MOLECULAR PHARMACOLOGY, 7, 209–218

Interaction of Several Coumarin Compounds with Human and Canine Plasma Albumin

ROBERT A. O'REILLY

WITH THE TECHNICAL ASSISTANCE OF CATHERINE H. MOTLEY

Departments of Medicine, Santa Clara Valley Medical Center, San Jose 95128, and the University of California School of Medicine, San Francisco 94122, and the Institute for Medical Research of Santa Clara County, San Jose, California 95128

(Received October 21, 1970)

SUMMARY

The interaction of several structural analogues of the anticoagulant drug sodium warfarin with human and canine plasma albumin was studied by equilibrium dialysis. The compounds studied were two precursor compounds, coumarin and 4-hydroxycoumarin; the anticoagulant drugs acenocoumarin, bishydroxycoumarin, ethyl biscoumacetate, and phenprocoumon; two metabolic products, 5-hydroxywarfarin and the warfarin alcohols; and the optical enantiomers (−)-S-warfarin and (+)-R-warfarin. The major source of the binding energy for the coumarin compounds was the coumarin nucleus itself. The precursor coumarins, which have almost no anticoagulant activity, showed a single binding site for albumin, while all the coumarin anticoagulant drugs studied showed two binding sites, suggesting that the side chains on the coumarin molecule provided anticoagulant activity not through an increase of binding energy but rather by the formation of a second binding site. There was little difference in the binding energy of the optical enantiomorphs of warfarin. The surprisingly high binding energy of the metabolite 5-hydroxywarfarin probably resulted from the formation of a hydrogen-bonded ring.

The binding of warfarin by human plasma albumin was 6 times greater than that by canine plasma albumin. This finding may explain why in man, with greater binding, phenylbutazone potentiates warfarin, but in dogs, with less binding, the enzyme-inducing effects of phenylbutazone predominate. The hydrophobic nature of the substituents on the 4-hydroxycoumarin molecule correlated directly with the binding strength of the compounds to plasma albumin and to the intracellular receptor site for anticoagulant activity.

INTRODUCTION

Analysis of the binding mechanism of a drug with plasma albumin can provide insight into its interaction with sites of biological activity. In previous studies the binding of the coumarin anticoagulant drug sodium warfarin and some of its known metabolic products with human plasma albumin was studied by continuous flow electrophoresis (1), equilibrium dialysis (2), and heat-burst microcalorimetry (3). In the present study the interaction of several other coumarin compounds with human plasma albumin was determined by equilibrium dialysis. The coumarin compounds were two precursor coumarins almost devoid of anticoagulant activity, four well-known anti-

This work was supported by United States Public Health Service Grant HE 8058-08.

coagulant drugs, two newly described metabolites of sodium warfarin in man (4), and the resolved optical isomers of the commercially available racemate of sodium warfarin (5). In addition, the binding of sodium warfarin to both human and canine plasma albumin was compared to examine further the discrepancy between man and dog in the therapeutic interaction of oral anticoagulants with phenylbutazone (6). With these diverse data, the molecular basis for the association of coumarin compounds with plasma albumin and sites of anticoagulant activity was analyzed.

