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# The Lone Tryptophan Residue of Human Serum Albumin as Part of the Specific Warfarin Binding Site

## Binding of Dicoumarol to the Warfarin, Indole and Benzodiazepine Binding Sites

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SUMMARY

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The interaction of warfarin and dicoumarol with tryptophan- and tyrosine-modified human serum albumin derivatives was investigated by equilibrium dialysis and circular dichroism measurements. The binding of warfarin to its specific high-affinity binding site is strongly reduced after the modification of the lone tryptophan residue of human serum albumin with three different reagents, while the modification of tyrosine residues has nearly no effect on the binding of warfarin to this site. It is concluded that the lone tryptophan residue is part of the warfarin binding site which is clearly separated from the specific indole and benzodiazepine binding site. Evidence is presented that dicoumarol interacts with the warfarin as well as the indole and benzodiazepine binding site of the human serum albumin molecule. A highly reactive tyrosine residue specifically involved in the indole and benzodiazepine binding site seems to be important for the dicoumarol binding, too. Its nitration with tetranitromethane differently affects the four induced circular dichroism bands of dicoumarol bound to human serum albumin. This effect has been qualitatively and quantitatively characterized by the resolution of the induced circular dichroism spectrum of dicoumarol bound to human serum albumin into the Gaussian component bands using a computer program. The identification of the lone tryptophan residue as a part of the warfarin binding site of human serum albumin is a significant step forward in localizing this important drug binding site.

## INTRODUCTION

Coumarin anticoagulants like warfarin, phenprocoumon, and dicoumarol are strongly bound to human serum albumin (1–4). There exists now increasing evidence that all monomeric coumarin anticoagulants (warfarin, phenprocoumon, acenocoumarin) interact with only one site of high affinity and several sites of clearly lower affinity (3–7). By contrast, the dimeric compound dicoumarol interacts with three sites

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of high affinity and some sites of much lower affinity (2, 4, 8). One of the three high-affinity binding sites of dicoumarol seems to be identical with the single high-affinity binding site of the monomeric compounds (9, 10). Since warfarin has been used to label the single high-affinity binding site of the coumarin anticoagulants (9, 11), many authors refer to this site as the "warfarin binding site" of human serum albumin (9, 11, 12). Besides its significance for the binding of the coumarin anticoagulants, this site is involved in the high-affinity binding of several other drugs to human serum albumin (9, 11–13). The nature of this site and especially its exact location within the human serum albumin primary structure are largely unknown.

There exists some evidence that the lone tryptophan residue of human serum albumin may be involved in the high-affinity binding of warfarin and dicoumarol (4, 10). Furthermore, since the binding of the coumarin anticoagulants to human serum albumin depends mainly on hydrophobic interactions (1, 4, 7, 8), the lone tryptophan residue as well as the tyrosine residues are strong candidates as important parts of the binding sites of the coumarin anticoagulants. Recently, the selective modification of the lone tryptophan residue of human serum albumin and of up to nine tyrosine residues has been reported (14–16). These modified albumin derivatives provide an excellent tool to investigate the participation of both amino acid residues in ligand binding sites of the human serum albumin molecule. In the present paper, both modifications were used to investigate whether the lone tryptophan residue and or tyrosine residues of the human serum albumin molecule are involved in the high-affinity binding sites of the protein for coumarin anticoagulants.

The significance of plasma protein binding of coumarin anticoagulants for their distribution, elimination, anticoagulant effect and pharmacokinetic drug-interactions is well known (17–19). Precise knowledge about properties and location of these binding sites in the human serum albumin molecule would be very useful for the understanding and possibly the prediction of phenomena like the changed binding during disease states or the displacement of coumarin anticoagulants by other drugs (17–19).

## MATERIALS AND METHODS

*Materials.* HSA[1] was obtained from Behringwerke, Marburg (electrophoretic purity 100%). Diazepam, $^{14}$C-diazepam, and chlordiazepoxide were gifts of Hoffmann-La Roche, Grenzach. Dicoumarol was obtained from Sigma, München. $^{14}$C-dicoumarol was from New England Nuclear (Boston) and was a generous gift of Drs. G. Levy (Amherst, N. Y.) and W. D. Wosilait (Colombia, MO). Warfarin was obtained from Merrell, Groß-Gerau and $^{14}$C-warfarin from Amersham, Buckinghamshire. The radiochemical purities were >99%. All other chemicals were of reagent grade.

*Preparation of HSA derivatives.* The modification of HSA with HNB bromide was reported in detail elsewhere (14). HSA was dissolved in 10 M urea adjusted to pH 4.4 by acetic acid, and a 1100-fold molar excess of HNB was added. Another amount of HSA was treated the same way but without adding HNB and used as control, called Urea-HSA. NPS chloride-modified HSA was prepared with a 22-fold molar excess of reagent in 20% acetic acid (16). For preparation of TNM-modified HSA I, II, and III a 4, 15, and 64-fold molar excess, respectively, of TNM was added to HSA dissolved in 0.05 M Tris buffer adjusted to pH 8.0. The modification was described in detail recently (16). A 1.8-fold molar excess of TNM was used for the modification of TNM-HSA IV. The reduction of TNM-HSA IV by a 6-fold molar excess of sodium hydrosulfite was performed according to Sokolovsky *et al.* (20). After modification, all HSA derivatives were purified by dialysis against distilled water, lyophylized, and stored at 0°. The degree of tryptophan and tyrosine residue modification of the HSA derivatives was determined spectrophotometrically and by amino acid analysis (14, 16).

