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Fluorimetric Analysis of the Binding of Warfarin to Human Serum Albumin

Equilibrium and Kinetic Study

VIVIANE MAES,[1,2] YVES ENGELBORGHS,[3] JOHAN HOEBEKE,[4] YVAN MARAS,[1] AND ANTOINE VERCRUYSSE[1]

Afdelingen Farmacognosie, Fytochemie en Toxicologie, Microbiologie en Hygiëne, Faculteit Geneeskunde en Farmacie, Vrije Universiteit Brussel, B-1090 Brussel, and Laboratorium voor Chemische en Biologische Dynamica, Katholieke Universiteit Leuven, B-3030 Heverlee, Belgium

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SUMMARY

Binding of warfarin to human serum albumin results in a red shift of the UV absorption maximum, suggesting that the binding site is a hydrophobic area of the protein. The enhancement of the fluorescence of warfarin upon binding to human serum albumin was used to study the binding equilibrium and the kinetics of this drug-protein interaction. From equilibrium fluorescence measurements, contributions from free and bound warfarin could be evaluated. From the resulting Scatchard plots, equilibrium constants ranging from 4.2×10^5 to 3.5×10^5 M^{-1} for temperatures from 8° to 37° were calculated. The reaction is slightly exothermic ($\Delta H = -1.2$ kcal·mole^{-1}) and strongly entropy-driven ($\Delta S = +21$ cal·mole^{-1}·K^{-1}). The reaction rate constants of the warfarin-albumin interaction were determined by the stopped-flow technique. The association rate constant varies from 2.2×10^5 to 7.7×10^5 M^{-1} sec^{-1} from 10° to 32°. The corresponding activation enthalpy is 9.0 kcal·mole^{-1}. These values are not consistent with a diffusion-controlled reaction. The dissociation of the complex was studied by making use of the direct competition between warfarin and phenylbutazone for the same binding site. The dissociation rate constant varies from 2.5 to 10.8 sec^{-1} in the same temperature range. Activation parameters obtained in the kinetic experiments correspond very well with the thermodynamic parameters calculated from the equilibrium study, validating the fluorescence approach to the equilibrium studies.

INTRODUCTION

Serum albumin, a multifunctional globular protein, has a limited number of binding sites for the transport of organic molecules. This protein binds a wide variety of endogenous substances such as hormones, fatty acids, bilirubin, and foreign molecules such as drugs. By the use of fluorescence probes, Sudlow *et al.* (1, 2) were able to label two specific drug-binding sites of the HSA[5] molecule: Site I is the high-affinity site for the coumarin anticoagulant warfarin; Site II is the benzodiazepine-binding site. Digitoxin seems to be a ligand for a third site of HSA. These important drug-binding sites bind several other drugs, and this may give rise to potentially important drug interactions.

In order to elucidate the binding mechanism of warfarin to its high-affinity site, the binding equilibrium and the kinetics of the interaction were studied. Binding was monitored by the enhancement of warfarin fluorescence upon binding to HSA (3).

The binding parameters of the warfarin-HSA interaction have been investigated by several authors with different techniques such as equilibrium dialysis (4–7), ultrafiltration (8), gel filtration (9), and fluorimetry (1, 10). This paper brings a new approach to the fluorimetric titrations, as the observed fluorescence is split into contributions from free and bound warfarin. From measurements at different temperatures the thermodynamic parameters were derived.

An important source of the information about the binding mechanism is the kinetic study: the measurement of the reaction rates of association and dissociation and their temperature dependence, from which the activation

[1] Afdelingen Farmacognosie, Fytochemie en Toxicologie, Faculteit Geneeskunde en Farmacie, Vrije Universiteit Brussel.

[2] Aangesteld Navorser bij het Nationaal Fonds voor Wetenschappelijk Onderzoek, België.

[3] Laboratorium voor Chemische en Biologische Dynamica, Katholieke Universiteit Leuven. Bevoegd verklaard Navorser bij het Nationaal Fonds voor Wetenschappelijk Onderzoek, België.

[4] Microbiologie en Hygiëne, Faculteit Geneeskunde en Farmacie, Vrije Universiteit Brussel.

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

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parameters of the complex can be calculated. Although several authors have studied the kinetics of the interaction between HSA and endogenous compounds such as oleate (11), haemin (12), and bilirubin (13–15), studies of reaction rate and activation parameters of drug-albumin interactions are rather scarce. A kinetic study of the binding of salicylazosulfapyridine to HSA (16) and recent work on the warfarin-HSA kinetics (17) have been reported.

