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# Optical Studies on the Mechanism of the Interaction of the Enantiomers of the Anticoagulant Drugs Phenprocoumon and Warfarin with Human Serum Albumin

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SUMMARY

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The interaction of the racemates and enantiomers of phenprocoumon and warfarin with human serum albumin was investigated using equilibrium dialysis and circular dichroism measurements. It was found that the human serum albumin molecule binds phenprocoumon stereospecifically, with about a 2-fold higher association constant for the $S(-)$ isomer. The stereospecificity of phenprocoumon binding is more pronounced than that of the warfarin enantiomers. Binding to human serum albumin induces Cotton effects in the enantiomers of phenprocoumon and warfarin, which superimpose upon the intrinsic Cotton effects of the drugs. The induced Cotton effects are similar in sign for the two phenprocoumon isomers, but dissimilar for the warfarin isomers. Therefore it is concluded that the orientation of the 4-hydroxycoumarin nucleus is the same for the phenprocoumon isomers, but different for the warfarin isomers, when bound to human serum albumin. This can explain differences in the stereoselective binding of the two drugs to human serum albumin.

## INTRODUCTION

Because the extent of binding of several classes of drugs to albumin correlates with their intrinsic activity, it has often been suggested that the drug-albumin complex may serve as a model for studying drug-receptor interactions. A case in point is the group of coumarin anticoagulants (1-3). The inadequacy of the use of albumin as a model for receptor sites was demonstrated for the enantiomers of warfarin, which vary widely in their intrinsic activities (4, 5) but differ only slightly in their affinities for human serum albumin (3, 6). For the structurally related drug phenprocoumon, however, the more strongly albumin-bound enantiomer also showed greater intrinsic activity (7). Therefore, when albumin binding is used as a model for drug-receptor interactions, parameters other than affinities may be important: for example, the mechanism of binding.

Circular dichroism measurements have proved extremly useful in gaining insight into the molecular mechanism of binding

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of drugs (8–10) and enantiomers of drugs (11, 12) to serum albumin. Thus, in the present study, we investigated the mechanisms of stereoselective binding of phenprocoumon and warfarin enantiomers to human serum albumin, using equilibrium dialysis and optical methods.

## MATERIALS AND METHODS

*Materials.* HSA[3] was obtained from Behringwerke, Marburg (*trocken, reinst;* electrophoretic purity, 100%). Racemic phenprocoumon and *S*(−)- and *R*(+)-phenprocoumon were gifts of Hoffman-La Roche, Basel. Racemic warfarin was obtained from Merrell, Gross-Gerau. *S*(−)- and *R*(+)-Warfarin were kind gifts from Dr. A. Breckenridge, Royal Postgraduate Medical School, London. 4-Hydroxycoumarin was obtained from Schuchardt, München. All other chemicals were of reagent grade. All solutions were made with deionized water.

*Circular dichroism measurements.* CD measurements were made at 27° with a Cary 61 CD spectropolarimeter calibrated with *d*-camphorsulfonic acid. All spectra were recorded in cylindrical cells with a 10-mm path length, using a full-scale deflection of 0.05° $\theta$ and a spectral bandwidth of 2 nm. Results are expressed as molar ellipticities, $[\theta]$ (degrees per square centimeter per decimole), calculated with reference either to the HSA concentration, using a molecular weight of 69,000, or to the total drug concentration or the concentration of drug bound. The reported molar ellipticities are difference values, using as blanks the molar ellipticities of HSA at the same wavelength. The solutions for CD measurements were prepared as described elsewhere (10). The final HSA concentration was always 30 $\mu$M (0.207%), and the drug concentrations were 30 $\mu$M or 60 $\mu$M. All solutions were prepared in M/15 phosphate buffer, adjusted to the desired pH with 1 M HCl or 1 M NaOH.

Anisotropy factors ($g$ values) were calculated following the method of Chignell (9), using the equation

$$g = \frac{[\theta]}{3300 \times \epsilon}$$

where $[\theta]$ is the molar ellipticity of the drugs and $\epsilon$ is the molar extinction coefficient. For the calculation of $g$ values in the presence of HSA, the molar extinction coefficients of the bound drugs were used and the molar ellipticities were calculated for the concentrations of bound drugs. In the case of the phenprocoumon and warfarin enantiomers in the presence of HSA, the intrinsic Cotton effects of the unbound enantiomers were subtracted from the observed ellipticities.