[1] Abbreviations used are: HSA, human serum albumin; HNB, 2-hydroxy-5-nitrobenzyl; NPS, o-nitrophenylsulfenyl; TNM, tetranitromethane; CD, circular dichroism.

*Circular dichroism measurements.* CD-measurements were performed with a Cary 61 CD-spectropolarimeter calibrated with *d*-camphorsulfonic acid. All solutions were prepared in 1/15 M phosphate buffer adjusted to pH 7.40. Results are expressed as molar ellipticities $[\theta]$ (deg × $cm^2$ × $dmol^{-1}$), calculated with reference to the HSA concentration, using a molecular weight of 69 000. The optical pathlength was 10 mm and the protein concentration 13.1 μM.

Difference spectra were obtained by substracting the effect of the corresponding HSA derivative as blank. The resolution of the difference spectra into the single Gaussian components was performed by a computer program based on the least-squares fitting of an arbitrary function to an observed spectrum (21). The single Gaussian components were defined by the position within the wavelength range, $x_{ok}$, the bandwidth, $\sigma_k$, and the molar ellipticity of the component at the extremum, $H_k$, which could be positive or negative. The number of components was limited by the magnitude of the left residuals compared to the observational errors (21).

*Equilibrium dialysis experiments.* The binding of $^{14}$C-diazepam, $^{14}$C-dicoumarol, and $^{14}$C-warfarin to HSA was determined by equilibrium dialysis, using a HSA concentration of 36.2 μM and varying concentrations of the drugs. All solutions were prepared with 1/15 M phosphate buffer, pH 7.4; 0.9 ml of the HSA solution was dialysed for 16 hours at 25° in the dark against 0.9 ml of the buffer containing the $^{14}$C-labeled drugs. One milliliter dialysis cells and cellophan dialysis membranes (Union carbide) were used. The radioactivity at both sides was determined by liquid scintillation spectrometry.

## RESULTS

The lone tryptophan residue of HSA can be selectively and completely modified with HNB bromide and NPS chloride (Table 1). Out of the 18 tyrosine residues of HSA only nine can be modified with TNM without denaturation of the protein (16). In the present study mainly three TNM-modified HSA derivatives were used, whose degree of modification is given in Table 1. In contrast to the tryptophan modification, the tyrosine modification is less selective (16, 22), so that besides the tyrosine residues appreciable amounts of the tryptophan residue are also modified by TNM (Table 1).

The binding of warfarin to the HSA derivatives was investigated by equilibrium dialysis. Curved Scatchard plots were obtained for all investigated HSA derivatives (Fig. 1). They could be resolved into two linear components according to the method of Pennock (24), each representing one set of binding sites. In agreement with other

TABLE 1

*Tyrosine and tryptophan residue modification in the HSA derivatives investigated*

| Albumin | Number of residues modified | | | |
|---|---|---|---|---|
| | Spectrophotometrically | | Amino acid analysis | |
| | Tyr | Trp | Tyr | Trp |
| TNM-HSA I | 2.2 | 0.2 | 2.3 | 0.3 |
| TNM-HSA II | 5.1 | 0.4 | 6.7 | 0.6 |
| TNM-HSA III | 7.8 | 1.0 | 10.7 | 1.0 |
| TNM-HSA IV | 0.9 | 0.0 | — | — |
| NPS-HSA | — | 1.4 | 0.0 | 1.0 |
| HNB-HSA | — | 1.1 | 0.5 | 1.0 |

The data are taken from Fehske *et al.* (14, 16).

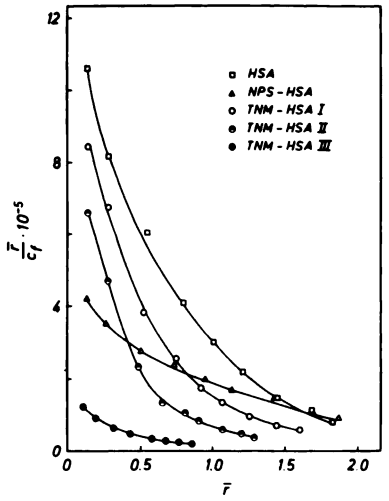


FIG. 1. *Scatchard plot of the binding of warfarin to HSA, NPS-HSA, and TNM-HSA I, II, and III*

Ordinate: $\bar{r}/c_f$ (liters per mole); $c_f$ = molar concentration of the free drug; $\bar{r}$ = moles of drug bound per mole of albumin. Abscissa: $\bar{r}$. Each point represents the mean of four determinations.