Our kinetic measurements were performed by the stopped-flow technique under psuedo-first order conditions, wherein warfarin concentration greatly exceeds that of HSA. Therefore the analysis of the data does not depend on the knowledge of the association constant. The association of warfarin with HSA was monitored by the fluorescence enhancement of the ligand. The dissociation rate constant was determined independently by making use of the competition between warfarin and phenylbutazone for the same binding site (5, 18, 19).

MATERIALS AND METHODS

Human serum albumin (essentially fatty acid-free, less than 0.005%; prepared from Fraction V human albumin) was obtained from Sigma Chemical Company (St. Louis, Mo.) (Lot 16C-7281). The concentration of HSA solutions was checked spectrophotometrically at 280 nm using an extinction coefficient of $E^{1\%}_{1cm} = 5.3$. All solutions were made in sodium phosphate buffer (0.01 M, pH 7.4, containing 0.9% NaCl). The albumin solutions (20 ml) were dialysed for 12 hr against buffer solution (1 liter) and filtered through a Millipore filter (pore size 0.22 μm) before use.

Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] was purchased from Sigma Chemical Company. Solutions of warfarin were prepared in the above buffer and concentrations were checked using the value of 1.39×10^4 for the molar absorption at 308 nm. Phenylbutazone was kindly provided by Drs. Keberle and Scheibli, of Geigy Pharmaceuticals. All other chemicals were of reagent grade. Optical density was measured in a Unicam SP 1750 spectrophotometer.

UV Difference Spectra

Difference spectra were obtained by the double-cell compensation technique of Herskovits (20). The spectra were recorded with an Aminco DW 2a UV-VIS spectrophotometer.

Warfarin in aqueous and ethanol media. A solution of 1 mM warfarin in 0.01 N NaOH was diluted to 50 μM (1:20) with water or with absolute ethanol. The baseline was set to zero with the ethanol blank (0.01 N NaOH/ethanol, 1:20) in the sample beam and the water blank (0.01 N NaOH/water, 1:20) in the reference beam. The difference spectrum was recorded with the aqueous warfarin solution in the reference beam and with the ethanol solution in the sample beam.

Warfarin, free and HSA-bound. The baseline was set to zero with buffer solution and HSA (15 μM) in double cells in both reference and sample beam. Difference spectra were recorded after addition of the same amount of warfarin to the buffer solution of the reference compartment and to the HSA solution of the sample compartment.

Equilibrium Binding Study

Fluorescence was measured with a JY-3C (Jobin-Yvon) spectrofluorometer, equipped with four thermally regulated cells and a magnetic stirrer in the cell compartment. Fluorescence was measured at 380 nm with excitation at 320 nm. Bandwidths of both excitation and emission were 4 nm. The use of fluorescence to determine the binding parameters of the warfarin-HSA interaction required three titration steps. Each titration was performed at least three times, at four different temperatures (8°, 18°, 24°, and 37°).

Determination of free warfarin fluorescence. A solution of 100 μM warfarin was added in small amounts to 2 ml of buffer solution. After mixing, fluorescence was measured.

Determination of bound warfarin fluorescence. A small amount of warfarin was titrated with HSA to determine the maximal fluorescence when all ligand was bound. Aliquots of a solution containing 200 μM HSA and 2 μM warfarin were added to 2 ml of a 2 μM warfarin solution, and fluorescence was measured. The contribution of HSA to the fluorescence was established in blank titrations; aliquots of a 200 μM HSA solution were added to 2 ml of buffer and the resulting fluorescence was measured under the same conditions.

Equilibrium titrations of HSA with warfarin. Small aliquots of a solution containing 100 μM warfarin and 2 μM HSA were added to 2 ml of a 2 μM HSA solution. After each addition, fluorescence was measured.

All fluorescence measurements were corrected for self-absorption of the solutions according to the method of Parker (21).

Kinetics

The kinetic experiments were performed on a temperature-controlled fluorescence stopped-flow instrument equipped with a 200-W Hanovia Xe-Hg lamp. The dead time of the stopped-flow, determined according to the method of Peterman (22) was 2.5 msec. The kinetic data were stored in a digital storage oscilloscope (Gould-Advance).

In a first series of experiments the association rate constant was determined at different temperatures (10°, 16°, 24°, and 32°) by mixing equal volumes of HSA and warfarin solutions. Final concentrations of 10 μM HSA and a range of 50–250 μM warfarin were used. The sample was excited at 320 nm (bandwidth 5 nm); for emission a Wratten 2B Kodak filter (>99% absorption beneath 390 nm) was used. The dissociation rate constant was obtained at the same temperatures by mixing solutions of phenylbutazone (final concentration 200–500 μM) and HSA, presaturated with warfarin (final concentration for both 10 μM). All measurements were performed at least three times for each concentration ratio.

