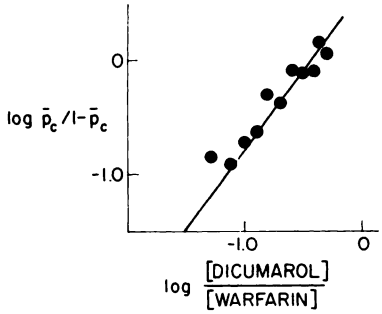

FIG. 9. *Hill plot for the replacement of warfarin by dicoumarol*

The concentrations were: HSA, 1×10^{-5} M; warfarin, 1×10^{-4} M; sodium phosphate buffer (pH 7.4), 0.1 M. Activation and emission wavelengths were 320 mμ and 400 mμ, respectively. Bandwidths for excitation and emission were 12 mμ. $\bar{p}_c$ = fractional occupation of warfarin-binding sites by dicoumarol.

TABLE 2

Quantum yields of dansylglycine, HSA, and the dansylglycine-HSA complex

The quantum yield of the dansylglycine-HSA complex was determined using a solution containing 1×10^{-5} M HSA, 1×10^{-5} M dansylglycine, and 0.1 M sodium phosphate buffer (pH 7.4). Under these conditions, ultrafiltration indicated that the concentration of free dansylglycine was 0.36×10^{-5} M. Quantum yields were calculated by the method of Chen and Kernohan (8).

Compound	Exciting wavelength	λ_{max} of emission	Quantum yield
	mμ	mμ	
HSA	290	335	0.077
Dansylglycine	350	580	0.051
Dansylglycine-HSA	290	480	0.235
Dansylglycine-HSA	350	480	0.443
Dansylglycine-HSA	290	335	0.046

TABLE 3

Quantum yield of warfarin dissolved in various solvents

Solvent dielectric constant	Solvent[a]	Quantum yield	Emission λ_{max}
Debye units			*mμ*
78.5	Water (0.05 M sodium phosphate, pH 7.4)	0.013	400
31.2	Methanol	0.016	400
25.8	Ethanol	0.033	400
19.2	1-Butanol	0.039	400
37.0	Ethylene glycol	0.047	400
32.0	Propylene glycol	0.061	400
42.5	Glycerol	0.104	392
3.0	Dioxane	0.022	412
36.7	Dimethylformamide	0.154	415
6.1	Ethyl acetate	0.010	400
	Cetrimide (0.05% in 0.05 M sodium phosphate, pH 7.4)	0.041	400

[a] Solutions were made by adding 0.1 ml of a stock solution (made up in 0.01 N NaOH) to 9.9 ml of solvent. Fluorescence was excited at 320 mμ.

others, e.g., methanol and ethyl acetate (Table 3). There appeared to be little correlation between the dielectric constant of a solvent and its effect on the fluorescence of warfarin (Table 3). The fluorescent yield of warfarin also increased in the presence of the cationic detergent cetyltrimethylammonium bromide (cetrimide) (Table 3), but only when the detergent was present in excess of the critical micellar concentration. No increase in fluorescent yield was observed in the presence of sodium lauryl sulfate, an anionic detergent. The fluorescent yield of dicoumarol was unchanged when the drug was dissolved in the solvents listed in Table 3. The addition of cetyltrimethylammonium bromide to aqueous solutions of dicoumarol also left the fluorescent yield of the drug unchanged.

DISCUSSION

When a symmetrical chromophore is perturbed by a nearby asymmetrical center, a Cotton effect may be generated and optical activity observed (26). If the asymmetrical center is present in the same molecule as the chromophore it perturbs, the resultant Cotton effect is termed intrinsic, whereas if it is present in another molecule, the term extrinsic is employed. Extrinsic Cotton effects are often generated by the binding of small chromophoric ligands to biological macromolecules, such as proteins and nucleic acids (27). Since such extrinsic Cotton effects apparently reflect the asymmetry of specific ligand-binding sites, they can be used to probe such sites.

The factors that govern the sign and magnitude of extrinsic Cotton effects have been recently discussed (1–4). Briefly, it may be stated that well-defined symmetry rules divide the space around a chromophore into regions of positive and negative contribution to a Cotton effect (26). The generation of an extrinsic Cotton effect must therefore depend on the ability of a ligand to take up a preferred orientation with respect to the binding site. At the same time the ligand-macromolecule complex must be rigid enough to preserve this special spatial arrangement.

The strong ultraviolet absorption maximum of dicoumarol (305–315 mμ) undoubtedly results from $\pi \rightarrow \pi^*$ transitions in the phenyl rings of the drug. The generation of a large negative extrinsic Cotton effect at 305 mμ when dicoumarol was bound to HSA must therefore have resulted from an interaction of these $\pi \rightarrow \pi^*$ transitions with an asymmetrical locus at the protein-binding site. If dicoumarol obeys the planar symmetry rule (26), the plane of the π-electron system would be the nodal plane. Placing an asymmetrical center on one side of the molecule will give a Cotton effect; moving it to the other side will reverse the effect. The generation of an extrinsic Cotton effect by the binding of dicoumarol to HSA may simply mean that one side of the drug molecule interacts preferentially with the protein binding site. The titration curve (Fig. 3) clearly shows that extrinsic Cotton effects were generated only when dicoumarol

was bound to the high-affinity sites; drug complexes with the low-affinity sites were optically inactive. Equilibrium dialysis measurements indicated that these low-affinity sites could not be saturated by adding a large excess of dicoumarol. O'Reilly, who has reported similar results for warfarin, has suggested (16) that drug binding to HSA may cause conformational changes that expose more binding sites. The inability of these sites to induce optical activity in dicoumarol suggests that they are relatively nonspecific.