TABLE 2

*Binding parameters of the interaction of warfarin with HSA, NPS-HSA, HNB-HSA and TNM-HSA I, II, III*

The association constants $k_1$ and $k_2$ and the number of binding sites on the albumin molecule $n_1$ and $n_2$ were calculated for 36 equilibrium dialysis experiments as described in the text.

| Albumin | $n_1$ | $10^{-4} \times k_1$ $[M]^{-1}$ | $n_2$ | $10^{-4} \times k_2$ $[M]^{-1}$ |
|---|---|---|---|---|
| HSA | 0.89 | 124 | 1.92 | 5.2 |
| TNM-HSA I | 0.75 | 120 | 1.48 | 5.6 |
| TNM-HSA II | 0.49 | 139 | 1.25 | 5.8 |
| TNM-HSA III | 0.24 | 40 | 1.05 | 3.9 |
| NPS-HSA | 0.96 | 36 | 1.80 | 5.4 |
| HNB-HSA | 0.38 | 42 | 2.05 | 4.5 |

authors (3, 6, 7), one high-affinity binding site and two sites of lower affinity were found for control HSA (Table 2). The modification of the lone tryptophan residue with NPS reduces the association constant of the high-affinity warfarin binding site by about 70% (Table 2). Interestingly, the modification of the lone tryptophan by HNB does not only reduce the association constant by about the same degree, but also the number of high-affinity binding sites from 0.96 to about half of this value. The unfolding of the HSA molecule in 10 M acidic urea solution during the HNB modification (14) can not be the reason for this specific effect, since unfolding alone does not influence the binding parameters of warfarin.

The association constant of the warfarin high-affinity binding site is affected only when about 8 tyrosine residues are modified (Table 2). The TNM modification of HSA also reduces the number of the high-affinity warfarin binding sites. Since this reduction parallels quite well the tryptophan modification by TNM (Table 1), it may represent rather an effect of the tryptophan modification by TNM than of the tyrosine modification. Accordingly, in TNM-HSA III, where the lone tryptophan is completely modified, the binding parameters of warfarin are very similar to those in HNB-HSA (Table 2).

The binding of warfarin to HSA generates extrinsic Cotton effects (5, 10, 25), with a positive band at 305 nm and a negative one at 275 nm (Table 3). The intensities of both bands are relatively small. The intensity of the band at 305 nm is unchanged after the HNB modification, but increases by about 100% in TNM-HSA I (Table 3). Both modifications do not change the positions of the maxima of both extrinsic CD bands.

For dicoumarol, as for warfarin, curved Scatchard plots were obtained from equilibrium dialysis experiments (Fig. 2), which could be resolved into two sets of binding sites (Table 4). In agreement with other authors (2, 4, 8), control HSA has three high-affinity binding sites for dicoumarol and four to five sites of lower affinity. The modification of the lone tryptophan residue of HSA by HNB reduces the number of high-affinity binding sites from 2.9 to 1.9, while the number of low-affinity binding sites is increased by one (Table 4). The unfolding during the HNB modification does not affect the dicoumarol binding, since the binding parameters of urea-HSA and control HSA do not differ. As found for the warfarin binding, the tryptophan modification by HNB and by NPS influences the dicoumarol binding differently. Only

TABLE 3

*Extrinsic Cotton effects of warfarin bound to HSA, Urea-HSA, HNB-HSA, and TNM-HSA I, II and III*

$[\theta]$ is the molar ellipticity, calculated with reference to the albumin concentration (13.1 μM). The warfarin concentration was 39.3 μM. The data are difference values, using the molar ellipticity of the albumins at the same wavelength as blank and represent means ± SEM of $n$ experiments. The values in parentheses are percent of the corresponding value of control HSA.

| Albumin | $n$ | $[\theta] \cdot 10^{-3}$ | |
|---|---|---|---|
| | | 305 nm | 275 nm |
| HSA | 6 | +8.66 ± 0.12 (100) | −9.81 ± 0.42 (100) |
| Urea-HSA | 3 | +7.64 ± 0.24 (88) | −9.75 ± 0.54 (99) |
| HNB-HSA | 6 | +7.40 ± 0.24 (85) | −4.15 ± 0.60 (42) |
| TNM-HSA I | 4 | +15.88 ± 0.06 (183) | −6.02 ± 0.96 (61) |
| TNM-HSA II | 4 | +10.47 ± 0.30 (121) | −2.41 ± 0.60 (25) |
| TNM-HSA III | 4 | +8.36 ± 0.42 (97) | −1.99 ± 0.84 (20) |

the HNB modification markedly reduces the association constant of one of the high-affinity binding sites, while the NPS modification does not affect the high-affinity binding of dicoumarol and increases only slightly the affinity of the low-affinity binding sites (Table 4).

In the case of the TNM modification, the modification of about 2.2 tyrosine residues (TNM-HSA I) has no remarkable influence on the binding parameters of dicoumarol, but further modification by TNM is followed by a distinct reduction of the number of the high-affinity binding sites from three to about two and by a reduction of the association constants of the remaining high-affinity binding sites (Table 4). The reduction in the number of the high-affinity binding sites parallels the effect of the tryptophan modification by TNM (Table 1).