RESULTS

UV difference spectra. Upon binding of warfarin to HSA there was a small shift of the warfarin absorbance maximum to longer wavelengths. A difference spectrum

was generated with a maximum at 333 nm and 2 minima at 289 and 301 nm, respectively (Fig. 1A). Dissolving warfarin in ethanol produced a similar shift of the absorbance maximum and a similar difference spectrum (Fig. 1B).

Equilibrium binding study. Under the chosen experimental conditions of the equilibrium titrations, the measured fluorescence consisted of only two components, the albumin fluorescence being negligible.

$$F = f_{w_f}[W_f] + f_{w_b}[W_b] \quad (1)$$

where f_{w_f} and f_{w_b} are the intrinsic molar fluorescence factors of free and bound warfarin, respectively and $[W_f]$ and $[W_b]$ are the molar concentrations of free and bound warfarin, respectively. Moreover, since the total ligand concentration was

$$[W_{tot}] = [W_f] + [W_b] \quad (2)$$

a set of two equations with two unknowns, the molar concentrations of free and bound ligand were obtained. Thus at each titration point free and bound warfarin could be calculated when the intrinsic fluorescence factors were known.

1. The slope of the plot of the observed fluorescence intensity versus the warfarin concentration represents the intrinsic molar fluorescence factor of the free warfarin. This factor decreases with increasing temperature.

2. In the titration of a small amount of warfarin with an excess of HSA, fluorescence tends to be maximal when all warfarin is bound. The maximal fluorescence value was obtained by extrapolation of the plot $1/F$ versus $1/[HSA]$ to $1/[HSA] = 0$, thus an infinite protein concentration. The ratio of this maximal value to the molar warfarin concentration used is the intrinsic molar fluorescence factor of bound warfarin. Comparison of both fluorescence factors indicated that upon binding to HSA the warfarin fluorescence at 380 nm increases about 13 times at the four temperatures.

3. The fluorescence values obtained in the equilibrium titrations (Fig. 2) and the corresponding total ligand concentrations were fitted to Eq. 1 and 2 from which free and bound ligand were derived. These binding data were plotted according to Scatchard (23), using the equation

$$\frac{r}{C} = K_A(n - r) \quad (3)$$

where r = number of moles ligand bound per mole of protein, C = molar concentration of free ligand, K_A = association constant, and n = number of binding sites per albumin molecule.

The Scatchard plots were linear for all temperatures with an intercept of about 0.95 at the r axis, indicating the presence of one binding site (Fig. 3). The slope, obtained by linear regression analysis, gives the value of the association constant. The association constant decreases slightly with rising temperature (Table 1). An enthalpy change $\Delta H = -1.2 \pm 0.2$ kcal·mole^{-1} was determined from a Van't Hoff plot with correlation coefficient 0.98. The free energy change ΔG, calculated from the thermodynamic relationship $\Delta G = -RT \ln K_A$ is given in Table 1. The entropy change, determined from $\Delta G = \Delta H - T\Delta S$, was highly positive ($\Delta S = +21$ cal·mole^{-1}·K^{-1}).

Kinetics. The kinetic curves clearly indicate that, upon binding of warfarin to HSA, warfarin fluorescence is enhanced (Fig. 4). The protein-ligand interactions can be

