Interaction of the Anticoagulant Drug Warfarin and Its Metabolites with Human Plasma Albumin

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ABSTRACT The interaction of the anticoagulant drug warfarin and its metabolites with human plasma albumin was studied by equilibrium dialysis. A 20-fold variation of buffer ionic strength (0.017–0.340) caused no significant change in the warfarin association constant. But the binding strength rose significantly as the pH was increased from 6.0 to 9.0 and then declined at pH 10.0. The 6-, 7-, and 8-hydroxywarfarin metabolites showed a 7- to 23-fold reduction in binding strength at pH 10.0. These data indicate that the molecular basis of the interaction is nonelectrostatic and that the introduction of polar hydroxyl groups on the coumarin nucleus by metabolism reduces its hydrophobic binding surface. The interaction was markedly exothermic and showed a positive entropy (increased molecular disorder), which suggests cooperative hydrogen and hydrophobic bonding as the molecular basis for the binding of warfarin to albumin.

The marked albumin binding and nonpolar character of warfarin explains the respective absence and presence of the unchanged drug in urine and plasma of warfarin-treated patients, while the more polar character and lesser albumin binding of the metabolites probably determines their absence in plasma and presence in urine. The relatively marked binding to albumin of the 4′-hydroxywarfarin metabolite suggests that it may occur in the plasma of warfarin-treated patients. The data suggest that a direct correlation exists between the interaction of warfarin with plasma albumin and the interaction with the warfarin receptor site.

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INTRODUCTION

In previous studies of the interaction of the coumarin anticoagulant warfarin sodium and human plasma albumin, the drug was strongly bound to plasma albumin when analyzed by equilibrium dialysis (1). While the strength of the interaction was studied as a function of temperature, no experiments were performed at different levels of buffer pH and ionic strength. Such experiments were performed in the present study which together with the thermodynamic data allowed us to analyze the molecular basis of the warfarin-albumin interaction.

Countercurrent distribution analysis of plasma of patients receiving clinical doses of warfarin shows essentially only the unchanged drug and similar analysis of urine reveals only hydroxylated metabolites of warfarin (2, 3). To determine if the absence of the metabolites in plasma and the absence of unchanged drug in the urine could be correlated with albumin binding, we also studied the four known metabolites by equilibrium dialysis. Based on these data, a hypothesis is presented for the pharmacodynamics of warfarin in man.

METHODS

Warfarin sodium

Material. The equilibrium dialysis technique, the crystalline human albumin and warfarin sodium, the preparation of the cellophane bags, the experimental techniques employed, the measurement of free and bound drug, and the determination of the standard free energy change were exactly as described previously (1). The low concentrations of warfarin sodium were varied over a 16-fold range (6–100 μmoles/liter), which evaluates mainly the association constant of the first warfarin anion bound (k_1). The concentration of

albumin used in all experiments was 0.4% (57.9 μM/liter). The experiments on the effect of the ionic strength of the medium were carried out over a 20-fold range of phosphate buffer, 0.0067–0.134 M (ionic strength 0.017–0.340), at pH 7.4 and 27°C. The experiments on the effect of the pH of the medium were carried out in sodium and potassium phosphate buffer for pH 6.0 and 7.4, in potassium phosphate–sodium borate buffer for pH 9.0, and in sodium borate–sodium carbonate buffer for pH 10.0, all at 0.170 ionic strength (μ) and 27°C. The molar extinction coefficient of warfarin showed no significant variation on raising the pH from 6 to 10. Experiments could not be be performed at a pH of < 6 because of marked precipitation of the drug.

Methods. Human plasma albumin,[1] prepared by repeated crystallization from Cohn fraction V, was used in all experiments. By cellulose acetate electrophoretic analysis, the albumin was 100% pure. The water content of the albumin, evaluated by heating a small sample in an oven at 110°C until a constant weight was obtained, was found repeatedly to be less than 1%; therefore, no correction for water content was required. The 0.4% albumin solution was prepared in phosphate buffer, 0.067 mole/liter, pH 7.4, unless otherwise specified. Two forms of warfarin, (3-(α-acetonylbenzyl)-4-hydroxy-coumarin) were used: a clathrate prepared by repeated crystallization of amorphous warfarin sodium from isopropyl alcohol (91.2% warfarin sodium), and an amorphous powder prepared by commercial methods (97.0% warfarin sodium, with 3.15% loss on drying to a constant weight (1).[2] When corrected for the difference in warfarin sodium content, the results obtained with the two forms of the drug did not vary significantly.

Dialysis bags were made from $\frac{18}{32}$ inch cellophane casing (Union Carbide Corp., Visking Div., Chicago, Ill.). The casings were cleaned by continuous rinsing in a recycling bath of deionized water for 8 hr and could then be stored up to 10 days in deionized water at 4°C. Just before use the bags were washed repeatedly with deionized water, followed by the phosphate buffer; they were not allowed to dry. With this technique the optical density of blank buffer carried through a run at the maximal wavelength of warfarin was always less than 0.005.