The binding of dicoumarol to HSA generates large extrinsic Cotton effects (4, 10), with a small positive band at 275 nm and some large negative bands between 290 and 350 nm (Fig. 3). The extrinsic Cotton effects at three different wavelengths, obtained by the titration of a fixed concentration of the HSA derivatives with dicoumarol (Fig. 4), were used for determining the parameters

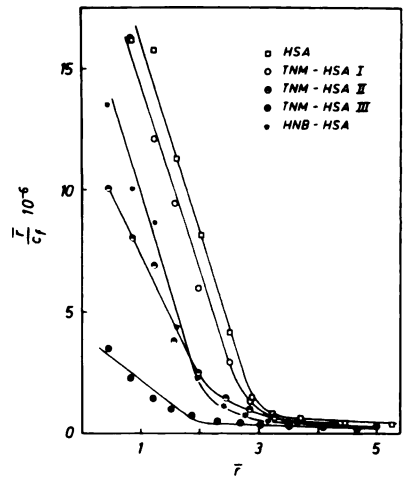


FIG. 2. *Scatchard plot of the binding of dicoumarol to HSA, HNB-HSA, and TNM-HSA I, II, and III*

Ordinate: $\bar{r}/c_f$ (liters per mole); $c_f$ = molar concentration of the free drug; $\bar{r}$ = moles of drug bound per mole of albumin. Abscissa: $\bar{r}$. Each point represents the mean of three determinations.

TABLE 4

*Binding parameters of the interaction of dicoumarol with HSA, HNB-HSA, NPS-HSA, and TNM-HSA I, II, and III*

The association constants $k_1$ and $k_2$ and the number of binding sites on the albumin molecule $n_1$ and $n_2$ were calculated for 33 equilibrium dialysis experiments as described in the text.

| Albumin | $n_1$ | $10^{-4} \times k_1$ $[M]^{-1}$ | $n_2$ | $10^{-4} \times k_2$ $[M]^{-1}$ |
|---|---|---|---|---|
| HSA | 2.90 | 760 | 4.30 | 7 |
| TNM-HSA I | 2.80 | 730 | 4.30 | 7 |
| TNM-HSA II | 2.25 | 490 | 4.80 | 8 |
| TNM-HSA III | 1.70 | 210 | 5.10 | 6 |
| HNB-HSA | 1.90 | 820 | 5.20 | 8 |
| NPS-HSA | 2.89 | 740 | 4.40 | 11 |

of the optically active binding sites (Table 5) according to the method of Rosen (26). Out of the three high-affinity binding sites of dicoumarol found by equilibrium dialysis (Table 4), only two could be detected by the Rosen method (Table 5), suggesting that only the binding to two of the three high-affinity binding sites induces extrinsic Cotton effects, which is in agreement with observations of Perrin *et al.* (10). While these optically active binding sites remain unaffected by the unfolding in urea solution (urea-HSA), the modification of the lone tryptophan residue by HNB reduces their number by one (Table 5), which is in agreement with the equilibrium dialysis measurements (Table 4). It was not possible to investigate the effect of the NPS modification on the optically active binding sites, since the modification itself generates a large positive Cotton effect within the wavelength range of the induced CD spectrum of dicoumarol (15, 16).

In contrast to the HNB modification, the modification with TNM changes the extrinsic Cotton effects of bound dicoumarol not only quantitatively but also qualitatively, since the extrinsic CD bands are affected in a different manner, e.g., the band at 292 nm is increased in TNM-HSA I and that at 320 nm is decreased (Fig. 4). To analyze this interesting observation, the extrinsic Cotton effects of dicoumarol bound to control HSA and to TNM-HSA I and II have been resolved into the Gaussian component bands using a computer program (21). The resolution into four components provided

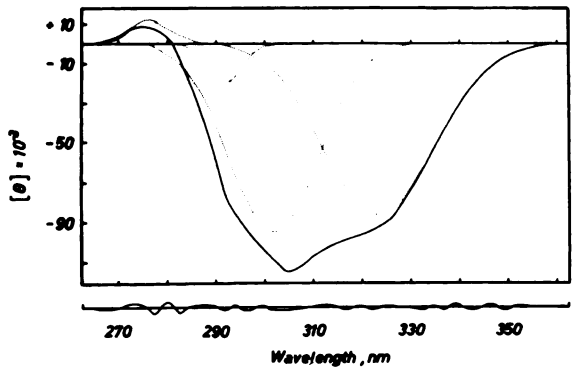


FIG. 3. *Gaussian analysis of the Extrinsic Cotton effects of dicoumarol bound to HSA*

Ordinate: molar ellipticity calculated with reference to the albumin concentration. Abscissa: wavelength. Shown are the observed spectrum (——), the calculated Gaussian components (· · · ·), and the residuals (lower part). The experiments were performed in 1/15 M phosphate buffer, pH 7.40, at an albumin concentration of 13.1 μM and a dicoumarol concentration of 39.3 μM.