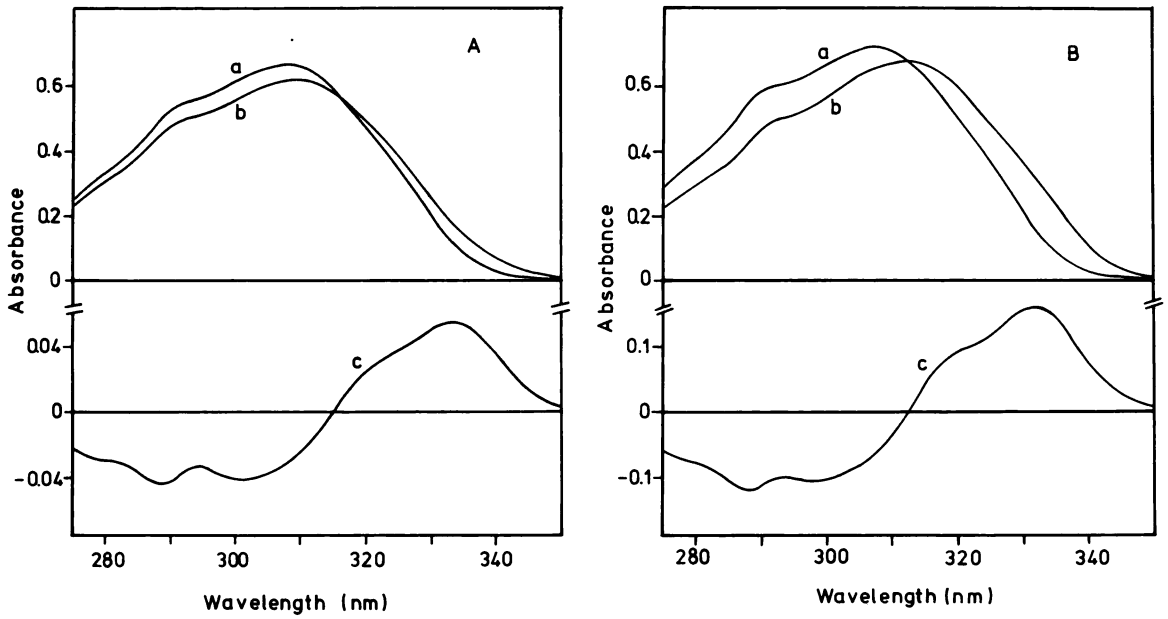

FIG. 1. *Ultraviolet difference spectra*

A. Ultraviolet difference spectra of HSA-bound versus free warfarin. *a*, Spectrum of warfarin versus buffer blank; *b*, spectrum of bound warfarin (HSA + warfarin) versus HSA blank; *c*, difference spectrum (see Materials and Methods). The concentration of warfarin was 44 μM; that of HSA, 15 μM.

B. Ultraviolet difference spectra of warfarin in ethanol versus aqueous solution. *a*, Warfarin in water (made alkaline with NaOH); *b*, warfarin in 95% ethanol (made alkaline with NaOH) (blanks represented the corresponding solvent); *c*, difference spectrum (see Materials and Methods). The concentration of warfarin was 50 μM.

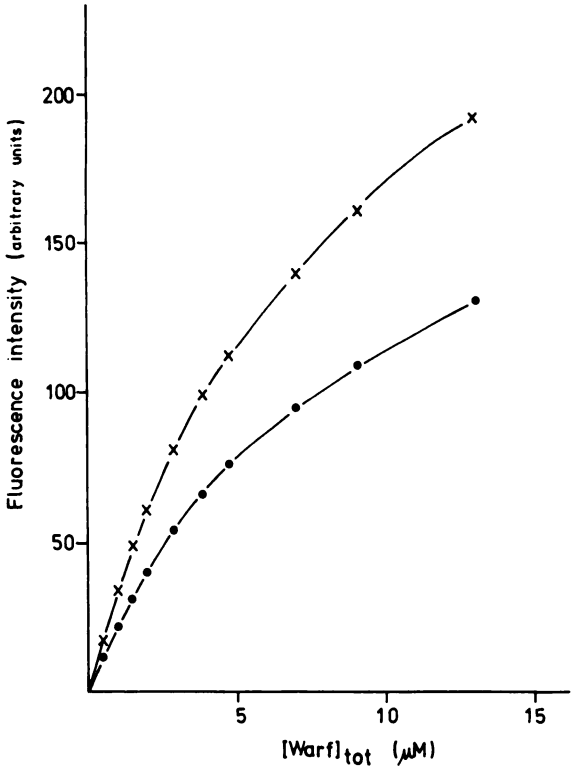

FIG. 2. *Increase in warfarin fluorescence upon binding to HSA: equilibrium titrations at two different temperatures*

The concentration of HSA was 2 μM. Each point represents the mean corrected value of at least three measurements at 8° (×) and 37° (●). Standard deviations were too small to be represented. The activation and emission wavelengths were 320 and 380 nm, respectively; bandwidths were 4 nm.

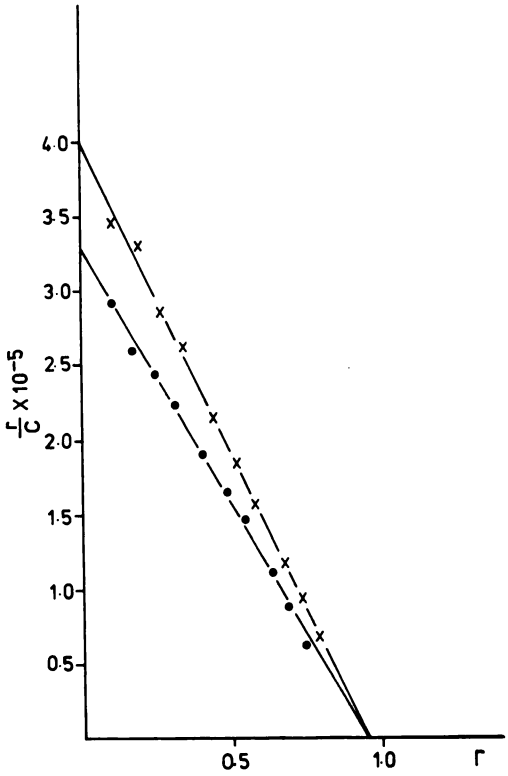

FIG. 3. *Scatchard plots for the binding of warfarin to HSA*

Binding was measured by fluorescence titration at 8° (×) and 37° (●). *r*, Number of moles of warfarin bound per mole of albumin; *C*, molar concentration of free warfarin.

described by the following mechanism:

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL$$

where P = free protein, L = free ligand, PL = protein-ligand complex and k_1 and k_{-1} = association and dissociation rate constants, respectively.