*Ultraviolet absorption measurements.* Ultraviolet absorption was measured with a Gilford spectrophotometer, model 2400. Molar extinction coefficients of the coumarins were determined with a drug concentration of 20 $\mu$M, either in phosphate buffer, pH 7.40, using the same buffer as reference, or in the presence of 30 $\mu$M HSA, using the same albumin solution as reference.

*Albumin binding measurements.* Binding of the anticoagulants to HSA was determined by equilibrium dialysis. The solutions were prepared in the same manner as for CD measurements. Portions (2 ml) of the solutions were transferred to dialysis cells and dialyzed for 16 hours at 25° against 2 ml of phosphate buffer, pH 7.40, using Cellophane dialysis membranes (Union Carbide). The drug concentrations were determined fluorometrically, by a modification of the method of Seiler and Duckert (13), as described previously (7).

## RESULTS

*Dialysis experiments.* The binding of *S*(−)-, *R*(+)-, and racemic phenprocoumon to HSA was investigated at six different drug concentrations, and the results were plotted according to Scatchard (14). The results (Fig. 1) were analyzed using a nonlinear least-squares curve-fitting procedure (15). The best curve-fitting model for all three derivatives was obtained by assuming $n_1 = 1$ and $n_2 = 4$. The calculated binding constants are summarized in Table 1. The calculated associa-

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

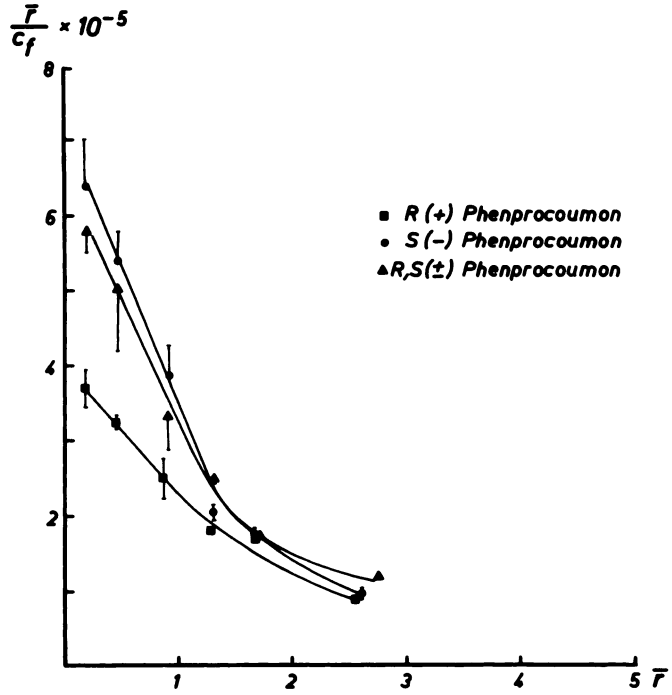


FIG. 1. *Scatchard plot of binding of phenprocoumon and its enantiomers to HSA*

Ordinate: $\bar{r}/c_f$ (liters per mole); $c_f$ = molar concentration of the free drug; $\bar{r}$ = moles of drug bound per mol of albumin. Abscissa: $\bar{r}$. Each point represents the mean ± standard error of four or five determinations. Standard errors for the $\bar{r}$ values are smaller than the symbols.

TABLE 1

*Binding constants of phenprocoumon and its enantiomers*

$\beta$ is the percentage of drug bound at total concentration $c$; $n$ is the number of binding sites in the HSA molecule; $k$ is the association constant.

| Phenprocoumon | $\beta$ ($n$ = 5) | | | $n_1$ | $k_1$ | $n_2$ | $k_2$ |
|---|---|---|---|---|---|---|---|
| | $c$ = 15 μM | $c$ = 30 μM | $c$ = 60 μM | | | | |
| | % ± SE | % ± SE | % ± SE | | $M^{-1} \times 10^{-4}$ | | $M^{-1} \times 10^{-4}$ |
| $S(-)$ | 94.08 ± 0.44 | 91.94 ± 0.52 | 84.06 ± 0.67 | 1 | 60.68 | 4 | 2.54 |
| $R(+)$ | 90.66 ± 0.21[a] | 87.84 ± 1.05[b] | 83.34 ± 0.58[c] | 1 | 35.45 | 4 | 2.50 |
| $RS(\pm)$ | 93.32 ± 1.06[c] | 90.92 ± 0.12[c] | 83.58 ± 0.12 | 1 | 46.74 | 4 | 3.33 |

[a] $p < 0.001$ compared with the $S(-)$ isomer.