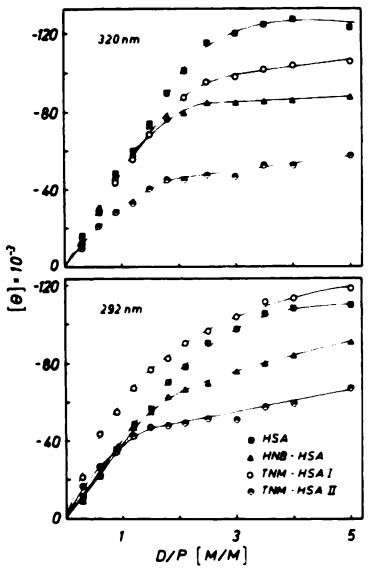


FIG. 4. *Relationship between the Extrinsic Cotton effects of dicoumarol bound to HSA, HNB-HSA, and TNM-HSA I, and II and the drug to protein ratio*

Ordinate: Molar ellipticity at 320 nm and 292 nm calculated with reference to the albumin concentration. Abscissa: molar dicoumarol/albumin ratio. Each point represents the mean of two determinations.

TABLE 5

*Binding parameters of the optically active dicoumarol binding sites of HSA, Urea-HSA, and HNB-HSA*

The association constants $k_1$ and the number of binding sites on the albumin molecule $n_1$ were calculated according to Rosen (35) at three different wavelengths, λ, each from 24 experiments ($k_2$ was always ≤5% of $k_1$, $n_2$ is always ≤1.0).

| Albumin | λ [nm] | $n_1$ | $10^{-5} \times k_1$ $[M]^{-1}$ |
|---|---|---|---|
| HSA | 292 | 1.91 | 105 |
| | 305 | 1.89 | 97 |
| | 320 | 2.00 | 87 |
| Urea-HSA | 292 | 1.92 | 112 |
| | 305 | 2.05 | 119 |
| | 320 | 2.08 | 98 |
| HNB-HSA | 292 | 1.15 | 109 |
| | 305 | 1.30 | 124 |
| | 320 | 1.25 | 130 |

the most probable solution (see Methods). A typical resolution of the induced CD spectrum of dicoumarol bound to control HSA is given in Fig. 3. A small positive band is located around 275 nm and three negative bands II, III, and IV are located around 293, 305, and 320 nm, respectively (Fig. 3). The location of the maxima of the resolved component bands is in good agreement with maxima and shoulders of the ultraviolet absorption spectra of dicoumarol in buffer or bound to HSA (Fig. 5), which gives further evidence that the observed Cotton effects are extrinsic in origin.

In a first series of resolutions the location of the Gaussian components, their magnitude and the bandwidth were unfixed (Table 6, upper part). When component I became so small that the resolution into four components became unreliable, the spectra

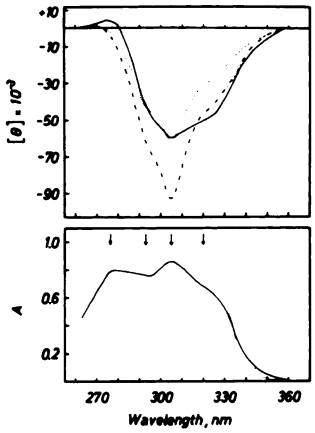


FIG. 5. *Comparison of the Extrinsic Cotton effects of dicoumarol and the UV-absorption spectrum*

Upper: Induced CD spectra of dicoumarol bound to HSA (——), TNM-HSA I (-·-·-), and TNM-HSA II (····) at an albumin and dicoumarol concentration of 13.1 μM. Lower: UV-absorption spectra of dicoumarol (····) and dicoumarol bound to HSA (——) using the phosphate buffer or the albumin spectrum as blank, respectively. The albumin concentration was 13.1 μM and the dicoumarol concentration 52.4 μM. The arrows indicate the positions of the extrema of the resolved Gaussian components of the induced CD spectrum of dicoumarol.

were only resolved into three components (Table 6). In the case of component II and III the location of the maxima of the resolved CD bands was very similar for control HSA and TNM-HSA I and II, found for six resolutions at two different dicoumarol concentrations (Table 6, upper part). By contrast, the maximum of component IV seems to be blue shifted in the case TNM-HSA I and II.

In order to get comparable data about the magnitude of the resolved CD bands, the location of the Gaussian component bands was fixed in a second series of resolutions (Table 6, lower part). For these resolutions, the RMS-values indicate a less close fitting of the spectra, but they still remain within the experimental error. In this series of resolutions, the concentration dependence of the magnitude of all four bands is clearly obvious, since it is nearly doubled by increasing the molar dicoumarol/HSA ratio from one to two (Table 6, lower part). After the modification of only 2.2 tyrosine residues (TNM-HSA I), the contribution of the four bands to the induced CD spectrum of dicoumarol is clearly changed. At a molar drug/HSA ratio of one,

TABLE 6

*Gaussian analysis of the Extrinsic Cotton effects of dicoumarol bound to HSA and TNM-HSA I, and II*

The following parameters are given for the Gaussian components I, II, III, and IV: The wavelength of the extremum, $x_{ok}$, the molar ellipticity at the extremum, $H_k$, the bandwidth, $\sigma_k$ (given as mean ± standard error of all components of the same group), and the error of the residuals after the resolution, RMS, at two different molar drug to HSA ratios (D/P).