Experimental. 10 ml of albumin-buffer solution was added inside the dialysis bag, and 15 ml of warfarin solution was added outside. To ensure efficient mixing, we prepared the bags with an enclosed air bubble and without tension. Dialysis was carried out in 50-ml glass tubes covered with Parafilm caps. The tubes were placed on a horizontal wrist-action shaker with a capacity of 24 tubes and rocked through a 5° arc at a frequency of 150 cpm. Equilibrium controls, consisting of warfarin-buffer solution outside and buffer solution inside the dialysis bags, were included in all experiments. To rule out leakage of albumin, we routinely tested the fluid outside the dialysis bags with 3% sulfosalicylic acid; in no instance was protein found outside the bags. Since warfarin, like most ligands, is adsorbed to some extent by the dialysis bags, calculation of the amount of warfarin actually bound by albumin requires a correction for bag binding. To determine the degree of adsorption to the bags, we carried out dialysis as described, but without the addition of albumin.

At equilibrium, the concentration of unbound warfarin on both sides of the membrane must be equal, and any increment of the drug in the protein compartment represents bound warfarin (1). The concentration of free warfarin anion in equilibrium with bound anion was determined by measuring the drug concentration outside the dialysis bag. The method used for determining warfarin content (3) will detect concentrations as low as 5 μmoles/liter and has an error of 2%. The amount of albumin-bound warfarin was determined by subtracting the free and bag-bound warfarin from the amount added initially. The accuracy of the method was verified in separate experiments with warfarin-^{14}C (4.35 μc/mg),[3] in which the labeled warfarin inside and outside the dialysis bags was determined by liquid scintillation counting. The results obtained by the two methods at all concentrations of warfarin varied less than 1%. Whether the drug was added initially to the inside or outside of the dialysis bag had no effect on the percentage of warfarin bound to albumin.

Metabolites

Material. The urinary metabolites of warfarin sodium in man and rat have been identified and synthesized (4, [3]). These were obtained as a white crystalline powder and diluted in buffer without any further purification.[4] All four metabolites at the concentrations studied (6–100 μmoles/liter) were insoluble at pH 7.4, but were readily soluble at pH 10.0. The competitive equilibrium dialysis experiments with 7-hydroxywarfarin, nonradioactive warfarin sodium, and warfarin-^{14}C were performed as described previously (5).

Methods. The conditions of the competitive equilibrium dialysis experiments were as follows: the pH was adjusted to 10.0 with the use of 0.15 M sodium borate in sodium carbonate buffer; the dialysis bags contained human albumin in a concentration of 0.4% (57.9 μM/liter) and ^{14}C-labeled warfarin sodium in concentrations varying from 2 (6.06 μM/liter) to 32 mg/liter (96.7 μM/liter). At equilibrium, aliquots of the solutions inside and outside the bag were analyzed for radioactivity in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Similar experiments were carried out with labeled warfarin sodium and albumin solutions inside the bags and either nonradioactive warfarin or nonradioactive 7-hydroxywarfarin, 32 mg/liter (96.7 μM/liter), in the dialysis tube outside the bag. At equilibrium for the labeled warfarin sodium, aliquots were collected and counted for radioactivity as before to determine the concentration of bound warfarin in the presence of either "cold" warfarin or "cold" 7-hydroxywarfarin. The diminution in the binding of warfarin sodium was used to calculate the association constants to albumin by means of the following equation of Edsall and Wyman (6):

$$k_B = \frac{1}{(B)}\frac{k_A}{k_{A'}} - 1$$

in which k_A is the association constant for labeled warfarin sodium alone, $k_{A'}$ is the warfarin sodium association constant in the presence of the "cold" compound, (B) is the molar concentration of the "cold" compound, and k_B is the apparent association constant of the nonradioactive (cold) compound for albumin.

The equilibrium dialysis experiments with the four metabo-

[1] Lot 22, obtained from Pentex Inc., Kankakee, Ill.

[2] Kindly supplied by the late Dr. Nathan Weiner, Endo Laboratories Inc., Garden City, N. Y.

[3] Hermodson, M., and K. P. Link. 1967. Personal communication.

[4] Kindly provided by Professor K. P. Link, University of Wisconsin School of Agriculture, and Dr. C. Schroeder, Wisconsin Alumni Research Foundation.

lites were carried out under exactly the same conditions as for warfarin sodium. The concentration of each metabolite was determined spectrophotometrically by measuring the optical density at its ultraviolet absorption maximum. The $\log_{10}$ of the molar extinction coefficient and the ultraviolet absorption maximum for each metabolite at pH 10.0 was 4.107 ±0.010 (302 mμ) for 6-hydroxywarfarin, 4.416 ±0.014 (330 mμ) for 7-hydroxywarfarin, 4.164 ±0.003 (304 mμ) for 8-hydroxywarfarin, and 4.179 ±0.005 (308 mμ) for 4′-hydroxywarfarin. The adherence to Beer's law was examined for each metabolite. Linear dependence of optical absorption upon concentration was observed in every instance up to the highest concentration of each metabolite used in the experiments.