[b] $p < 0.01$ compared with the $S(-)$ isomer.

[c] Not significant; $p > 0.05$.

tion constants suggest a greater affinity of the $S(-)$ isomer for the high-affinity binding site, whereas the second set of binding sites seems to lack stereospecificity. The results for racemic phenprocoumon always fell between those found for the two isomers (Fig. 1 and Table 1).

Binding of the warfarin enantiomers to HSA was investigated under similar experimental conditions. It was found that the $S(-)$ isomer is bound slightly but insignificantly more strongly to HSA (Table 2).

*Circular dichroism measurements.* The intrinsic CD spectra of the phenprocoumon and warfarin isomers are shown in the upper parts of Figs. 2 and 3. The intrinsic Cotton effects of the two $S(-)$ and

TABLE 2

*Binding data for warfarin enantiomers*

$\beta$ is the percentage of drug bound at total concentration $c$.

| Warfarin | $\beta$ | |
|---|---|---|
| | $c$ = 15 $\mu$M | $c$ = 60 $\mu$M |
| | % ± *SE* | % ± *SE* |
| *S*(−) | 89.88 ± 0.36 | 77.69 ± 0.31 |
| *R*(+) | 89.08 ± 0.61[a] | 76.73 ± 0.40[a] |

[a] Not significant; $p > 0.05$.

the two *R*(+) enantiomers are of equal sign and similar in wavelength position and intensity, which supports the designated configuration of the enantiomers (16, 17). In buffer at pH 7.40, racemic phenprocoumon and racemic warfarin did not show any intrinsic Cotton effects.

Binding to HSA changes the circular dichroism spectra of both phenprocoumon enantiomers (Fig. 2, upper). The changes in Cotton effects observed with binding to HSA are similar in sign and wavelength position for both enantiomers (Fig. 2, lower). Similar effects were found for the binding of racemic phenprocoumon to HSA (Fig. 2, lower). The molar ellipticities at the wavelengths of the CD maxima of the drugs are summarized in Table 3. The *g* values, or anisotropy factors, of the isomers in buffer or bound to HSA (corrected for binding-induced changes in the molar extinction coefficients of the drugs) differ in the same way as molar ellipticities (Table 3).

The CD spectra of the warfarin enantiomers also change upon binding to HSA (Fig. 3, upper), but in a substantially different way from phenprocoumon. Whereas the wavelength positions are similar for the phenprocoumon and warfarin isomers alone, the signs of the induced Cotton effects differ for the two drugs. Furthermore, whereas the induced Cotton effects of *R*(+)-warfarin and racemic warfarin are qualitatively similar, there are differences in the signs of the induced effects between *R*(+)- and *S*(−)-warfarin (Fig. 3, lower). The molar ellipticities and anisotropy factors of warfarin and its enantiomers in buffer and bound to HSA are summarized in Table 4.

In experiments using phenprocoumon and warfarin isomer concentrations of 30 $\mu$M instead of 60 $\mu$M, but with the same HSA concentration, qualitatively similar changes in the intrinsic CD spectra of all four derivatives were found.

Binding to HSA of 4-hydroxycoumarin, the precursor of the anticoagulants used induces Cotton effects in the symmetrical molecule (Fig. 4). The wavelength positions of the observed Cotton effects can be compared with the wavelength positions of the CD spectra of the phenprocoumon and warfarin isomers in buffer and bound to HSA. The sign of the induced CD maximum near 310 nm of 4-hydroxycoumarin bound to HSA was reported by Perrin and Nelson (18) to be negative at pH 7.40. We repeated our measurements several times, and always found positive values. A possible explanation could be that Perrin and Nelson (18) used different concentrations and another albumin than those in this study.

## DISCUSSION

It is obvious from the dialysis experiments that the high-affinity binding site of HSA binds the phenprocoumon isomers stereoselectively. From the limited data available it seems that the second set of binding sites lacks stereoselectivity. Thus differences in the extent of binding between the enantiomers will be apparent only at low drug to albumin ratios, where the drug is bound predominantly to the high-affinity site. This explains the finding that stereoselective binding of a given concentration of the phenprocoumon isomers could be observed only at high albumin concentrations (7). The stereospecificity of the binding of the phenprocoumon isomers is more prounounced than that of the warfarin isomers, as found in this study (Table 2) and reported by O'Reilly (3) and by Sellers and Koch-Weser (6).