| Albumin | D/P M/M | I | | II | | III | | IV | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $x_{ok}$ nm | $H_k$ $[\theta] \times 10^{-3}$ | $x_{ok}$ nm | $H_k$ $[\theta] \times 10^{-3}$ | $x_{ok}$ nm | $H_k$ $[\theta] \times 10^{-3}$ | $x_{ok}$ nm | $H_k$ $[\theta] \times 10^{-3}$ | RMS $[\theta] \times 10^{-3}$ |
| HSA | 1 | 276.1 | +4.4 | 293.1 | −28.1 | 304.3 | −38.1 | 322.1 | −47.4 | 0.63 |
| HSA | 2 | 276.7 | +10.8 | 291.9 | −19.3 | 301.8 | −94.2 | 323.4 | −85.5 | 0.74 |
| TNM-HSA I | 1 | — | — | 293.9 | −46.9 | 306.1 | −43.6 | 317.9 | −46.2 | 0.94 |
| TNM-HSA I | 2 | 273.5 | +7.7 | 293.5 | −75.0 | 305.8 | −67.1 | 319.6 | −81.2 | 1.00 |
| TNM-HSA II | 1 | — | — | 293.1 | −30.0 | 305.7 | −29.4 | 316.1 | −32.4 | 0.84 |
| TNM-HSA II | 2 | — | — | 292.0 | −43.4 | 304.7 | −49.4 | 319.2 | −45.6 | 1.71 |
| | | $\sigma_k$: 4.4 ± 0.3 nm | | $\sigma_k$: 5.9 ± 0.6 nm | | $\sigma_k$: 6.3 ± 0.7 nm | | $\sigma_k$: 13.5 ± 0.7 nm | | |
| HSA | 1 | 276.4 | +3.7 | 292.9 | −33.5 | 305.4 | −28.6 | 319.7 | −51.0 | 1.59 |
| HSA | 2 | 276.4 | +9.4 | 292.9 | −62.5 | 305.4 | −53.1 | 319.7 | −93.4 | 3.07 |
| TNM-HSA I | 1 | 276.4 | +0.2 | 292.9 | −46.3 | 305.4 | −52.8 | 319.7 | −44.6 | 1.05 |
| TNM-HSA I | 2 | 276.4 | +7.1 | 292.9 | −72.4 | 305.4 | −70.0 | 319.7 | −80.6 | 1.20 |
| TNM-HSA II | 1 | 276.4 | +0.1 | 292.9 | −33.7 | 305.4 | −35.8 | 319.7 | −30.2 | 1.01 |
| TNM-HSA II | 2 | 276.4 | +4.3 | 292.9 | −46.6 | 305.4 | −45.0 | 319.7 | −46.1 | 1.18 |
| | | $\sigma_k$: 7.5 ± 0.7 nm | | $\sigma_k$: 6.6 ± 0.3 nm | | $\sigma_k$: 5.7 ± 0.1 nm | | $\sigma_k$: 13.6 ± 0.3 nm | | |

the positive component I is largely reduced, component IV is slightly reduced, but the magnitudes of component II and III are markedly increased, the latter by nearly 100% (Table 6, lower part). At a molar dicoumarol/HSA ratio of two, the changes are qualitatively similar. After modification of about 5 tyrosine residues (TNM-HSA II), the magnitudes of all four components are reduced (Table 6, lower part).

The results reported give some evidence that the tryptophan and the tyrosine modification affect two different high-affinity binding sites of dicoumarol, one of which seems to be identical with the warfarin site. The other one could be identical with the specific indole and benzodiazepine binding site (12), which is already quantitatively modified in TNM-HSA I (16). Thus, we have tested both assumptions by displacement experiments using $^{14}$C-warfarin and $^{14}$C-diazepam. The results are summarized in Table 7. While both investigated benzodiazepine derivatives, chlordiazepoxide and diazepam, do not displace bound warfarin even at a 4.5-fold molar excess, warfarin is strongly displaced by dicoumarol (Table 7). Diazepam, on the other hand, is displaced by chlordiazepoxide, but not by warfarin

TABLE 7

*Displacement of diazepam and warfarin from HSA by other drugs*

The HSA concentration was 36.2 μM, the diazepam concentration 40.0 μM, and the warfarin concentration 72.4 μM. Under these conditions the percentage bound of diazepam was 84.9 ± 0.4 and that of warfarin was 79.7 ± 0.2 ($n$ = 7). Displacement is expressed in percent of these control experiments. The molar drug/albumin ratios of the displacing drugs were 1.5 and 4.5 (1.0 and 3.0 for dicoumarol). Each value is the mean ± SEM of four determinations.

| Bound drug | Displacing drug | % Displacement | |
|---|---|---|---|
| | | D/P 1.5 | D/P 4.5 |
| Warfarin | Diazepam | 2.5 ± 1.2 | 2.1 ± 0.8 |
| | Chlordiaz-epoxide | 1.5 ± 0.2 | 2.5 ± 0.1 |
| | Dicoumarol | 20.7 ± 1.3 | 62.7 ± 0.1 |
| Diazepam | Chlordiaz-epoxide | 14.7 ± 0.4 | 29.0 ± 0.9 |
| | Dicoumarol | 3.8 ± 1.1 | 19.6 ± 3.8 |
| | Warfarin | 0.8 ± 0.8 | 0.5 ± 0.4 |

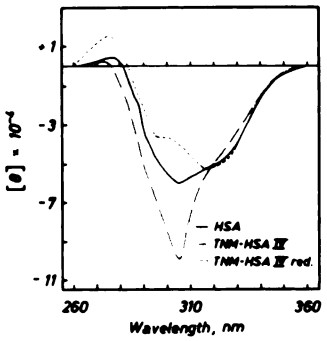


FIG. 6. *The Extrinsic Cotton effects of dicoumarol bound to HSA, TNM-HSA IV, and reduced TNM-HSA IV*

Ordinate: molar ellipticity calculated with reference to the albumin concentration. Abscissa: wavelength. The albumin and dicoumarol concentration was 13.1 μM.

and the low concentration of dicoumarol. However, a three-fold molar excess of dicoumarol significantly displaces bound diazepam (Table 7).