Calculations

The binding constants for the interaction were analyzed as before (1) by means of the Scatchard equation for the law of mass action (7) $\bar{v}/A = kn - k\bar{v}$, where $\bar{v}$ is the molar ratio of bound warfarin to albumin, A is the molar concentration of free warfarin at equilibrium, k is the average association for the binding at each site, and n is the average maximum number of binding sites on the albumin molecule. For each compound studied $\bar{v}$ and $\bar{v}/A$ and their standard errors at every concentration used were calculated.[5] A regression line was calculated for each set of data by the method of least squares, and kn (equal to the first anion bound or k_1) and n were determined by extrapolation as in the previous study (1). The values of $\bar{v}/A$ plotted against $\bar{v}$ give a straight line when all the binding sites are independent and equivalent. Deviations from linearity can arise from interaction of the bound anions or from the occurrence of binding at more than one class of sites with different association constants. As $\bar{v}/A$ approaches zero as a limit, the intercept on the $\bar{v}$ axis (abscissa) is n, and as $\bar{v}$ approaches zero as a limit, the intercept on the $\bar{v}/A$ axis (ordinate) is kn the classical association constant for the first anion bound. The standard free energy change of binding for the first anion bound (ΔF_1^0) was determined from k_1 by the general thermodynamic relationship $\Delta F_1^0 = -RT \ln k_1$, where R is the gas law constant and T is the absolute temperature.

RESULTS

The effects of varying the buffer ionic strength on the interaction of warfarin and human plasma albumin at pH 10.0 and 27°C are reported in Tables I and II. Raising the ionic strength from 0.017 to 0.340 resulted

[5] In our previous study (1) an arithmetic error occurred and all values of $\bar{v}$ and $\bar{v}/A$ were low by a factor of 2.5 because in the calculation of $\bar{v}$, the albumin molarity was calculated incorrectly on a volume of 25 ml, the total volume both inside and outside the bags, rather than on a correct basis of a 10 ml volume for the albumin solution, the volume inside the bags. Hence $\bar{v}$ and therefore $\bar{v}/A$ were too low by a factor of 25/10 or 2.5. We are grateful to Doctors D. E. Guttman and M. C. Meyer of the State University of New York at Buffalo School of Pharmacy for bringing this fact to our attention.

TABLE I

*Effect of Buffer Ionic Strength (μ) on Mean Binding Data for Each Concentration of Warfarin Studied**

No. of experiments	Initial warfarin concentration		Final warfarin concentration: Bound		Unbound	Bag-bound	$\bar{v}$ ±SE‡	$\bar{v}/A$ ±SE§ × 10^{-4}
	mg/liter	*μM/liter*	*%*	*μM/liter*	*μM/liter*	*μM/liter*		*liter/mole*
					μ 0.017			
5	2	6.1	77.0	4.7	1.3	0.2	0.20 ± <0.01	16.67 ±0.25
8	4.1	12.2	77.9	9.5	2.6	0.1	0.40 ± <0.01	15.78 ±0.50
10	8.2	24.6	75.2	18.5	5.3	0.8	0.80 ± <0.01	15.20 ±0.48
9	16.1	48.4	63.9	31.2	16.6	0.6	1.38 ± <0.01	8.38 ±0.20
7	32.9	99.7	46.4	46.0	51.5	2.2	1.98 ± <0.01	3.85 ±0.17
					μ 0.170			
13	4	12.1	79.3	9.6	2.2	0.3	0.40 ± <0.01	17.88 ±0.15
12	8	24.2	74.8	18.1	5.3	0.8	0.78 ± <0.01	14.83 ±0.08
10	16	48.4	66.3	32.1	14.7	1.6	1.38 ± <0.01	9.68 ±0.03
10	32	96.9	45.4	44.0	49.5	3.4	1.90 ± <0.01	4.10 ±0.02
					μ 0.340			
4	4	12.1	80.2	9.7	2.4	0.0	0.43 ± <0.01	17.98 ±0.85
4	8	24.2	74.0	17.9	6.3	0.0	0.78 ± <0.01	12.38 ±0.33
5	16.2	48.8	62.9	30.7	18.1	0.0	1.33 ± <0.01	7.33 ±0.02
4	32.5	98.4	44.8	44.0	52.9	1.5	1.90 ± <0.01	3.60 ±0.01

* Temperature 27°C., pH 7.4.

‡ $\bar{v}$, average molar ratio of bound warfarin to albumin, determined by dividing data in fifth column (molar concentration of bound warfarin) by 23.2 μM/liter (the product of the concentration of the albumin solution, 57.9 μM/liter, and its relative volume, 10 ml/25 ml).

§ $\bar{v}/A$, average molar ratio of bound warfarin, divided by the concentration of unbound warfarin (A) at equilibrium, determined by dividing data of eighth column ($\bar{v}$) by data of sixth column (A), and expressed in units of liters per mole (liter/mole).