The binding of phenprocoumon and warfarin isomers to HSA produces new Cotton effects in addition to the intrinsic Cotton effects (Figs. 2 and 3; Tables 3 and 4). These effects could be explained by several different mechanisms. (a) Drug-induced changes in the intrinsic Cotton effects of

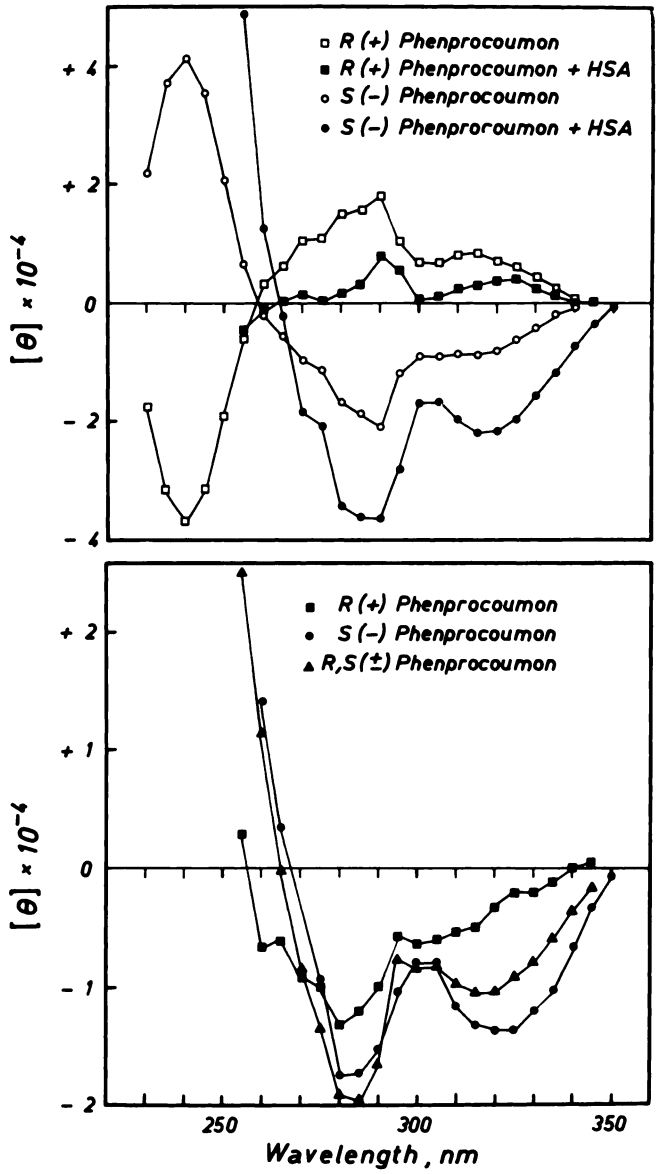


FIG. 2. *Circular dichroism spectra of phenprocoumon and its isomers in buffer and in the presence of HSA*
Upper: CD spectra of the phenprocoumon enantiomers (60 μM) in buffer and bound to HSA, with the Cotton effects of HSA subtracted in the latter case. Lower: CD spectra of racemic phenprocoumon (60 μM) and the phenprocoumon isomers (60 μM) bound to HSA. In the case of racemic phenprocoumon the Cotton effects of HSA alone have been subtracted, and in the case of the isomers the Cotton effects of HSA and of the isomers alone have been subtracted. Ordinates: molar ellipticity calculated with respect to the HSA concentration (30 μM). Abscissae: wavelength. Each point represents the mean of five determinations.

the albumin: this can be excluded, since the induced CD maxima correspond with the intrinsic CD maxima of the isomers. (b) A change in pertubation of the enantiomers of the drug by its own asymmetrical carbon atom: this can also be excluded, since the induced CD spectrum of the symmetrical 4-hydroxycoumarin molecule is qualitatively similar regarding the wavelength positions of the three forms (Fig. 4)