Interestingly, the large increase of the induced Cotton effects of dicoumarol at 292 and 305 nm can also be seen in TNM-HSA IV (Fig. 6), where only one tyrosine residue is modified (Table 1). After reduction of the nitro-group in TNM-HSA IV by hydrosulfite, the increase of the extrinsic Cotton effects is abolished (Fig. 6). The treatment of native HSA with hydrosulfite changes the extrinsic Cotton effects of dicoumarol only slightly.

## DISCUSSION

The present results clearly show that the modification of the lone tryptophan residue of HSA by HNB and NPS strongly reduces the binding of warfarin to its single high-affinity binding site. Interestingly, both modifications affect the warfarin binding differently. In contrast to the tryptophan modification, the effects of the tyrosine modification by TNM on the binding of warfarin are less dramatic. In TNM-HSA I, where the specific indole and benzodiazepine binding site is completely modified (16), no effects on the warfarin binding have been observed. This observation and the failure of diazepam and chlordiazepoxide to displace bound warfarin leave no doubt that

the warfarin and the benzodiazepine binding site of HSA represent two clearly distinct areas of the protein (11). Assuming that the reduction of the number of the warfarin high-affinity binding sites found in TNM-HSA II and III is due to the tryptophan modification by this reagent, as has been suggested above, then the tryptophan modification by TNM equals more the modification by HNB than that by NPS, since TNM as well as HNB modification reduce affinity and number of binding sites, while NPS reduces only the affinity.

Up to this point, the following conclusions can be drawn:

(1) The high-affinity binding sites of the HSA molecule for warfarin and for the benzodiazepines are two clearly separated areas of the protein.
(2) The lone tryptophan residue of the HSA molecule is involved in the high-affinity warfarin binding site.

The reasons for the different influences of the three tryptophan modifications on the warfarin binding must be the different chemical nature of the resulting reaction products. The modification of tryptophan residues with NPS chloride results in 2-thioaryl derivatives, which undergo no subsequent reactions (23). The 2-thioaryl substituent is not hindered from freely rotating, which may be the reason that warfarin still fits into the binding site and only its affinity is reduced. The first step of the tryptophan modification by HNB bromide is the electrophilic attack of HNB bromide at position 3, resulting in a 3-substituted indolenine derivative (27). In a subsequent reaction the phenolic oxygen of the substituent can cyclize with position 2 of the indolenine derivative (27). Since the modification creates a new asymmetric center at position 3 of the tryptophan nucleus, two diastereoisomeric cyclization products will be formed (27). Possibly only one of the two diastereoisomeric products is able to block the binding site completely, which would explain the reduction of the warfarin high-affinity binding sites by only 50%. Interestingly, after modification of position 3 of the indole nucleus, the reaction with TNM further results in a 2-dinitromethylene-3-nitro derivative (28). As found for the HNB modification, where positions 2 and 3 are also substituted, the modification by TNM seems to reduce the number of the warfarin high-affinity binding sites, depending on the degree of the tryptophan modification.

As found for the warfarin binding site, one of the high-affinity dicoumarol binding sites disappears after the tryptophan modification by HNB or by TNM. This observation and the large displacement of bound warfarin by dicoumarol give further evidence that one of the two optically active high-affinity dicoumarol binding sites and the warfarin binding site of HSA are identical. Also similar to warfarin, the effects of the tryptophan modifications by HNB or by NPS on the dicoumarol binding are different. But in contrast to warfarin, the NPS modification is without any detectable effect on the dicoumarol binding, while the affinity of warfarin is reduced. Obviously, the freely rotating NPS substituent is not able to prevent or to reduce the binding of dicoumarol to this site, which could indicate a slightly different binding of dicoumarol to this site. Chignell (4) has suggested that both 4-hydroxycoumarin rings of the dicoumarol molecule are attached to the protein in the case of binding to HSA. Assuming that only one of both rings is located at the tryptophan site, then the other ring may be able to retain the molecule bound, even after the modification of the tryptophan site by NPS. Such a mechanism may be possible only when the substituent at the tryptophan residue is not very bulky or is not hindered from freely rotating.