EXPERIMENTAL PROCEDURE

Materials. Equilibrium dialysis, preparation of cellophane bags, experimental procedures, measurement of free and bound drug, and determination of the free energy change were performed essentially as described previously (1, 2). The low concentrations of the coumarin compounds were varied over a 16-fold range, to evaluate only the association constant of the first class of binding sites. Human plasma albumin (lot 27, Pentex), prepared by repeated crystallization from Cohn's Fraction V, was used in all experiments except when the comparative binding of sodium warfarin (Coumadin, Panwarfin) to human and canine plasma albumin was studied. In these experiments unchanged Fraction V of human and canine plasma albumin (Pentex) was used. The concentration of albumin used in all experiments was 0.4% (57.9 μM). The coumarin compounds, obtained as amorphous powders from academic and commercial sources, were used at 95% purity by weight. The compounds were as follows: coumarin, Fritsche Brothers and Wisconsin Alumni Research Foundation; 4-hydroxycoumarin and bishydroxycoumarin (Dicumarol), Abbott Laboratories; ethyl biscoumacetate (Tromexan) and acenocoumarin (Sintrom), Geigy Pharmaceuticals; phenprocoumon (Marcumar), Hoffmann–La Roche; S(−)-D-warfarin, R (+)-L-warfarin, and the racemic mixture (±)-S,R-warfarin, Endo Laboratories and Wisconsin Alumni Research Foundation; and 5-hydroxywarfarin and the side chain alcohol of warfarin, Dr. W. F. Trager, University of California School of Pharmacy, San Francisco. The experiments were carried out at 27° in 0.067 M sodium phosphate buffer at pH 7.4 and ionic strength 0.170 or in 0.067 M sodium borate–sodium carbonate buffer at pH 10.0 and ionic strength 0.181. Dialysis bags were made from $^{15}/_{16}$-inch Visking cellophane casings. The casings were cleaned by continuous rinsing in a recycling bath of deionized water for 8 hr and could be stored up to 10 days in deionized water at 4°. Just before use, the casings were washed repeatedly with deionized water, followed by the buffer used in the experiment; they were not allowed to dry. With this technique the optical density of a buffer blank carried through an experimental run was always less than 0.015 at the wavelength of the maximum extinction coefficient for the coumarin compound studied.

Experimental methods. The albumin-buffer solution (10 ml) was placed inside the dialysis bag, and 15 ml of the coumarin-buffer solution were placed outside the bag. To ensure efficient mixing, the bags were prepared with an enclosed air bubble and without tension. Dialysis was performed in 50-ml glass tubes covered with caps of Parafilm. The tubes were placed on a horizontal wrist-action shaker with a capacity of 24 tubes and rocked through a 5-degree arc at a frequency of 150 cycles/min. Equilibrium control bags, consisting of coumarin-buffer solution outside and buffer solution inside, without any albumin, were included in all runs. To detect leakage of albumin by the bags, the outside fluid was tested with 3% sulfosalicylic acid; in no instance was protein found outside the bags. To determine the degree of adsorption of the coumarin compounds to the bags, dialysis was carried out as described above without the addition of albumin. As binding to the bag did not occur with any of the coumarin compounds studied, no correction for bag binding was required in the experimental calculations.

At equilibrium the concentrations of the unbound coumarin compound on both sides of the membrane are equal, and any increment of the drug in the protein compart-

ment represents binding of the coumarin compound to the albumin (1, 2). The amount of unbound coumarin compound was determined by measuring the coumarin concentration outside the dialysis bag. The amount of coumarin compound bound to the albumin was determined by subtracting the unbound compound from the amount added initially. Whether the coumarin compound was added initially to the inside or outside of the dialysis bag had no effect on the amount of the compound bound to albumin. Most of the compounds were sparingly soluble at pH 7.4 but were freely soluble at pH 10. Previous studies with warfarin showed little difference in binding strength between pH 10 and 7.4 (2). As coumarin was freely soluble at pH 7.4 and unstable at pH 10, it was studied at the former pH. The concentrations of the anticoagulant drugs (ethyl biscoumacetate, acenocoumarin, bishydroxycoumarin, phenprocoumon, and warfarin) were determined spectrophotometrically by measuring the optical density at their ultraviolet absorption maxima (7). The values of the $\log_{10}$ ± standard error of the molar extinction coefficient and the ultraviolet absorption maximum for the coumarin compounds were, at pH 7.4, coumarin, 4.034 ± 0.011 (276 mμ); and at pH 10, 4-hydroxycoumarin, 4.170 ± 0.006 (286 mμ); warfarin alcohol, 4.178 ± 0.008 (308 mμ); 5-hydroxywarfarin, 4.107 ± 0.007 (302 mμ); (−)-S-warfarin, 4.160 ± 0.012 (308 mμ); and (+)-R-warfarin, 4.166 ± 0.003 (308 mμ). The adherence to Beer's law was examined for each compound; linear dependence of optical density was observed up to the highest concentration used in the experiments.