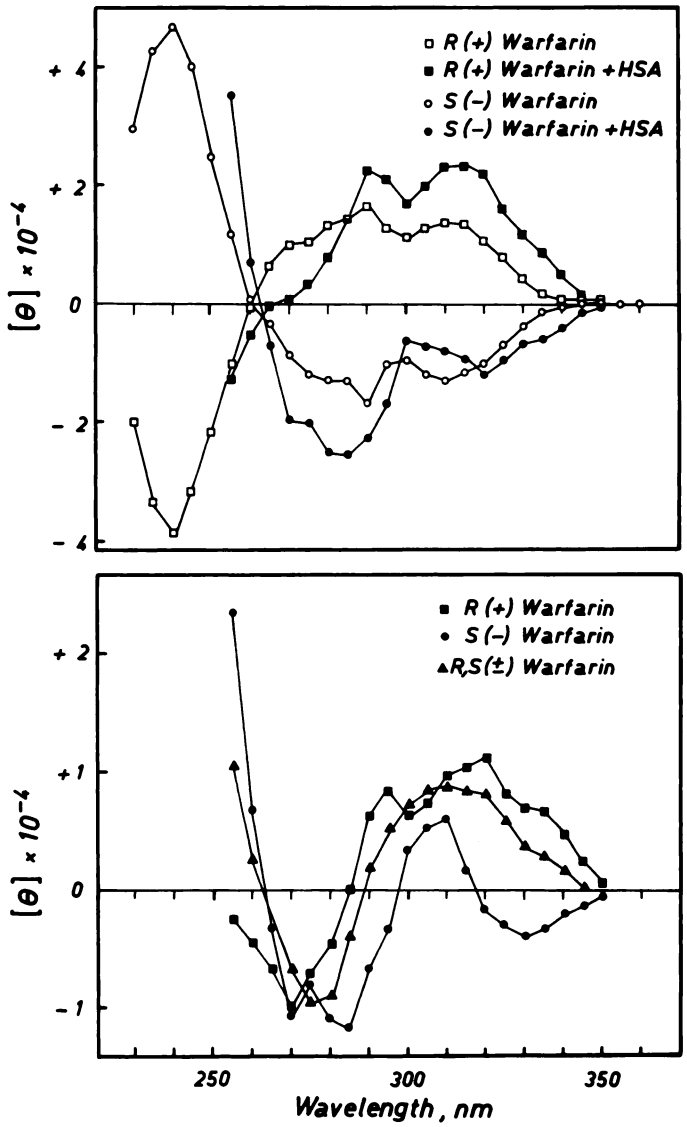


FIG. 3. *Circular dichroism spectra of warfarin and its isomers in buffer and in the presence of HSA*
Upper: CD spectra of the warfarin enantiomers (60 μM) in buffer and bound to HSA, with the Cotton effects of HSA subtracted in the latter case. Lower: CD spectra of racemic warfarin (60 μM) and the warfarin isomers (60 μM) bound to HSA. In the case of racemic warfarin the Cotton effects of HSA alone have been subtracted, and in the case of the isomers the Cotton effects of HSA and of the isomers alone have been subtracted. Ordinates: molar ellipticity calculated with respect to the HSA concentration (30 μM). Abscissae: wavelength. Each point represents the mean of three determinations.

and since the signs of the binding-induced effects are mainly similar for each pair of enantiomers. (c) A binding-induced change in the molar extinction coefficients of the drugs, causing different intrinsic Cotton effects of the isomers when bound: this mechanism is unlikely, since both the anisotropy factors and the molar ellipticities change in a similar way when the drugs are bound to albumin. (d) A perturbation of the electronic transitions of the drugs bound by an asymmetrical locus of the protein, which superimposes upon the intrinsic Cotton effects of the enantiomers:

TABLE 3

*Optical data for HSA-phenprocoumon complex*

[θ] is the molar ellipticity calculated with respect to the drug concentration (60 μM); *g* is the anisotropy factor calculated with respect to the drug concentration (60 μM) for the isomers in buffer and with respect to the concentration of bound drug for drugs in the presence of HSA.

| Phenprocoumon | λ | Buffer, pH 7.40 | | | HSA, 30 μM | | |
|---|---|---|---|---|---|---|---|
| | | [θ] | *n* | *g* | [θ] | *n* | *g* |
| | *nm* | $\times 10^{-4} \pm SE$ | | $\times 10^{5}$ | $\times 10^{-4} \pm SE$ | | $\times 10^{5}$ |
| *s*(−) | 310 | −0.40 ± 0.03 | 8 | −8.3 | −0.98 ± 0.03[a] | 8 | −24.5 |
| | 290 | −0.99 ± 0.03 | 8 | −27.8 | −2.03 ± 0.09[a] | 8 | −78.6 |
| | 270 | −0.49 ± 0.02 | 8 | −40.1 | −0.76 ± 0.02[a] | 8 | −70.1 |
| *R*(+) | 310 | +0.35 ± 0.01 | 8 | +6.9 | +0.19 ± 0.02[a] | 8 | −10.8 |
| | 290 | +0.90 ± 0.02 | 8 | +23.9 | +0.16 ± 0.05[a] | 8 | +0.6 |
| | 270 | +0.47 ± 0.01 | 8 | +37.5 | −0.15 ± 0.02[a] | 8 | −23.8 |
| *RS*(±) | 310 | | | | −0.53 ± 0.01 | 6 | −13.4 |
| | 290 | | | | −0.84 ± 0.01 | 6 | −32.6 |
| | 270 | | | | −0.45 ± 0.03 | 6 | −45.6 |

[a] $p < 0.001$ compared with the values for the isomers in buffer.