Table II

Standard Free Energy Change of Binding for Warfarin and Human Plasma Albumin at 27°C: Effect of pH and Ionic Strength of Buffer and Effect of Metabolism

Compound	Buffer pH	Buffer Ionic strength	Number of binding sites	Association constant (k_1) × 10^{-4}	$\text{Log}_{10}\, k_1$	Standard free energy change ($\Delta F_1{}^0$)
				liter/mole	*liter/mole*	*kcal/mole*
Warfarin	6.0	0.170	2.2	16.13	5.21	−7.16
Warfarin	7.4	0.170	2.6	23.00	5.36	−7.37
Warfarin	9.0	0.170	1.7	37.60	5.58	−7.66
Warfarin	10.0	0.170	1.7	16.41	5.22	−7.18
Warfarin	7.4	0.017	2.5	19.19	5.28	−7.25
Warfarin	7.4	0.340	2.3	20.78	5.32	−7.30
6-hydroxywarfarin	10.0	0.170	2.0	2.37	4.37	−5.99
7-hydroxywarfarin	10.0	0.170	0.4	0.71	3.85	−5.29
8-hydroxywarfarin	10.0	0.170	1.6	1.85	4.07	−5.58
4′-hydroxywarfarin	10.0	0.170	1.6	10.13	5.01	−6.88
Average			1.9			

k_1, average association constant for the first warfarin anion bound; $\Delta F_1{}^0$, standard free energy change for the first warfarin anion bound.

in less than 2% variation in log k_1 and $\Delta F_1°$. The number of binding sites remained essentially unchanged and averaged 1.9 for all the compounds.

The results of varying the pH of the buffer (27°C and ionic strength 0.170) are reported in Table III; the results at pH 7.4 are seen in Table I under μ0.170. The values for k_1, n, and $\Delta F_1°$ are shown in Table II. At pH 6.0 k_1 was 161,250 liter/mole; it rose with increasing pH until the maximum k_1 of 376,000 liter/mole was reached at pH 9.0. At pH 10.0, k_1 fell to 164,000 liter/mole. The values for log k_1 and $\Delta F_1°$ for the pH levels used varied 7% and 0.50 kcal/mole, respectively.

The results of the competitive dialysis experiments (27°C., pH 10.0, and ionic strength 0.170) are given in

Table III

*Effect of Buffer pH on Mean Binding Data for Each Concentration of Warfarin Studied**

No. of experiments	Initial warfarin concentration		Final warfarin concentration: Bound		Unbound	Bag-bound	$\bar{v}$ ±SE	$\bar{v}/A$ ±SE × 10^{-4}
	mg/liter	*μM/liter*	*%*	*μM/liter*	*μM/liter*	*μM/liter*		*liter/mole*
					pH 6.0			
5	4	12.1	75.2	9.1	3.0	0.0	0.40 ± <0.01	13.03 ±0.40
3	8	24.2	73.5	17.8	6.4	0.0	0.72 ± <0.01	12.03 ±0.38
6	16.1	48.8	61.9	30.2	17.2	1.4	1.35 ± 0.03	7.82 ±0.08
5	32.6	97.6	46.7	46.1	50.8	0.7	2.00 ± 0.02	3.93 ±0.08
					pH 9.0			
12	4	12.1	84.6	10.4	1.7	0.0	0.45 ± <0.01	25.15 ±0.15
14	8.2	24.2	76.9	19.0	5.2	0.0	0.83 ± <0.01	15.75 ±0.03
15	16.3	48.8	57.6	28.4	19.8	0.6	1.23 ± 0.02	6.23 ±0.02
12	32.7	97.6	43.5	43.1	53.8	0.7	1.85 ± 0.03	3.58 ±0.01
					pH 10.0			
18	2.0	6.1	78.7	4.8	1.3	0.0	0.21 ± <0.01	15.43 ±0.68
8	4.1	12.4	70.6	8.7	3.3	0.4	0.37 ± <0.01	11.40 ±0.35
8	8.2	24.8	67.7	16.8	7.4	0.6	0.73 ± <0.01	9.38 ±0.33
15	16.3	48.8	54.6	26.9	21.5	0.4	1.15 ± 0.03	5.40 ±0.28
12	32.6	97.6	34.5	34.1	62.5	1.0	1.48 ± 0.02	2.35 ±0.02

See Table I for explanation of the abbreviations.
* Temperature 27°C, ionic strength of buffer 0.170.

TABLE IV

*Effect of Nonradioactive Warfarin and of Nonradioactive 7-Hydroxywarfarin on Mean Binding Data for Each Concentration of Warfarin-¹⁴C-Studied**

No. of experiments	Initial warfarin concentration		Final warfarin concentration				$\bar{v}$ ±SE	$\bar{v}/A$ ±SE × 10^{-4}
			Bound		Unbound	Bag-bound		
	mg/liter	*μM/liter*	%	*μM/liter*	*μM/liter*	*μM/liter*		*liter/mole*
			Nonradioactive warfarin: 32 mg/liter (96.9 μM/liter)					
6	4	12.1	33.9	4.1	7.8	0.2	0.18 ± <0.01	2.38 ±0.02
7	8.2	24.8	32.7	8.1	16.3	0.4	0.35 ± <0.01	2.13 ±0.03
7	16.2	48.8	29.6	14.5	33.9	0.4	0.63 ± <0.01	1.85 ±0.02
4	32.5	97.6	25.9	25.5	71.4	0.8	1.10 ± <0.01	1.53 ±0.01
			7-hydroxywarfarin 32 mg/liter (96.9 μM/liter)					
6	2.1	6.2	71.9	4.5	1.7	0.0	0.20 ± <0.01	11.80 ±0.55
7	4.0	12.1	66.9	8.3	3.8	0.0	0.35 ± <0.01	9.70 ±0.33
9	8.4	24.4	59.4	15.1	9.3	0.0	0.65 ± <0.01	6.98 ±0.18
12	16.4	48.4	49.1	24.3	24.1	0.0	1.05 ± <0.02	4.30 ±0.13
7	31.4	96.0	34.9	34.2	61.8	0.0	1.48 ± 0.08	2.40 ±0.15

See Table I for explanation of abbreviations.
* Temperature 27°C, pH 10.0, ionic strength of buffer 0.170.