The rate at which free protein disappears during complexation was calculated as follows:

$$\frac{d[P]}{dt} = \frac{d[PL]}{dt} \quad (4)$$

$$= k_1[P][L] - k_{-1}[PL] \quad (5)$$

$$= k_1([L_0] - [PL])[P] - k_{-1}([P_0] - [P]) \quad (6)$$

where $[P_0]$ and $[L_0]$ are the total concentrations of protein and ligand, respectively.

Under experimental conditions in which ligand is in large excess over protein, $[L_0] - [PL] \simeq [L_0]$, so Eq. 6 becomes

$$\frac{d[P]}{dt} = (k_1[L_0] + k_{-1})[P] - k_{-1}[P_0] \quad (7)$$

which determines a pseudo-first order reaction with an observed rate constant $k_{obs} = k_1[L_0] + k_{-1}$.

The kinetic data of the fluorescence changes versus time were transferred to a semilogarithmic plot (Fig. 4), from which k_{obs} was calculated for five saturating concentrations of ligand. The plot of k_{obs} versus ligand concentration was a straight line for all temperatures (Fig. 5); from the slope determined by linear regression analysis, the association rate constant was obtained (Table 2).

To obtain the dissociation rate constant of the warfarin-HSA complex, the decrease in warfarin fluorescence was monitored after mixing the complex with a large excess of the competitor phenylbutazone. The concentration of phenylbutazone was such that after the dissociation of the HSA-warfarin complex the reassociation of warfarin was minimal.

The k_{obs}, the observed rate constant of the fluorescence decrease, was calculated from semilog plots for two concentrations of phenylbutazone in large excess over HSA (Fig. 6). The k_{obs} values are independent of the phenyl-

TABLE 1

Binding and thermodynamic parameters of warfarin-HSA interaction

$\Delta H = -1.2 \pm 0.2$ kcal·mole^{-1}; $\Delta S = +21$ cal·mole^{-1}·K^{-1}; n, number of binding sites. Values in parentheses are the correlation coefficients of the respective linear regressions.

Temperature	$K_A \times 10^{-5}$	n	ΔG
	M^{-1}		kcal·mole^{-1}
8°	4.20 ± 0.08 (0.998)	0.95	−7.2
18	3.97 ± 0.07 (0.999)	0.94	−7.5
27	3.59 ± 0.10 (0.996)	0.88	−7.6
37	3.46 ± 0.09 (0.997)	0.94	−7.8

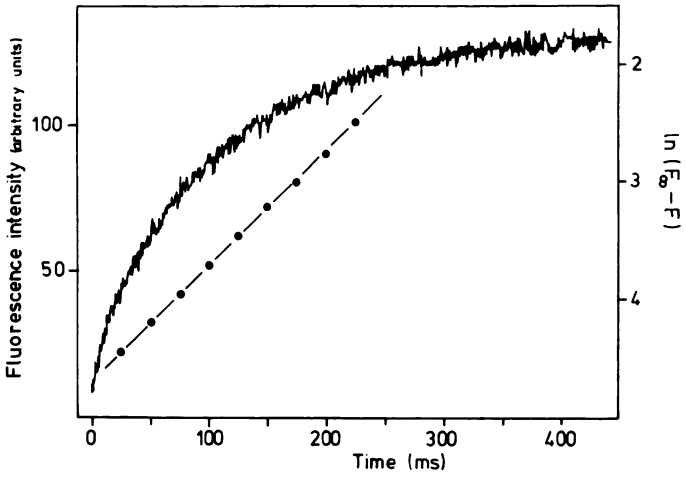

FIG. 4. *Representative stopped-flow kinetic curve of the warfarin-HSA association and the corresponding linear semilogarithmic plot*

The final HSA and warfarin concentrations were 10 μM and 50 μM, respectively. The excitation wavelength was 320 nm, and fluorescence was measured above 390 nm. Measurement was performed at 10°. The slope of the semilogarithmic plot yields the observed rate constant k_{obs} for the corresponding warfarin concentration.