The inability of both 4-hydroxycoumarin and warfarin to generate extrinsic Cotton effects on binding to HSA must mean that both coumarin rings of dicoumarol are necessary for optical activity. O'Reilly *et al.* have proposed (17) that electrostatic interactions play only a minor role in the binding of warfarin to HSA, and suggested instead that hydrophobic and hydrogen bonding are more important. However, it has been found that both electrostatic and hydrophobic interactions are often important for the generation of extrinsic Cotton effects by drug-protein complexes (1–4). Dicoumarol has two enolic hydroxyl groups, making possible a 2-point electrostatic interaction of the drug with HSA. If such an interaction were reinforced by short-range dispersive forces involving the planar benzene rings, it is quite possible that dicoumarol could maintain a preferred orientation to the HSA binding site. Steric factors are also important, since the introduction of a carbethoxy group into the bridging methylene group of dicoumarol to give ethyl biscoumacetate resulted in a loss of optical activity.

Fluorescence. Although equilibrium dialysis indicated (Fig. 1) that HSA has three binding sites with a high affinity for dicoumarol, energy transfer between drug and protein tryptophan occurred at only one of these sites (Fig. 4). A single binding site also seemed to be involved in the quenching of HSA fluorescence by warfarin (Fig. 5). The fluorescence titration curve for warfarin also showed that some quenching occurred when the drug became bound to other sites (Fig. 5). This may have meant that some energy transfer also occurred at these sites. Alternatively, the binding of warfarin could have decreased the fluorescence of HSA by changing the conformation of the protein (14). O'Reilly has suggested that such conformational changes occur at high warfarin to HSA ratios (16).

The re-emission by warfarin of energy absorbed from the HSA tryptophans permitted the calculation of a mean effective transfer distance of 34.5 Å by the method of Förster (20–22). This value is an estimate of the distance between the single tryptophan residue of HSA (28) and the bound warfarin molecule. It should be emphasized, however, that this distance is only an approximation, since several assumptions have been made in order to use Eq. 1. For example, it has been assumed that the orientation of tryptophan is random with respect to the drug, and that the refractive index of the protein is 1.6. Nevertheless, as Chen and Kernohan have pointed out (8), an error of 100% in the estimation of $J\bar{\nu}/n^2\bar{\nu}_0^2$ would result in only a 10% error in R_0. These same reservations also apply to the value of 23.7 Å calculated as the mean effective transfer distance between the tryptophan of HSA and bound dansylglycine. If HSA is an anhydrous sphere of mol wt 69,000, it would have a diameter of 56 Å (29). The difference between the transfer distances of warfarin and dansylglycine is therefore sufficiently large to suggest that these molecules are bound at different sites. The inability of warfarin to displace dansylglycine bound to HSA certainly supports this hypothesis.

Although warfarin and dansylglycine do not share the same binding site, both ligands are competitively displaced from HSA by dicoumarol (Figs. 8 and 9). However, HSA has three high-affinity sites for dicoumarol (Fig. 1), so that one could be shared by warfarin while a second was being shared by

dansylglycine. An alternative explanation may be that warfarin and dansylglycine occupy binding sites sufficiently close together so that dicoumarol can interact with both sites simultaneously. Certainly the circular dichroism studies suggest that both coumarin rings of dicoumarol take part in the binding of the drug to HSA. Flanagan and Ainsworth have suggested that the trypan blue molecule can simultaneously occupy two sites that also bind 1-anilinonaphthalene-8-sulfonic acid (11).

The multiple binding sites for dicoumarol on HSA may also explain why fluorescence titration (Fig. 4) and dansylglycine displacement (Fig. 8) gave association constants different from that obtained by equilibrium dialysis (Fig. 1). It should be pointed out, however, that the association constant from the equilibrium dialysis experiments was calculated on the assumption that the binding sites were homogeneous and may therefore only represent an average value.

The fluorescence of warfarin increased when the drug was dissolved in certain organic solvents, although there was a poor correlation between dielectric constant and quantum yield (Table 3). However, Corn and Berberich have shown (30) that the fluorescence of warfarin decreases in acid solution, so that it is possible that these measurements were complicated by ionization of the drug. The large increase in fluorescent yield observed when warfarin was dissolved in glycerol undoubtedly was due to solvent viscosity rather than solvent polarity (31). In spite of these results, the increase in the fluorescent yield of warfarin on binding to HSA, together with the blue shift in the fluorescence emission maximum of the drug (Table 1), does suggest that warfarin is located in a hydrophobic region of the protein (32). The increase in the fluorescent yield of warfarin when the drug entered the cetrimide micelle also indicated that a hydrophobic environment can enhance fluorescence. These results are in accord with the work of O'Reilly *et al.*, who have shown, by means of heat burst calorimetry (17), the existence of hydrophobic interactions in the warfarin-HSA complex. Unlike warfarin, the fluorescent yield of dicoumarol did not increase when the drug was dissolved in organic solvents or aqueous solutions of detergents. This explains why the fluorescent yield of dicoumarol did not increase on binding to HSA.

These experiments illustrate the usefulness of such techniques as circular dichroism and fluorescence spectroscopy for the study of drug interactions with serum albumin. As separation and isolation techniques improve so that other biomolecules become available, spectroscopic techniques will undoubtedly play an important role in studying their interactions with drug molecules.

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