Table IV and Fig. 1. The data for the radioactive warfarin alone are almost identical to those listed in Table III under pH 10.0. The common point near the abscissa and different slopes for the displacement of radioactive warfarin by nonradioactive warfarin (96.9 μM/liter) and 7-hydroxywarfarin (96.9 μM/liter) are characteristic of competition for common binding sites (competitive displacement) on the albumin molecule (8). From the degree of diminution of binding of the warfarin-¹⁴C, the logs of the association constant for warfarin and 7-hydroxywarfarin were 5.17 and 3.82, respectively. By direct equilibrium dialysis the logs of the association constant for warfarin and 7-hydroxywarfarin were 5.22 and 3.85, respectively.

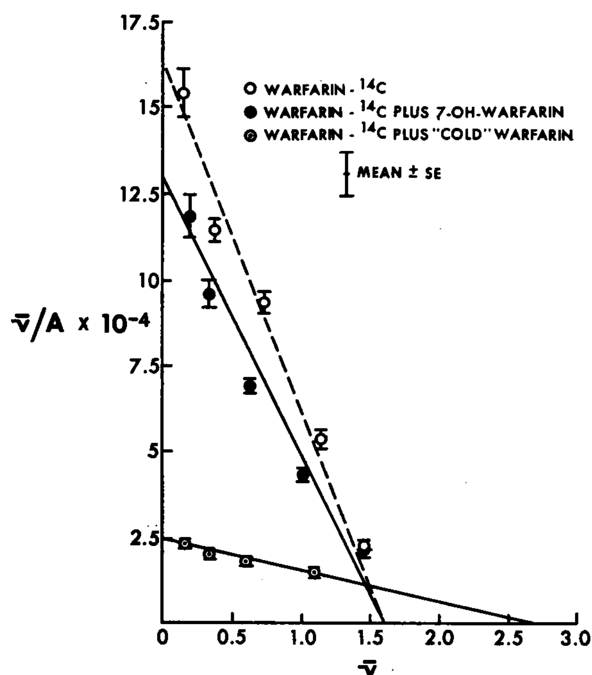

FIGURE 1 Scatchard plot of data in competitive equilibrium dialysis studies of the binding of warfarin-¹⁴C sodium and human plasma albumin in the presence and absence of either nonradioactive warfarin sodium or nonradioactive 7-hydroxywarfarin. The nearly common intercept and the markedly different slopes indicate the competitive nature of the albumin binding of the radioactive and nonradioactive compounds.

The mean binding data for the four metabolites (27°C, pH 10.0, and ionic strength 0.170) are reported in Table V and in Figure 2. The 6-, 7-, and and 8-hydroxywarfarin, in which the hydroxyl group is inserted on the benzpyran part of the coumarin nucleus, showed markedly less binding than unchanged warfarin, with k_1 7–23 times less for these metabolites (Table II). But k_1 for 4′-hydroxywarfarin, in which the hydroxyl group is present on the benzyl group of the side chain, was only ½-fold less bound than the unchanged drug. The $\Delta F_1°$ was 1.2–1.9 kcal/mole less for the 6-, 7-, and 8-hydroxywarfarin than for unchanged warfarin. The $\Delta F_1°$ was only 0.3 kcal/mole less than the unchanged drug for the 4′-metabolite.

DISCUSSION

Equilibrium dialysis was used in these experiments because it directly determines albumin binding and allows reasonable representation of physiologic conditions. This method is preferred to ultrafiltration, which may introduce some error because of accumulation of protein at the membrane surface and may therefore adversely af-