Calculations. The binding constants for the interaction were analyzed by means of the Scatchard equation for the law of mass action, $\bar{\nu}/A = kn - k\bar{\nu}$, where $\bar{\nu}$ is the molar ratio of bound coumarin to albumin, A is the molar concentration of free coumarin at equilibrium, k is the average association constant for the binding at each site, and n is the average number of binding sites on the albumin molecule (1, 2). For each compound studied, $\bar{\nu}$ and $\bar{\nu}/A$ and their standard errors at each concentration studied were calculated. A regression line was calculated for each set of data by the method of least squares, and kn (equal to the first coumarin anion bound, or k_1) and n were determined by extrapolation (1, 2). The values of $\bar{\nu}/A$ plotted against $\bar{\nu}$ give a straight line when the binding sites are independent and equivalent. As $\bar{\nu}/A$ approaches zero as a limit, the intercept on the $\bar{\nu}$-axis (abscissa) is n, and as $\bar{\nu}$ approaches zero as a limit, the intercept on the $\bar{\nu}/A$ axis (ordinate) is k_1, the association constant for the first anion bound. The standard free energy change for the first anion bound ($\Delta F_1°$) was determined from k_1 by the general thermodynamic relationship $\Delta F_1° = -RT \ln k_1$, where R is the gas law constant and T is the absolute temperature. About 600 separate experiments were carried out, which averaged about 50 experiments per compound and 10 experimental points for each concentration of every compound studied.

RESULTS

The values for n, k_1, and $\Delta F_1°$ are shown in Table 1. The $\Delta F_1°$ for coumarin was −5.81 kcal/mole, and for 4-hydroxycoumarin, −6.72 kcal/mole, an increment of about −0.9 kcal/mole. The number of binding sites for both compounds was 0.9 (Fig. 1). Acenocoumarin, a monocoumarin anticoagulant drug had a $\Delta F_1°$ of −7.09 kcal/mole, slightly less than that of (±)-S,R-warfarin (−7.16 kcal/mole), while the other monocoumarin anticoagulant, phenprocoumon, had a $\Delta F_1°$ of −7.44 kcal/mole, somewhat greater than warfarin. These three monocoumarin anticoagulants had an average number of binding sites of 1.9. Ethyl biscoumacetate, a dicoumarin anticoagulant, had a $\Delta F_1°$ of −6.54 kcal/mole. Another dicoumarin anticoagulant, bishydroxycoumarin, had a $\Delta F_1°$ of −7.37 kcal/mole. The dicoumarin anticoagulants had an average number of binding sites of 1.8.

The $\Delta F_1°$ for warfarin alcohol, −7.07 kcal/mole, was nearly the same as that for (±)-S,R-warfarin, −7.16 kcal/mole. The $\Delta F_1°$ for warfarin alcohol at pH 7.4 was larger than at pH 10; this was also true for (±)-S,R-warfarin (2). The $\Delta F_1°$ at pH 10 of 5-hydroxywarfarin was the largest of any

TABLE 1

Binding data for coumarin compounds and plasma albumin

Plasma albumin	Coumarin compound	Average daily dose for therapeutic hypoprothrombinemia	pH of buffer	No. of binding sites[a]	Association constant, k_1[a]	$\text{Log}_{10} k_1$	Standard free energy change ($\Delta F_1°$)
		mg			$M^{-1} \times 10^{-4}$	M^{-1}	*kcal/mole*
Human crystalline	Coumarin	0	7.4	0.9 ± 0.1	1.7 ± 0.2	4.24	−5.81
	4-Hydroxycoumarin	1500	10	0.9 ± 0.1	7.9 ± 0.3	4.90	−6.72
	Ethyl biscoumacetate	150	10	1.6 ± 0.2	5.9 ± 0.1	4.77	−6.54
	Acenocoumarin	5	10	2.1 ± 0.1	14.7 ± 0.4	5.17	−7.09
	Bishydroxycoumarin	75	10	2.0 ± 0.1	23.1 ± 0.5	5.36	−7.37
	Phenprocoumon	4	10	1.9 ± 0.2	26.6 ± 0.8	5.42	−7.44
	Warfarin alcohol	50	10	1.3 ± 0.1	14.5 ± 0.1	5.15	−7.07
	Warfarin alcohol	50	7.4	1.8 ± 0.1	15.6 ± 0.2	5.19	−7.14
	5-Hydroxywarfarin	0	10	2.0 ± 0.1	27.2 ± 0.3	5.43	−7.45
	(±)-S,R-Warfarin[b]	7	7.4	2.6 ± 0.2	23.0 ± 0.2	5.36	−7.37
	(±)-S,R-Warfarin[b]	7	10	1.7 ± 0.2	16.4 ± 0.7	5.22	−7.16
	(−)-S-Warfarin	6	10	1.7 ± 0.1	24.4 ± 0.3	5.39	−7.39
	(+)-R-Warfarin	30	10	1.9 ± 0.1	20.6 ± 0.2	5.31	−7.28
Human Fraction V	(±)-S,R-Warfarin	7	10	1.5 ± 0.2	42.1 ± 1.8	5.62	−7.71
Canine Fraction V	(±)-S,R-Warfarin	7	10	2.6 ± 0.2	7.4 ± 0.1	4.87	−6.68