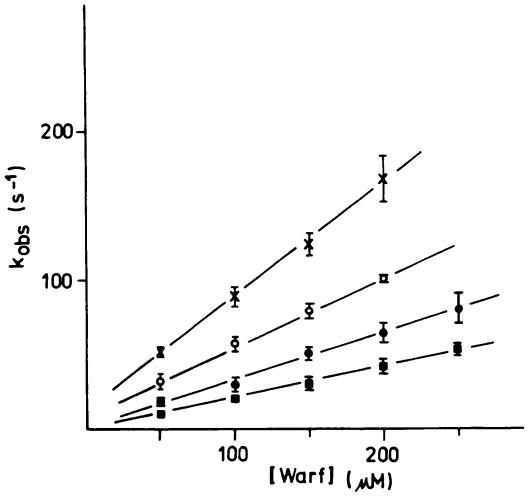

FIG. 5. *Association of warfarin to HSA*

Plots of the observed rate constant k_{obs} versus warfarin concentration at different temperatures: 10° (■), 16° (●), 24° (○), and 32° (×). The HSA concentration was 10 μM. Each point represents the average of at least three stopped-flow measurements. Standard deviations are presented. The slope yields the association rate constant.

butazone concentration and represent the dissociation rate constant k_{-1} of the HSA-warfarin complex. The values k_1 and k_{-1} at four different temperatures allowed the calculation of the activation parameters according to the following equations:

$$k = \frac{k_B \cdot T}{h} \cdot e^{-\Delta G^*/RT} \quad \text{(Eyring equation)} \qquad (8)$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad (9)$$

and thus

$$\ln \frac{k}{T} = \left(\ln \frac{k_B}{h} + \frac{\Delta S^*}{R} \right) - \frac{\Delta H^*}{R} \cdot \frac{1}{T} \quad \text{(Arrhenius plot)} \qquad (10)$$

where k_B is Boltzmann's constant, h is Planck's constant, R is the gas constant, ΔG^* is the activation free energy, ΔH^* is the activation enthalpy, and ΔS^* is the activation entropy. The results of these calculations are presented in Table 2.

Combination of the activation parameters yielded the over-all reaction parameters

$$\Delta H_{\text{over-all}} = \Delta H^*_{on} - \Delta H^*_{off} = -1.6 \text{ kcal} \cdot \text{mole}^{-1} \qquad (11)$$

$$\Delta S_{\text{over-all}} = \Delta S^*_{on} - \Delta S^*_{off} = +17 \text{ cal} \cdot \text{mole}^{-1} \cdot K^{-1} \qquad (12)$$

DISCUSSION

The binding of warfarin to HSA results in a UV difference spectrum very similar to the one obtained when the drug is dissolved in ethanol. As ethanol has a lower dielectric constant and is less polar than water, it may be suggested that the spectrum results from the movement of the ligand molecule from an aqueous to a more hydrophobic region in the albumin molecule. An analogous similarity of spectra was also observed for phenylbutazone (24, 25).

Although bound warfarin fluoresces much more strongly than the free product, the fluorescence of the free ligand is far from negligible under experimental conditions. It is therefore important to split the observed fluorescence intensity into contributions of free and bound warfarin. By solving Eq. 1 and Eq. 2, free and bound ligand concentrations were calculated at each titration point. The resulting linear Scatchard plots clearly indicate that there is only one binding site for warfarin which is responsible for the observed fluorescence changes. In accordance with our results, other fluorimetric studies indicate the presence of one strong binding site (1, 10). With other techniques such as equilibrium dialysis (4–7), ultrafiltration (8), and gel filtration (9) one or more classes of low-affinity binding sites are observed. Most authors agree on the existence of one high-affinity site for warfarin on the HSA molecule. It has been calculated that, at clinically encountered plasma warfarin concentrations, 95% of the bound warfarin occupies this site (7). The primary binding constants reported in the literature are rather divergent. We took into account the fluoresence contribution of free ligand; therefore, our results are higher than the values of Chakrabarti (10). Since some fatty acids compete for the same

TABLE 2

Reaction rate and activation parameters of warfarin-HSA interactions

ΔH^*_{on} = 9.0 ± 0.5 kcal·mole^{-1} (0.996); ΔH^*_{off} = 10.6 ± 0.5 kcal·mole^{-1} (0.998); ΔS^*_{on} = −2 cal·mole^{-1}·K^{-1}; ΔS^*_{off} = −19 cal·mole^{-1}·K^{-1}. Values in parentheses represent correlation coefficients of the respective linear regressions.