TABLE V

*Mean Binding Data for Each Concentration of Warfarin Metabolites Studied**

No. of experiments	Initial warfarin concentration		Final warfarin concentration				$\bar{\nu}$ ±SE	$\bar{\nu}/A$ ±SE × 10^{-4}
			Bound		Unbound	Bag-bound		
	mg/liter	*μM/liter*	%	*μM/liter*	*μM/liter*	*μM/liter*		*liter/mole*
					6-hydroxywarfarin			
10	4	12.1	33.7	4.1	8.0	0.0	0.18 ± <0.01	2.20 ±0.08
11	8	24.2	30.8	7.5	16.7	0.0	0.33 ± <0.01	2.08 ±0.10
10	16.3	49.0	27.2	13.2	36.8	0.0	0.60 ± 0.01	1.63 ±0.08
6	32	96.9	22.8	22.1	74.8	0.0	0.94 ± 0.02	1.28 ±0.05
					7-hydroxywarfarin			
15	4	12.1	10.9	1.3	10.8	0.0	0.06 ± <0.01	0.53 ±0.03
15	8	24.2	8.5	2.1	22.1	0.0	0.09 ± <0.01	0.40 ±0.03
17	16	48.4	7.7	3.8	44.6	0.0	0.16 ± 0.01	0.38 ±0.02
16	32	96.9	5.9	5.7	91.1	0.0	0.25 ± 0.03	0.28 ±0.01
					8-hydroxywarfarin			
9	4	12.1	28.3	3.4	8.7	0.0	0.15 ± <0.01	1.75 ±0.05
10	8	24.2	25.2	6.1	18.1	0.0	0.28 ± <0.01	1.45 ±0.08
9	16	48.4	21.5	10.4	38.0	0.0	0.45 ± 0.02	1.20 ±0.05
5	32	96.9	18.7	18.1	78.8	0.0	0.78 ± 0.03	1.00 ±0.02
					4'-hydroxywarfarin			
9	4	12.1	63.8	7.7	4.4	0.0	0.35 ± <0.01	8.05 ±0.25
9	8	24.2	57.7	14.0	10.2	0.0	0.60 ± <0.01	6.23 ±0.23
7	16	48.4	47.0	22.7	25.7	0.0	0.98 ± 0.03	3.90 ±0.30
6	32	96.9	31.4	30.4	66.5	0.0	1.33 ± 0.02	1.98 ±0.05

See Table I for explanation of abbreviations.
* Temperature 27°C., pH 10.0, ionic strength of buffer 0.170

fect ligand-protein bonds (9). The possible sources of error in the dialysis experiments were membrane adsorption of drug, a Donnan effect, denaturation of the protein and pH changes during dialysis, and alteration in the membrane pore size. There was no evidence of any of these physicochemical changes probably because equilibrium of the drug and its metabolites was achieved at all concentrations within 4 hr. The membrane adsorption of unchanged warfarin in these experiments was about 1% of the free drug concentration, which is significantly lower than that observed previously (1). None of the warfarin metabolites showed any evidence of bag binding. Direct measurement at the end of the dialysis runs showed no significant Donnan effect detectable by the volume inside the bag, no pH change, and no measurable leak of protein across the membrane. Close adherence to Beer's law for every compound indicated no evidence of self-aggregation of the drug and its metabolites in the concentrations studied. The equilibrium dialysis technique is often criticized because the ions of the relatively strong buffer solutions used to eliminate the Donnan effect of the protein can compete for the binding sites (10). In the present experiments at pH 7.4, a 10-fold reduction and 2-fold elevation of buffer ion concentration did not significantly change the binding constant.

Variation in the ionic strength of the buffer results in a change in the binding strength of a ligand-protein interaction if electrostatic forces are present (11). Changes in ionic strength can also alter the dielectric constant of the medium and hence the binding interaction, but with the moderately dilute solutions used in these studies the dielectric constant probably did not vary significantly. A lack of change of binding affinity during variation in ionic strength ruled out any significant ionic interaction between warfarin and albumin. This is not surprising as warfarin is a weakly acidic anion (pKa 5.1) and has no strongly ionizing groups. Most previous studies on anion-albumin binding that have shown marked electrostatic interactions (12) were performed with compounds (organic dyes and sulfa drugs) with strong ionizing groups (sulfates).

The data clearly indicate that on raising the pH from 6.0 to 9.0 the strength of the warfarin-albumin interaction surprisingly increased. With a rising pH and hence an increasing negative charge on the protein, a substantial decrease in the ability to attract anions might be anticipated (13). At a pH of 6, the basic amino acids ar-

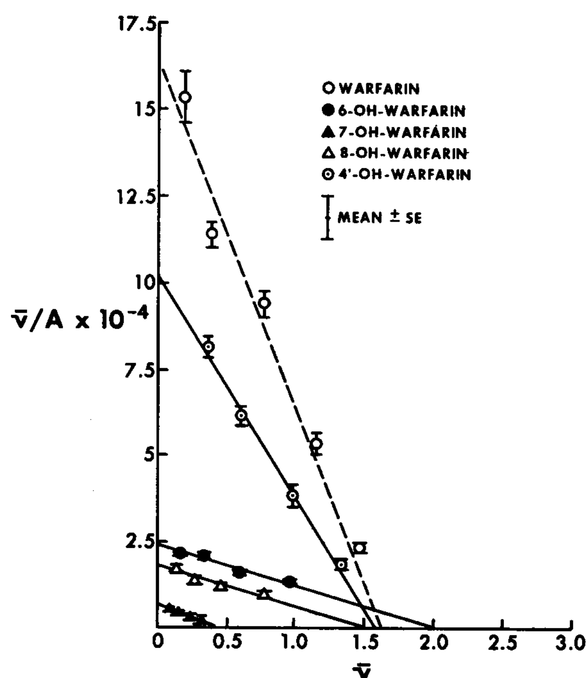

FIGURE 2 Scatchard plot of data in equilibrium dialysis studies of the binding of warfarin sodium and four of its metabolites with human plasma albumin. The intercepts on the $\bar{v}$ axis equals the number of binding sites, which for most of the compounds was nearest the whole integer of 2.

ginine, histidine, and lysine combine with a hydrogen ion and hence have a net positive charge, whereas the acidic amino acids, such as glutamic acid, release a hydrogen ion and form carboxylate ions (14). Since the isoelectric point of plasma albumin is near pH 5, the number of carboxylate ions at pH 6 exceeds the number of quarternary nitrogen atoms and thus the net charge on the protein is negative. Nevertheless, the presence of the positively charged nitrogen atoms apparently forms a focus of attachment for the anionic warfarin. Thus, one would expect the binding to decrease at a pH where the quarternary nitrogen atoms lose their hydrogen ions. The region of decreased binding of warfarin and albumin (above pH 9) corresponds to the region in which the ϵ-amino group of lysine loses its hydrogen ion (pH 9.4–10.6) (14).