[a] Mean ± standard error.

[b] Values derived from ref. 2.

coumarin compound tested, −7.45 kcal/mole. The number of binding sites for the metabolic products of warfarin averaged 1.7. The number of binding sites for both racemic warfarin, (±)-S,R-warfarin, and warfarin alcohol declined as the pH was raised from 7.4 to 10.

There was a slight difference in $\Delta F_1°$ between the two enantiomorphs of warfarin, −7.28 and −7.39 kcal/mole, and the racemic mixture of warfarin, −7.16 kcal/mole. The number of binding sites averaged 1.8 for the enantiomorphs and 1.7 for racemic warfarin at pH 10, an insignificant difference.

The association constant of racemic warfarin was about 6 times greater for human Fraction V than for the canine fraction, and the $\Delta F_1°$ was −7.71 and −6.68 kcal/mole, respectively. The average number of binding sites for the two species of plasma albumin was 2.1. The $\Delta F_1°$ for binding of racemic warfarin to Fraction V of human albumin, −7.71 kcal/mole, was considerably greater than that for binding to crystalline human albumin, −7.16 kcal/mole.

DISCUSSION

The major source of the energy of binding of the coumarin anticoagulant drugs to human plasma albumin is the coumarin part of the molecule itself (−5.8 kcal/mole). The addition to coumarin of the enolic hydroxyl group at position 4, necessary for anticoagulant activity, contributes less than −1.0 kcal/mole of binding energy. The bulky substitutions at position 3, whether an acyl group or another coumarin nucleus, contribute an average of only −0.5 kcal/mole additional binding energy. Yet these substitutions are mandatory for significant anticoagulant activity, as 4-hydroxycoumarin itself has only 5% of the biological activity of bishydroxycoumarin (8).

The most striking difference between the precursor coumarin compounds and the coumarin anticoagulant drugs in binding to plasma albumin is the number of binding sites. The precursor coumarins had an average number of 0.9, while the anticoagulant drugs averaged 1.9 binding sites. This bivalency of plasma albumin for the anticoagulants occurred whether the drug was a

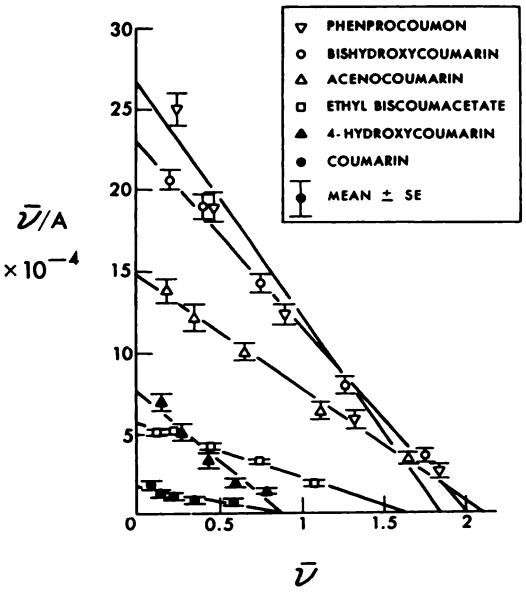


FIG. 1. *Scatchard plot of data for binding of four coumarin anticoagulant drugs and two precursor coumarin compounds to human plasma albumin*