The large qualitative changes of the induced CD spectrum of dicoumarol observed after TNM modification seem to be specific effects of the tyrosine modification by this reagent, since they can be found even in TNM-HSA I when only 2.2 tyrosine residues are modified and the lone tryptophan is unchanged. Since in TNM-HSA I some extrinsic CD bands seem to be increased and others decreased, a computer program was employed to resolve the induced CD spectrum into the single components. This method was first shown by Daniel and Yang in the case of the binding of 8-anilino-1-naphthalenesulfonate to bovine serum albumin (29). The resolution shows that the

small positive band at 275 nm (component I) is partially reduced in TNM-HSA I and II and disappears in TNM-HSA III and HNB-HSA, which correlates quite well with the degree of the tryptophan modification by both reagents. Therefore, we conclude that this band is mainly originated by the binding of dicoumarol to the warfarin binding site of HSA.

The magnitude of the extrinsic CD band at 320 nm (component IV) is reduced in HNB-HSA and all three TNM-HSA derivatives. This corresponds well to the decrease of the amount bound, which was found by equilibrium dialysis and by the Rosen method. It seems therefore that this band is originated at both optically active dicoumarol binding sites. Comparable observations were made for the binding of dicoumarol to bovine serum albumin, where an extrinsic CD band at 303 nm is associated only with the binding to one site while a band at 318 nm is clearly associated with the binding to several sites (30).

The observation that the increase of the bands at 292 nm (component II) and at 305 nm (component III) is maximal in TNM-HSA IV, where only the highly reactive tyrosine residue is modified (16), strongly suggests that the nitration of this residue is responsible for the observed increase of the extrinsic Cotton effects of dicoumarol. The nitration of the tyrosine residue will increase its lipophilicity (31). Since hydrophobic forces seem to play a major role in the interaction of dicoumarol with HSA (1, 4, 7, 8), we suggest that due to the nitration of the highly reactive tyrosine the hydrophobic interactions of dicoumarol with the albumin can increase. Due to this mechanism the distance between chromophore (dicoumarol) and the asymmetric center of the albumin will decrease, which would explain the increase of the induced CD bands at 292 and 305 nm (4, 32). The observation that the increase of the induced Cotton effects is completely abolished in TNM-HSA IV-red fits very well in this theory, since the reduction of the nitrotyrosine to an aminotyrosine (20) will decrease the lipophilicity again (31). The absorption bands of the 4-hydroxycoumarin derivatives at 305 nm and at 292 nm seem to be depending on electronic transitions in the $\alpha$-$\beta$-unsaturated, hydroxylated lactone structure of the molecule (5, 8, 25). Since only the induced Cotton effects at those two wavelengths are increased after the nitration of the highly reactive tyrosine residue, we suggest that the $\alpha$-$\beta$-unsaturated lactone structure plays an important role in the binding of dicoumarol to this second optically active binding site, in which the highly reactive tyrosine is involved in some way.

These findings and our previous observations on the diazepam and *L*-tryptophan binding (16) clearly demonstrate that the highly reactive tyrosine residue of HSA must be involved in the second optically active dicoumarol as well as in the indole and benzodiazepine binding site of the HSA molecule. The simplest explanation would be that both binding sites are identical, especially since dicoumarol can displace diazepam (Table 7) as well as *L*-tryptophan (33) from their binding to HSA. On the other hand, the nitration of the highly reactive tyrosine residue strongly reduces the diazepam and *L*-tryptophan binding (16) but rather enhances that of dicoumarol, as it can be concluded from the CD measurements. Both observations do not completely agree with the hypothesis of identical sites. Therefore, we conclude that both sites are at least located very close together so that the nitration of this tyrosine residue can affect both sites and dicoumarol bound to one site can prevent the binding of other drugs to the diazepam site.

Interestingly, the extrinsic Cotton effects of warfarin bound to the TNM-modified HSA derivatives give some evidence that warfarin also interacts with this site. Only the extrinsic Cotton effects of warfarin at 275 nm decrease in a manner which can be explained by the modification of the lone tryptophan involved in the high-affinity warfarin binding site, with a large decrease in the case of TNM-HSA III and HNB-HSA. By contrast, the Cotton effects at 305 nm remain unaffected in HNB-HSA, but increase in the case of TNM-HSA I and II as do the extrinsic Cotton effects of dicoumarol at 305 nm. The most likely explanation is that the extrinsic Cotton effects at

305 nm are not generated at the high-affinity binding site of warfarin, but at one of the secondary sites, which is presumably identical with the second optically active high-affinity dicoumarol binding site.

In summary, the lone tryptophan residue of HSA is involved in the high-affinity binding site of HSA for the coumarin anticoagulants, and the coumarin anticoagulant dicoumarol binds to this site as well as to the specific indole and benzodiazepine binding site of the HSA molecule. Since the lone tryptophan residue is located at position 213 in loop 4 of the HSA secondary structure and the amino acid sequence 145–150 in loop 3 is thought to form the main part of the indole and benzodiazepine binding site (15, 16, 34, 35), an independent binding of two ligands to both areas might be possible. The assumed location of both specific binding sites of HSA is in good agreement with the idea that the major binding sites of HSA are located in loops 3 and 4 (36, 37).

The warfarin binding site of HSA has been proposed as a model for the pharmacological receptor of the coumarin anticoagulants in the liver (1, 5, 12). It will be the task of future work in this area to find out whether or not a tryptophan residue is also involved in the receptor site in the liver.

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