While the increase in warfarin-albumin interaction with the increase in pH from 7.4 to 9.0 is consistent with the uncovering of new binding sites on the albumin molecule, it is inconsistent with an electrostatic mechanism: an increasing number of negative charges on the protein should lead to a decrease in binding (14). Changes in the availability of tyrosine hydroxyl groups and in the configuration of the protein molecule occur in human plasma albumin at pH levels above 7, arising apparently from a reversible swelling or unfolding of the molecule (13). It seems likely that the change in configuration of albumin with rising pH is not the result of dissociation of any specific side chain or of any change in the helical content (15) but rather an effect of a swelling or unfolding induced by the strong electrostatic repulsions that develop in the protein molecule as its negative charge increases beyond -10 at pH 7.6 and higher (16). In our previous study of the warfarin-albumin interaction (1), the very act of complex formation with anions apparently increases the net negative charge of the albumin and makes more sites available for further binding (16). This mechanism could account for the continued failure of all attempts to find an actual plateau on graphs for the number of ions of warfarin bound, or any other anion noncovalently bound to albumin, plotted against the free ion concentration (1). The increase in binding strength of an anion to albumin when the pH is raised above 7.4 also occurs with bilirubin (17), L-tryptophan (18), testosterone (19), thyroxine (20), penicillin (21), organic dyes (13), and sulfa drugs (12).

The metabolites bound to human plasma could not be subjected to continuous flow electrophoresis to prove their exclusive binding to the albumin fraction. However, this has been demonstrated for unchanged warfarin, and no instance has been reported of a drug bound to albumin whose metabolites were bound significantly to a nonalbumin fraction (1). Additionally, the competition of unchanged warfarin and one of its metabolites for albumin could be evaluated by competitive equilibrium dialysis, as the relative ability of analogues to displace a compound from albumin can be taken as a measure of the relative affinity of the analogues for albumin (6). The close agreement of the association constant for 7-hydroxywarfarin determined by direct dialysis and by competitive dialysis indicates the absence of any significant nonalbumin binding for this warfarin metabolite (8).

The 6-, 7-, and 8-hydroxywarfarin metabolites were bound to albumin 7- to 23-fold less than the unchanged drug. Unlike unchanged warfarin, the metabolites appeared to have only a single class of binding sites that were of the same order of binding magnitude as the lower class of sites of the unchanged drug. However, the limited availability of the metabolites prevented study of them at higher concentrations. The 4′-hydroxywarfarin metabolite showed only a slight decrease in binding strength from that of unchanged warfarin. As shown in Fig. 3, the 6, 7, and 8 positions of the warfarin molecule occur on the benzpyran portion of the coumarin nucleus, while the 4′ position is located on the benzyl group of the side chain. The binding energy of the coumarin nucleus metabolites (Table II) was about 1.2–1.9 kcal/mole less than unchanged warfarin at pH

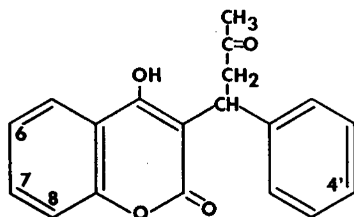

FIGURE 3 Structural formula of warfarin. The numbers indicate the positions for hydroxylation by metabolism which yields the four known metabolites of the drug. The benzpyran nucleus (4-hydroxycoumarin) is seen on the left and the acetonyl-benzyl side chain is seen on the right side of the molecule.

10 and 27°C. This indicates that under physiologic conditions of pH 7.4 and 37°C the benzpyran portion of the warfarin molecule contributes at least 2.0 kcal/mole of the 7.5 kcal/mole of its binding energy to albumin. Similarly, in studies of corticosteroids increasing the number of polar substituents by hydroxylation and hydrogenation depressed the binding to plasma albumin (22).

The binding of warfarin to human plasma albumin (complex formation) at pH 7.4 and 37°C results in a free energy change of about − 7.6 kcal/mole. About 40% of the source of this free energy change (− 3 kcal/mole) is the result of the favorable enthalpy of the reaction (1), which has been confirmed by direct calorimetric measurement.[6] The remaining part of the free energy change is derived from a favorable entropy change (+ 15 electrostatic units). Simplistically, one would expect the entropy change on complex formation to be markedly negative because of a restriction of translational and rotational entropy accompanying the decrease in the number of ions in solution (23). It is unlikely that the positive entropy change is the result of significant disorientation of the albumin molecule, at least for the first warfarin anion bound, as the reaction is exothermic whereas a process of unfolding with the breaking of bonds would be an endothermic reaction (24). However, the role of the aqueous solvent must be considered. When a compound is dissolved in a polar solvent, the solvation of ions and polar groups is a large factor in reducing the entropy of the system (25). Because the albumin and warfarin molecules are hydrated on solution in aqueous solvent, there should be an increase in the number of molecular species on binding because of the release of water of solvation from both the warfarin and albumin molecules; hence, one would expect an increase in entropy for the interaction (25).