The intercepts on the $\bar{\nu}$-axis equal the number of binding sites, which for the coumarin nucleus and 4-hydroxycoumarin (solid symbols) were nearest the whole integer of 1. The average number of binding sites for the anticoagulant drugs (open symbols), both the monocoumarins (acenocoumarin and phenprocoumon) and the dicoumarins (bishydroxycoumarin and ethyl biscoumacetate), was nearest the whole integer of 2.

monocoumarin (warfarin, acenocoumarin, and phenprocoumon) or a dicoumarin (ethyl biscoumacetate and bishydroxycoumarin). The metabolic products of warfarin also had nearly 2 binding sites for albumin (2). Those metabolites bearing the hydroxyl group on the coumarin nucleus (6-, 7-, or 8-hydroxywarfarin) had an association constant for human plasma albumin 6–23 times less than unchanged warfarin, and are devoid of anticoagulant activity (2). Those bearing the hydroxyl group on the acetonyl side chain (warfarin alcohol) or on the phenyl side chain (4′-hydroxywarfarin) had an association constant similar to unchanged warfarin and about one-third and one-fifteenth the anticoagulant activity of unchanged warfarin, respectively (4, 9).

The most surprising result for the metabolic products of warfarin was the binding strength of 5-hydroxywarfarin, which was greater than that of unchanged warfarin or of any coumarin compound tested. It is possible that the close proximity of the two hydroxyl groups at positions 4 and 5 on the coumarin nucleus allows the formation of a hydrogen-bonded ring (4). This ring formation would yield a compound with a pK_a lower than that of unchanged warfarin; at pH 7.4 or higher it would ionize more readily and bind more avidly to albumin (10). The discrepancy between the binding strength of 5-hydroxywarfarin and its lack of biological activity (4) would be explicable by the loss, through ring formation, of the keto-enol tautomerism of the 4-hydroxycoumarin function that is mandatory for anticoagulant activity (9). The average number of binding sites decreased significantly for both warfarin alcohol and racemic warfarin when the pH was raised from 7.4 to 10.0. This suggests that the binding sites on the albumin molecule are not rigid or preformed, but are influenced by the environment or by competition with the buffer anion (11).

There was little difference in the binding energy for the optical enantiomorphs of warfarin, yet the anticoagulant activity for (−)-S-warfarin form is several times greater than for (+)-R-warfarin in man[1] and the rat (12). This may indicate the relative inadequacy of using plasma albumin as a model for binding to intracellular receptor sites for any drug. This inadequacy of the albumin molecule may result from the two-dimensional nature of its interactions, since the interactions with intracellular receptor sites are three-dimensional and discrimination between optical isomers readily occurs.

The comparative study of the binding of warfarin by canine and human plasma albumin stemmed from the opposite effect of phenylbutazone on anticoagulant activity in the two species (13). Phenylbutazone is a stimulator of drug metabolism by hepatic microsomes that lessens the hypoprothrombinemic effect of the oral anticoagulants in the dog but enhances it in man (14). In man phenylbutazone competes with the

[1] R. A. O'Reilly, unpublished data.

anticoagulant for binding sites to plasma albumin, and thereby increases the delivery of the coumarin compounds to the liver (15). It has been suggested that the species difference could be explained by a greater affinity of human plasma albumin for the anticoagulant drug (6). Thus, the 6-fold lesser binding of warfarin by canine albumin reported here suggests that the phenylbutazone-induced stimulation of warfarin metabolism may be more important in the dog, while the greater binding with human albumin indicates that the phenylbutazone effect on warfarin transport may be dominant in man.

The stronger binding of racemic warfarin at pH 10 by Fraction V of human plasma albumin than by the purer crystalline form was surprising. However, this finding has also been reported for the binding of 2-(4'-hydroxybenzeneazo)benzoic acid, which is frequently used as a standard for the analysis of albumin (16). This discrepancy could result from a decrease in the number of binding sites or an increase in polymer formation during the crystallization process (17).