TABLE 4

*Optical data for HSA-warfarin complex*

[θ] is the molar ellipticity calculated with respect to the drug concentration (60 μM); *g* is the anisotropy factor calculated with respect to the drug concentration (60 μM) for the isomers in buffer and with respect to the concentration of bound drug for drugs in the presence of HSA.

| Warfarin | λ | Buffer, pH 7.40 | | | HSA, 30 μM | | |
|---|---|---|---|---|---|---|---|
| | | [θ] | *n* | *g* | [θ] | *n* | *g* |
| | *nm* | $\times 10^{-4} \pm SE$ | | $\times 10^{5}$ | $\times 10^{-4} \pm SE$ | | $\times 10^{5}$ |
| *S*(−) | 310 | −0.65 ± 0.02 | 6 | −13.8 | −0.47 ± 0.02[a] | 6 | −8.9 |
| | 290 | −0.79 ± 0.02 | 6 | −20.3 | −1.10 ± 0.03[a] | 6 | −34.2 |
| | 270 | −0.44 ± 0.02 | 6 | −29.6 | −0.91 ± 0.05[a] | 6 | −70.3 |
| *R*(+) | 310 | +0.66 ± 0.02 | 6 | +12.9 | +1.05 ± 0.03[a] | 6 | +23.2 |
| | 290 | +0.80 ± 0.05 | 6 | +18.9 | +1.11 ± 0.02[a] | 6 | +32.8 |
| | 270 | +0.46 ± 0.01 | 6 | +29.7 | 0.00 ± 0.06[a] | 6 | −9.2 |
| *RS*(±) | 310 | | | | +0.44 ± 0.02 | 6 | +13.6 |
| | 290 | | | | +0.11 ± 0.01 | 6 | +4.8 |
| | 270 | | | | −0.32 ± 0.04 | 6 | −35.7 |

[a] $p < 0.001$ compared with the values for the isomers in buffer.

such extrinsic Cotton effects seem to be the most likely explanation.

The phenprocoumon and warfarin enantiomers exhibit three extrinsic CD bands in the wavelength region between 350 and 250 nm. The wavelength positions of these CD maxima correspond to those of the intrinsic CD maxima of the enantiomers and to three bands in the ultraviolet absorption spectrum reported for warfarin (19). A qualitatively similar ultraviolet spectrum was found for 4-hydroxycoumarin (19) and this substance also shows extrinsic Cotton effects at all three wavelength postions (Fig. 4). Therefore we conclude that the three ellipticity bands of the drugs observed between 350 and 250 nm are due to electronic transitions in the 4-hydroxycoumarin moieties of the substances. The ultraviolet absorption band near 290 nm cannot be found for the coumarin ring alone (19), but only appears when a hydroxyl

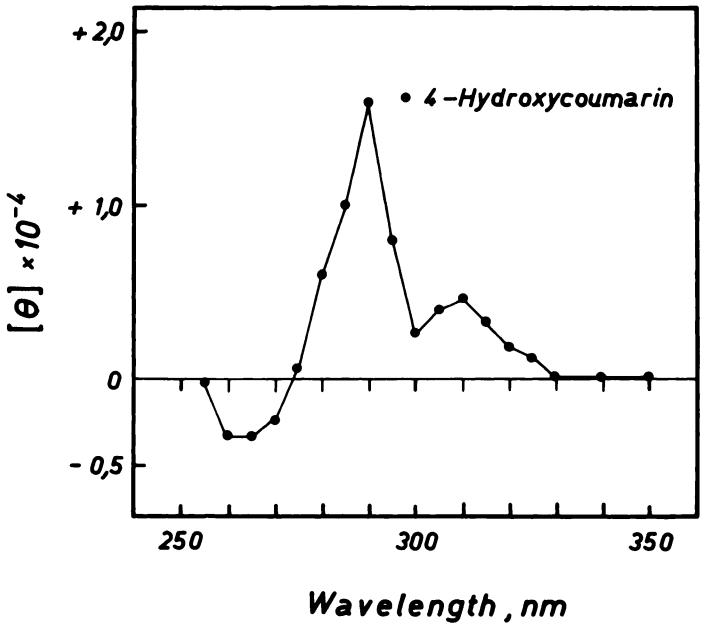


FIG. 4. *Circular dichroism spectrum of 4-hydroxycoumarin (60 μM) in the presence of HSA*