Temperature	$k_1 \times 10^{-5}$	k_{-1}	$K_A \times 10^{-4}$
	M^{-1} sec^{-1}	sec^{-1}	M^{-1}
10°	2.2 ± 0.1 (0.999)	2.5 ± 0.1	8.8 ± 0.8
16	3.2 ± 0.2 (0.996)	3.9 ± 0.2	8.2 ± 0.9
24	4.6 ± 0.1 (0.999)	6.1 ± 0.3	7.5 ± 0.5
32	7.7 ± 0.3 (0.998)	10.8 ± 0.3	7.1 ± 0.5

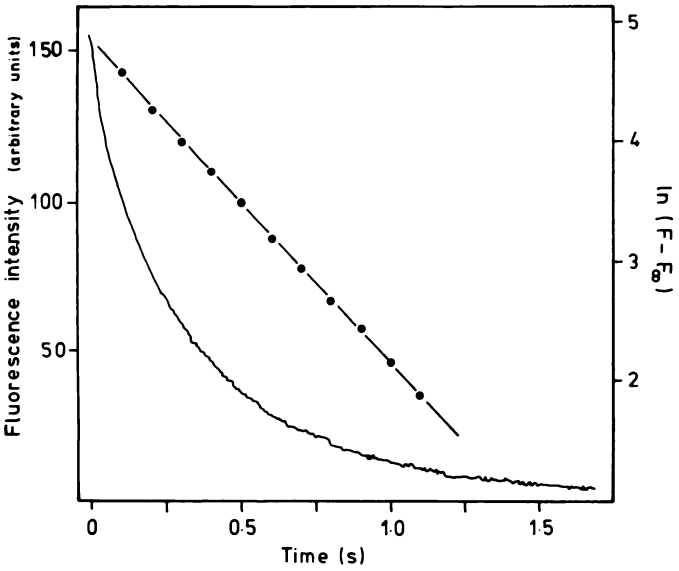

FIG. 6. *Representative stopped-flow kinetic curve of the dissociation of the warfarin-HSA complex in the presence of phenylbutazone, and the corresponding semilogarithmic plot*

The final concentrations of both HSA and warfarin were 10 μM; the final concentration of phenylbutazone was 0.5 mM. The excitation wavelength was 320 nm, and fluorescence was measured above 390 nm; temperature was 10°.

binding site on HSA (19), the fatty acid-free HSA used in our experiments may have added to the higher values of K_A as compared with other fluorimetric results.

By repeating the entire experimental procedure at four different temperatures the thermodynamic parameters could be calculated. The binding constant decreases with rising temperature: the reaction is slightly exothermic. The contribution of the entropy in the free energy change is more important and emphasizes the hydrophobic character of the binding, since O'Reilly (26) demonstrated that electrostatic interactions do not contribute to the entropy factor. For many interactions between organic molecules and albumin, a positive entropy has been reported: azo dyes (27), penicillins (28), and phenylbutazone (25). Our warfarin data are comparable to the values obtained by other authors (Table 3). O'Reilly and Kowitz (4) calculated a ΔH of −3.48 kcal·mole^{-1} and a ΔS of + 11 cal·mole^{-1} ·K^{-1} from the temperature dependence of the binding constant. In a microcalorimetric study O'Reilly *et al.* (29) obtained a ΔH of −3.14 kcal·mole^{-1}. The exothermy of the reaction was more pronounced and was ascribed to hydrogen bonding. From gel filtration measurements at different temperatures, Oester *et al.* (9) derived a ΔH of −2.55 kcal·mole^{-1} and a ΔS of 16 cal·mole$^{-1}K^{-1}$.

The value of the association rate constant (Table 2) is too low to define a diffusion controlled reaction, for which a rate constant of more than 3×10^7 M^{-1}sec^{-1} is expected (30). Moreover, the value of the activation enthalpy of association (9 kcal·mole^{-1}) excludes the possibility of diffusion control, as the expected value for such a reaction is about 4 kcal·mole^{-1} (30).

Not every encounter between protein and drug results in a complex. Definitive binding must therefore be accompanied by a supplementary process. Sterical factors may be involved. A conformational change in the protein cannot be excluded. Possible reasons for the higher ΔH^*_{on} are dehydration of the reactants and/or bond disruption in the protein upon formation of the activated complex. Transition from this activated state to the final complex is accompanied with a large entropy gain.

It is interesting to compare these data with the results of the competitor phenylbutazone. The over-all reaction entropy is considerably larger (25). Part of this entropy is already gained in the transition from reactants to activated state.[6] The activation parameters of dissociation from complex to activated state are very similar for both compounds. The difference in ΔS^*_{on} of the two competitors probably lies in the liberation of a different number of water molecules upon formation of the respective activated complexes. Phenylbutazone competitively displaces dansylglycine from HSA (24), whereas warfarin does not displace this probe (3). This suggests that warfarin and phenylbutazone share only a part of a rather large binding site on the HSA molecule. The difference in ΔS^*_{on} may then originate from the different parts of their binding site.