The strength of binding to albumin and to the intracellular receptor site for anticoagulant activity correlates with the hydrophobic nature of the substituents introduced onto the 4-hydroxycoumarin nucleus by hepatic metabolism or by organic synthesis (1, 2, 18), In the formation of bishydroxycoumarin the introduction of another hydrophobic group, the second 4-hydroxycoumarin function, greatly increases both the anticoagulant activity and the binding to albumin. In ethyl biscoumacetate the addition of the hydrophilic ethyl acetate ester function to the bishydroxycoumarin molecule decreases both the anticoagulant activity and the binding to albumin. In phenprocoumon the introduction of an ethylbenzyl function, a hydrophobic group, to 4-hydroxycoumarin greatly increases the anticoagulant activity and the binding to albumin. In warfarin the introduction of the acetonylbenzyl function, a less hydrophobic group, results in less anticoagulant activity and less binding to albumin compared with phenprocoumon. In acenocoumarin the introduction of an acetonylnitrobenzyl function, a hydrophobic group similar to acetonylbenzyl, results in anticoagulant activity and binding to albumin similar to warfarin.

Similarly, a marked *decrease* in the hydrophobic character of the coumarin anticoagulants, by introduction through metabolism of a hydroxyl group in position 6, 7, or 8 on the coumarin nucleus of warfarin or ethyl biscoumacetate, abolishes the anticoagulant activity and greatly reduced the binding to albumin (2, 19). The hydrophobic contribution to binding by the phenyl side chain of warfarin is somewhat less than that of the coumarin nucleus, as the introduction in that position by metabolism of a hydroxyl group (4'-hydroxywarfarin) reduces the loss of anticoagulant activity and produces a smaller decrease in binding to albumin. The hydrophobic contribution to binding by the acetonyl side chain of warfarin is markedly less than that of the coumarin nucleus, as the reduction by metabolism of the ketone function to form a diastereoisomeric mixture of alcohols leads to the least reduction of both anticoagulant activity and binding to albumin. In 5-hydroxywarfarin the hydrophobic nature of the compound is enhanced through the formation of a ring structure by hydrogen bonding, but the anticoagulant activity is abolished through the loss of the enolic hydroxyl group at position 4.

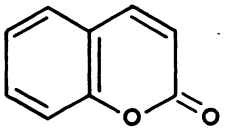
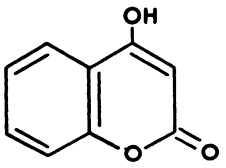
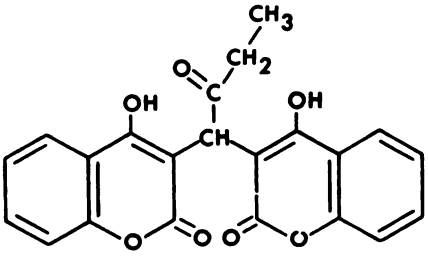
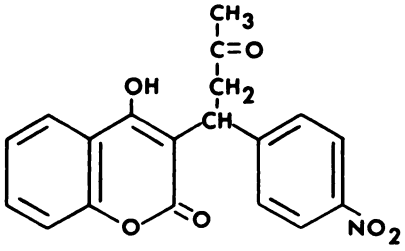
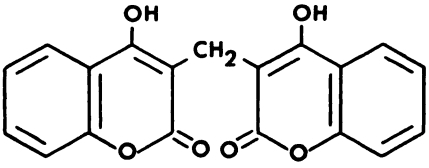
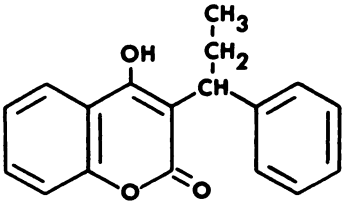
Hydrogen bonding is said to be an important source of binding energy for the interaction of sodium warfarin and human plasma albumin because of the favorable enthalpy change observed (3). Hydrogen bonding is suggested when the sulfur analogue of a compound is bound less strongly to its receptor site than is the oxygen analogue (20). The sulfur analogue of bishydroxycoumarin shows a 10-fold reduction of anticoagulant activity in the rabbit (21). Studies with circular dichroism have demonstrated hydrophobic bonding between the warfarin and human plasma albumin (22). These observations reinforce the previous conclusion, based on thermodynamic considerations, that the binding of coumarin anticoagulants to plasma albumin involves the formation of both hydrogen and hydrophobic bonds (1–3).