Ordinate: molar ellipticity calculated with respect to the HSA concentration (30 μM). Abscissa: wavelength. Each point represents the mean of six determinations. The molar ellipticities calculated for the drug concentration at the wavelengths of the maxima of the reported spectrum are: 310 nm, $[\theta] = +0.11 \pm 0.01 \times 10^4$; 290 nm, $[\theta] = +0.75 \pm 0.03 \times 10^4$; 265 nm, $[\theta] = -0.02 \pm 0.01 \times 10^4$ (means and standard errors of nine determinations).

group is introduced at position 4 of the coumarin ring (19). Therefore, it was concluded that the ultraviolet absorption band near 290 nm is due to the 4-hydroxyl group attached to the coumarin ring (19).

For phenprocoumon similar Cotton effects were induced in both enantiomers and in the racemate (Fig. 2, lower). Such observations have been reported for the enantiomers of two other drugs (11, 12), and were explained by sterically similar complexes of both enantiomers with the albumin (12). Therefore the orientation of the perturbed chromophore of the phenprocoumon isomers, the 4-hydroxycoumarin ring, must be identical in the complexes with HSA. If this is the case, the orientation of the other substituents at the asymmetrical carbon atom must be different for the complexes of each enantiomer with HSA. These substituents specificially contribute to the binding mechanism and binding energy of the coumarin derivatives (3, 20, 21), possibly by hydrophobic interactions with the albumin. An optimal interaction of the phenprocoumon side chains with the albumin surface probably is possible for only one of the two isomers. This could explain the much higher association constant of $S(-)$-phenprocoumon.

In the case of warfarin the signs of the extrinsic Cotton effects of the two enantiomers are partially different (Fig. 3, lower, and Table 4), especially for the CD band near 290 nm. Since this band is probably due to the 4-hydroxyl group attached to the coumarin ring (19), this observation suggests that the orientation of the 4-hydroxybenzene moiety, or possibly of the coumarin ring itself, differs in the complexes of the two enantiomers with HSA. Thus the orientation of the side chains, and therefore their interaction with the albumin surface, may be similar. This could explain the finding that the affinities of the two enantiomers of warfarin for HSA differ only slightly (3, 6). At present we cannot explain why the signs of some of the CD bands are similar for the two warfarin enantiomers (Fig. 3, upper). Possibly

the signs of these bands are not influenced by the different orientations of the isomers when bound. A comparable observation was made with the enantiomers of oxazepam hemisuccinate (12). A similar orientation of the 4-hydroxycoumarin ring of the two warfarin isomers when bound would undoubtedly result in similar extrinsic Cotton effects, as is the case for the isomers of phenprocoumon.

The signs of the extrinsic Cotton effects induced in the phenprocoumon and warfarin derivatives differ markedly (Figs. 2 and 3; Tables 3 and 4). Only the negative CD band near 270 nm can be found for all derivatives investigated. These differences in extrinsic Cotton effects probably do not depend on differences in the asymmetrical configuration of the enantiomers, since the intrinsic Cotton effects of the enantiomers of phenprocoumon and warfarin are very similar in sign and wavelength position (Figs. 2 and 3). This excludes large differences in the electronic structure of the drugs produced by the different side chains. Therefore the observed differences between the extrinsic Cotton effects of the bound drugs seem to have their origin in specific differences in the conformation of their complexes with HSA. Two mechanisms could explain these observations. First, binding of warfarin changes the HSA conformation and thus influences the position of the asymmetrical center of the protein, resulting in changed symmetry rules (22) for the bound warfarin molecules. Dialysis studies (23) and fluorescence quenching studies (21) have suggested that warfarin may affect the HSA conformation. Second, it is possible that the signs of the induced Cotton effects are influenced by the lipophilic nature of the ligands. Such an observation was made for four structurally related sulfonamides bound to bovine serum albumin (24), in which the signs of one extrinsic CE band changed with increasing hydrophobic character of the drugs. In the case of the coumarins the two more lipophilic (1) derivatives [phenprocoumon (Fig. 2 and Table 3) and dicoumarol (21, 25)] have negative induced Cotton effects. The sign of the extrinsic Cotton effects changes to positive if the derivatives are more hydrophilic, as shown for warfarin (Fig. 3 and Table 4) and 4-hydroxycoumarin (Fig. 4).