The sizeable negative enthalpy and the relative insensitivity to changes in ionic strength indicate that electrostatic factors do not play a role in the warfarin-albumin interaction and that the formation of one or more hydrogen bonds contributes significantly to the noncovalent interaction (26). The positive entropy probably results from hydrophobic bonding in which nonpolar groups on the molecular surfaces of the albumin and the warfarin are brought together, thereby reducing their contact with water (27). The extrusion of water between the warfarin anions and the side chains of the protein lining the binding sites brings the two surfaces closer together and thus facilitates the formation of distance-sensitive hydrogen bonds (28). It has been shown experimentally that hydrophobic interactions enhance the formation of hydrogen bonds (29). The loss of binding energy for the 6-, 7-, and 8-hydroxywarfarin metabolites could be the result of decreased hydrophobic bonding of the benzpyran nucleus because of the introduction of the polar hydroxyl group by metabolism. The relative lack of reduction of binding energy of the 4′-hydroxywarfarin metabolite would be the result of the preservation of the hydrophobic binding surface of the benzpyran nucleus. The 4-hydroxy group of the benzpyran nucleus of unchanged warfarin functions as an enolate group; it is a likely group for hydrogen bond formation of the warfarin molecule to albumin and to its receptor site as loss of that group results in loss of anticoagulant activity (30).

The interaction of drugs with proteins represents an important aspect of drug metabolism because of the interactions with *a*) receptor proteins which may result in pharmacologic activity of the drug, *b*) an enzyme responsible for the degradation of the drug, and *c*) inert proteins of blood (albumin) or tissue which may be responsible for the distribution and transport of the drug and may limit its activity or metabolism (31). All three of these aspects of drug metabolism have been studied with warfarin in man: the first by the prothrombinopenic response (2), the second by the half-life of the drug in plasma and its correlation with the duration of the anticoagulant effect (32), and the third by the warfarin-albumin interaction described herein. Administration of the 6-, 7-, and 8-hydroxywarfarin metabolites to rats does not produce an anticoagulant effect (4). This agrees with the earlier observations of Link et al. (30) that the introduction of any chemical group by synthesis into the 5, 6, 7, or 8 positions of the benzpyran nucleus of a 4-hydroxycoumarin anticoagulant resulted in a marked loss of its anticoagulant activity. Because of the marked albumin binding of 4′-hydroxywarfarin, the intact benzpyran nucleus of 4′-hydroxywarfarin, and the marked biologic activity of warfarin compounds synthesized with substituents in the 4′ position (Sintrom and Coumachlor) (33), one would predict this metabolite to be biologically active and to cause prothrombino-

[6] O'Reilly, R. A., J. Ohms, and C. Motley. 1968. Unpublished observations.

penic effect in man and rat. These findings suggest that a direct correlation exists between the interaction of warfarin with plasma albumin and with its receptor site.

Thus, the high degree of albumin binding and nonpolar character of warfarin would account for its high levels in plasma, low levels in urine, and small volume of distribution (2), and the low protein binding and more polar character of the warfarin metabolites would account for their low levels in plasma and high levels in urine (3). However, these findings should not be extrapolated as generalizations for human metabolism. The clearance of some sulfate ester metabolites of pregnenolone and its derivatives is actually slower than that of the unchanged compound, the volume of distribution is smaller, and the half-lives in plasma are longer (34). The lack of increased sensitivity of response to coumarin anticoagulants in patients with uremia suggests that the metabolites are devoid of anticoagulant activity and that the unchanged drug is not cleared by the normal human kidney (35). We reported a metabolite of warfarin with an ultraviolet absorption maximum of 320 mμ in the urine of man (3). Since the ultraviolet absorption maxima of the four known warfarin metabolites are 302, 304, 308, and 330 mμ, the previous maximum of 320 mμ probably represents more than one urinary metabolite of the drug.

Recently, a photofluorometric method for the determination of warfarin was described (36). This method consistently gives higher values than the ultraviolet spectrophotometric method (3) for plasma of warfarin-treated patients, but gives identical values when warfarin is added to plasma in vitro.[7] The marked binding to albumin and marked fluorescence[7] of the 4′-hydroxywarfarin metabolite suggest that it may occur in the plasma of warfarin-treated patients which would account for the consistently higher readings for warfarin obtained by the fluorometric method. The similar physical properties of warfarin and its metabolite 4′-hydroxywarfarin (marked albumin binding and an identical ultraviolet absorption maxima of 308 mμ) may explain the previous inability to separate the two by countercurrent distribution with spectral measurement at 308 mμ and therefore to wrongly conclude that the spectrophotometric assay for unchanged warfarin in biologic specimens was specific (3).

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[7] O'Reilly, R. A. 1968. Unpublished observations.

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