REFERENCES

1. R. A. O'Reilly, *J. Clin. Invest.* **46**, 829 (1967).
2. R. A. O'Reilly, *J. Clin. Invest.* **48**, 193 (1969).
3. R. A. O'Reilly, J. I. Ohms and C. H. Motley, *J. Biol. Chem.* **244**, 1303 (1969).
4. R. J. Lewis and W. F. Trager, *J. Clin. Invest.* **49**, 907 (1970).
5. B. D. West, S. Preis, C. H. Schroeder and K. P. Link, *J. Amer. Chem. Soc.* **83**, 2676 (1961).
6. H. S. Marver and R. Schmid, *Gastroenterology* **55**, 282 (1968).
7. R. A. O'Reilly and P. M. Aggeler, *Pharmacol. Rev.* **22**, 35 (1970).
8. S. Roseman, C. F. Huebner, R. Pankratz and K. P. Link, *J. Amer. Chem. Soc.* **76**, 1650 (1954).
9. W. M. Barker, M. A. Hermodson and K. P. Link, *J. Pharmacol. Exp. Ther.* **171**, 307 (1970).
10. K. H. Palmer, M. S. Fowler, M. E. Wall, L. S. Rhodes, W. J. Waddell and B. Baggett, *J. Pharmacol. Exp. Ther.* **170**, 355 (1969).
11. R. Lovrien, *J. Amer. Chem. Soc.* **85**, 3677 (1963).
12. J. N. Eble, B. D. West and K. P. Link, *Biochem. Pharmacol.* **15**, 1003 (1966).
13. R. M. Welch, Y. E. Harrison, A. H. Conney and J. J. Burns, *Clin. Pharmacol. Ther.* **10**, 817 (1969).
14. R. A. O'Reilly and P. M. Aggeler, *Proc. Soc. Exp. Biol. Med.* **128**, 1080 (1968).
15. P. M. Aggeler, R. A. O'Reilly, L. Leong and P. E. Kowitz, *N. Engl. J. Med.* **276**, 496 (1967).
16. J. V. Pastewka and A. T. Ness, *Clin. Chim. Acta* **12**, 523 (1965).
17. D. Watson, *Clin. Chim. Acta* **15**, 121 (1967).
18. W. Scholtan, *Arzneimittel-Forschung* **18**, 505 (1968).
19. J. J. Burns, M. Weiner, G. Simson and B. B. Brodie, *J. Pharmacol. Exp. Ther.* **108**, 33 (1953).
20. H. G. Mautner, *Pharmacol. Rev.* **19**, 107 (1967).
21. C. Mentzer and P. Meunier, *Bull. Soc. Chim. Biol.* **25**, 379 (1943).
22. C. F. Chignell, *Mol. Pharmacol.* **6**, 1 (1970).

Appendix 1

Means of binding data for each concentration of coumarin compound studied

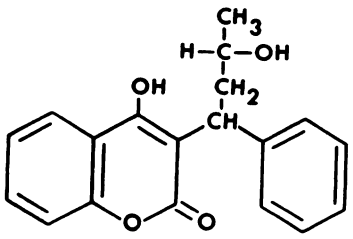
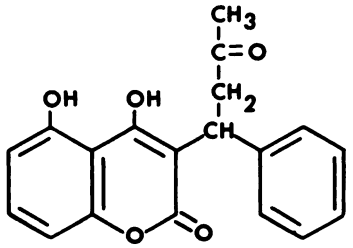
Structural formula	Compound	No. of experiments	Initial coumarin concentration	Final unbound coumarin concentration	Bound
			μM	μM	%
	Coumarin nucleus[a]	13	6.5	4.6	28.9
		9	12.9	9.9	23.4
		7	25.8	21.2	18.0
		6	51.7	43.8	15.3
		4	103.4	90.0	13.0
		39			
	4-Hydroxy-coumarin[b]	16	5.9	2.3	61.8
		10	11.7	5.4	53.6
		18	23.4	13.5	42.8
		16	46.9	33.3	29.1
		12	93.8	75.7	19.3
		72			
	Ethyl biscoum-acetate[b]	16	5.2	2.4	54.5
		16	10.4	4.7	54.6
		12	20.9	10.5	49.7
		8	41.8	24.8	40.7
		8	83.5	58.6	29.8
		60			
	Acenocoumarin[b]	8	5.4	1.3	75.1
		7	10.8	2.8	73.8
		8	21.5	6.5	69.6
		7	43.0	17.3	59.8
		10	86.1	48.1	44.1
		40			
	Bishydroxy-coumarin[b]	22	5.7	1.0	82.7
		15	11.3	2.1	81.3
		9	22.6	5.3	76.7
		9	45.2	16.0	64.6
		7	90.4	50.3	44.8
		62			
	Phenprocoumon[b]	11	6.7	1.0	84.9
		10	13.5	2.5	81.5
		7	27.0	7.2	73.3
		5	53.9	23.4	56.6
		5	107.8	64.4	40.3
		38			