The value of k_{-1}, calculated as the ordinate intercept in the plot k_{obs} versus warfarin concentration (Fig. 5) agrees very well with the value obtained independently from our kinetic measurements based on the warfarin-phenylbutazone competition. The intercepts at the two lowest temperatures approach the zero value and are not reliable. Linear regression analysis leads to intercepts of 8.6 sec^{-1} at 24° and 12.3 sec^{-1} at 32°. The agreement with the k_{-1} values presented in Table 2 proves the validity of our method.

The consistency of the thermodynamic values (ΔH and ΔS) obtained by the fluorescence equilibrium study with those calculated from the fast kinetic measurements strengthen the validity of our analysis of the equilibrium data. There is some discrepancy between the association constants derived from the equilibrium measurements and those obtained in the kinetic study. The possible explanation that in the dissociation study the excess of phenylbutazone binds to its secondary sites, causing an increase in the k_{-1} value, is excluded, as the k_{-1} calculated independently as the ordinate intercept corresponds with the experimental values. Moreover, we may conclude that binding of phenylbutazone to its secondary sites has no effect on the conformation of the primary HSA-warfarin complex. Another explanation is that there are two conformations of the albumin molecule, whereby the association rate constant of one form greatly exceeds that of the other and the dissociation rate constants are identical. In the equilibrium study a weighted average affinity will be found. In the kinetic experiments the binding to the high-affinity site of one conformation is very fast and occurs within the mixing time without being detected. The existence of two pH-dependent conformations of the albumin molecule has indeed been described (7). A pH-dependence study of the kinetics and an accurate amplitude analysis are necessary to examine this hypothesis.

Our kinetic data show no evidence for deviation of linearity (k_{obs} versus warfarin concentration) as observed

[6] V. Maes, Y. Engelborghs, J. Hoebeke, and A. Vercruysse, unpublished results.

TABLE 3

Primary binding and thermodynamic parameters reported for warfarin-HSA interaction at pH 7.4

Temperature	n_1	$K_1 \times 10^{-5}$	ΔH	ΔS	Method	Reference
		M^{-1}	$kcal \cdot mole^{-1}$	$cal \cdot mole^{-1} \cdot K^{-1}$		
3°	1	1.54	−3.48	+11.2	Equilibrium dialysis	4
15	1	1.40				
27	1	0.92				
37	1	0.87				
3	2	3.85	−3.48	+11	Equilibrium dialysis	5
15	2	3.50				
27	2	2.31				
37	2	2.17				
25	0.89	12.4			Equilibrium dialysis	6
25	1	9			Equilibrium dialysis	7
22	1.25	3.32			Ultrafiltration	8
6	1.38	3.21	−2.55	+16.1	Gel filtration	9
15	1.38	2.78				
25	1.38	2.40				
37	1.38	2.03				
42	1.38	1.90				
22	1.30	0.81[a]			Fluorimetry	10
22	0.90	2.50			Fluorimetry	1

[a] Value reported for a HSA concentration of 5 μM.

by Rietbrock and Lassman (17). At all temperatures, our values for k_{obs} are much larger than the maximal rate (k_2) calculated by these authors. However, it should be noted that in their analysis the rate constant k_2 is determined as the reciprocal of a small intercept with a large relative error. In our experiments the dissociation rate constant was determined completely independently of the association study, whereas Rietbrock and Lassman (17) calculated their rate constant (k_{-2}) from the intercept, which is again a small value.

The kinetic analysis presented here allows the calculation of the association constant independently from equilibrium binding studies and without the need for the determination of free ligand concentrations. The fact that warfarin is highly bound to HSA strongly decreases the rate of clearance of the drug. Indeed, only free ligand is available for hepatic metabolism or biliary secretion (the urinary excretion of warfarin is negligible) (31).

If the dissociation rate constant is very small, this dissociation step may be rate-limiting for the clearance. One way of determining whether the dissociation rate constant of warfarin binding is a limiting factor for the clearance is to compare the dissociation half-time to the mean transit time for plasma through the organ of clearance (16). The dissociation half-time of warfarin bound to its primary site is about 0.06 sec, whereas the mean transit time through liver is about 10 sec. It is clear that the dissociation rate is not a limiting factor for warfarin clearance.

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Send reprint requests to: Dr. Viviane Maes, Dienst Toxicologie, Farmaceutisch Instituut V.U.B., Laarbeeklaan, 103, B-1090 Brussels, Belgium.