The $S(-)$ isomers of warfarin and phenprocoumon exhibit many times the anticoagulant activity of the $R(+)$ isomers (4, 5, 7). Since the coumarin-HSA complex may be used as a model for the interaction of the coumarins with the receptor site (1–3), our results may also explain differences in the intrinsic activities of the enantiomers. We have suggested that the complexes of the phenprocoumon isomers with HSA differ in the orientation of the side chains, whereas the complexes of the warfarin enantiomers differ at least in the orientation of the 4-hydroxyl group. Both moieties of the molecules, the side chains and the 4-hydroxyl group, are mandatory for high anticoagulant activity (1). It seems possible that similar differences in orientation of these moieties in complexes with the receptor site are responsible for the different anticoagulant activities of the isomers. Furthermore, the different oxidative metabolism described for the warfarin isomers (hydroxylation preferentially in position 6 or 7 of the coumarin ring) (5) could also be due to a different orientation of the 4-hydroxycoumarin rings of the warfarin enantiomers when bound to the metabolizing enzyme system.

These results may be helpful in understanding different binding mechanisms and different biological activities of the coumarin anticoagulants.

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## REFERENCES

1. O'Reilly, R. A. (1973) *Ann. N. Y. Acad. Sci.*, 226, 293–308.
2. O'Reilly, R. A. (1969) *J. Clin. Invest.*, 48, 193–202.
3. O'Reilly, R. A. (1971) *Mol. Pharmacol.*, 7, 209–218.
4. O'Reilly, R. A. (1974) *Clin. Pharmacol. Ther.*, 16, 348–354.
5. Lewis, R. J., Trager, W. F., Chan, K. K., Breck-

enridge, A., Orme, M., Roland, M. & Schary, W. (1974) *J. Clin. Invest.*, **53**, 1607–1617.
6. Sellers, E. M. & Koch-Weser, J. (1975) *Pharmacol. Res. Commun.*, **7**, 331–336.
7. Jähnchen, E., Meinertz, T., Gilfrich, H. J., Groth, U. & Martini, A. (1976) *Clin. Pharmacol. Exp. Ther.*, **20**, 342–349.
8. Chignell, C. F. (1970) *Adv. Drug Res.*, **5**, 55–94.
9. Chignell, C. F. (1969) *Mol. Pharmacol.*, **5**, 455–462.
10. Müller, W. E. & Wollert, U. (1973) *Naunyn-Schmiedebergs Arch. Pharmacol.*, **278**, 301–312.
11. Perrin, J. H. (1973) *J. Pharm. Pharmacol.*, **25**, 208–212.
12. Müller, W. E. & Wollert, U. (1975) *Mol. Pharmacol.*, **11**, 52–60.
13. Seiler, K. & Duckert, F. (1968) *Thromb. Diath. Haemorrh.*, **19**, 389–396.
14. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.*, **51**, 660–672.
15. Fletcher, J. E., Ashbrook, J. D. & Spector, A. A. (1973) *Ann. N. Y. Acad. Sci.*, **226**, 69–81.
16. West, B. D., Preis, S., Schroeder, C. H. & Link, K. P. (1961) *J. Am. Chem. Soc.*, **83**, 2676–2678.
17. West, B. D. & Link, K. P. (1964) *J. Heterocycl. Chem.*, **2**, 93–94.
18. Perrin, J. H. & Nelson, D. A. (1972) *Life Sci.*, **11**, Pt. 1, 277–283.
19. Brand, J. G. & Toribara, U. Y. (1972) *Mol. Pharmacol.*, **8**, 751–758.
20. Garten, S. & Wosilait, W. D. (1971) *Biochem. Pharmacol.*, **20**, 1661–1168.
21. Chignell, C. F. (1970) *Mol. Pharmacol.*, **6**, 1–12.
22. Schellman, J. A. (1968) *Acc. Chem. Res.*, **1**, 144–151.
23. O'Reilly, R. A. (1967) *J. Clin. Invest.*, **46**, 829–837.
24. Müller, W. E. & Wollert, U. (1976) *Biochem. Pharmacol.*, **25**, 1459–1464.
25. Perrin, J. H., Vallner, J. J. & Nelson, D. A. (1975) *Biochem. Pharmacol.*, **24**, 769–774.