[a] Temperature, 27°; pH 7.4; ionic strength of buffer, 0.170.

[b] Temperature, 27°; pH 10; ionic strength of buffer, 0.181.

Appendix 2

Means of binding data for each concentration of warfarin metabolite studied

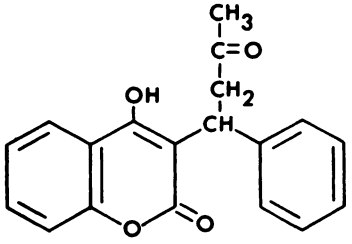
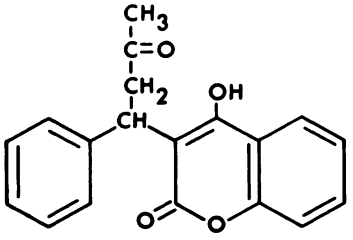
Structural formula	Compound	No. of experiments	Initial coumarin concentration	Final unbound coumarin concentration	Bound
			μM	μM	%
	Warfarin alcohol[a]	10	6.1	1.5	75.5
		10	12.3	3.7	70.0
		10	24.5	10.3	58.0
		6	49.0	26.9	45.1
		6	98.1	69.7	28.9
		42			
	Warfarin alcohol[b]	12	6.1	1.4	76.3
		10	12.3	3.2	74.2
		10	24.5	8.3	66.0
		5	49.0	22.8	53.5
		4	98.1	61.0	37.8
		41			
	5-Hydroxy-warfarin[a]	7	5.9	0.8	86.5
		11	11.7	2.1	82.2
		9	23.4	5.3	77.2
		6	46.9	16.6	64.6
		3	93.8	52.2	44.3
		36			

[a] Temperature 27°; pH 10; ionic strength of buffer, 0.181.
[b] Temperature, 27°; pH 7.4; ionic strength of buffer, 0.170.

Appendix 3

Means of binding data for each concentration of optical enantiomers of warfarin studied

Temperature, 27°; pH 10; ionic strength of buffer, 0.181.

Structural formula	Compound	No. of experiments	Initial coumarin concentration	Final unbound coumarin concentration	Bound
			μM	μM	%
	(−)-S-Warfarin	12	6.2	1.0	83.7
		12	12.3	2.3	81.1
		10	24.7	7.3	70.4
		12	49.4	22.1	55.3
		6	98.7	60.7	38.5
		52			
	(+)-R-Warfarin	6	6.2	1.2	80.1
		9	12.3	2.6	78.5
		7	24.7	6.7	72.7
		5	49.4	20.8	57.9
		4	98.7	59.4	39.8
		31			

APPENDIX 4

Means of binding data to Fraction V of human and canine plasma albumin for each concentration of (±)-S,R-warfarin studied

Temperature, 27°; pH 10; ionic strength of buffer, 0.181.

Plasma albumin	No. of experiments	Initial warfarin concentration	Final unbound coumarin concentration	Bound
		μM	μM	%
Human	16	5.8	0.6	89.4
	15	11.5	1.5	86.7
	9	23.0	5.0	78.3
	5	46.1	16.3	64.6
	45			
Canine	12	5.8	2.2	62.9
	10	11.5	4.6	60.1
	8	23.0	10.0	56.7
	8	46.1	23.4	49.2
	8	92.2	54.2	41.2
	46			