MOLECULAR PHARMACOLOGY, 27:263-270

Interaction of Warfarin with Human Serum Albumin

A Stoichiometric Description

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Received August 27, 1984; Accepted November 6, 1984

SUMMARY

Reversible binding of warfarin to defatted serum albumin was studied by equilibrium dialysis at pH 7.4, in a 66 mM sodium phosphate buffer at 37°. The binding isotherm could be described by two stoichiometric binding constants, K_1 in the range 141,000 to 192,000 M^{-1} and K_2 at 39,000 to 57,000 M^{-1}. At least two additional molecules could be bound but gave indeterminate binding constants. The product $K_3 \times K_4$ was about 4.7×10^7 M^{-2}. Different site models were possible, either one high affinity and several low affinity sites, or two high affinity sites, cooperative, independent, or anticooperative, together with two low affinity sites. Binding affinity for the first warfarin molecule did not vary with pH in the interval from 6 to 9. The affinity decreased with increasing concentrations of sodium sulfate, sodium chloride, and calcium chloride, depending upon ionic strength. Specific effects of chloride and calcium ions were not observed. Light absorption spectra indicated that the warfarin *anion* was bound to albumin. All observations were consistent with a binding process involving albumin and the warfarin anion, without participation of hydrogen ions and not influenced by the N-B conformational transition of albumin.

INTRODUCTION

Binding of coumarin anticoagulants to human serum albumin has been the topic of numerous reports, partly because these drugs are strongly bound and can be displaced by other drugs, and partly because one representative of the group, warfarin, is used as a marker substance for a specific binding site on the albumin molecule (1, 2). Most authors have analyzed their data in terms of a site model with one high affinity site for binding of warfarin, as deduced from binding measurements at different ligand/protein ratios. Klotz (3) has made it clear, however, that conclusions of the presence of a binding site cannot usually be made from a binding isotherm alone unless supported by evidence from sources other than binding equilibrium studies. It further has been found that albumin binding isotherms generally fail to show a level of saturation (4); the presence of one high affinity site or a limited number of sites hardly can be postulated for any ligand binding to human serum albumin if evidence is based exclusively upon the shape of binding isotherms.

Recent work in our laboratory has demonstrated that pairwise interaction of three ligands, warfarin, phenylbutazone, and bilirubin, cannot be explained by the presence of one simple warfarin site, since warfarin binding is competitive with phenylbutazone as well as with bilirubin, while phenylbutazone and bilirubin can be bound independently (5). A full description of the binding of a drug to serum albumin and its interaction with other ligands, in qualitative and quantitative respects, cannot be based at present upon a site model. A stoichiometric binding concept, ignoring location of ligand molecules within the complexes with albumin, can be applied successfully to primary and multiple binding equilibria of single drugs as well as to co-binding of two ligands (5). This paper presents a stoichiometric description of warfarin-albumin binding equilibria, including variation with pH and salt concentrations. Light absorption spectra for warfarin-albumin are analyzed. It is discussed whether an albumin model with one high affinity site for binding of warfarin can be established on the basis of these observations.

MATERIALS AND METHODS

Human serum albumin was obtained from AB Kabi, Stockholm, Sweden (lot 75953) and was defatted with charcoal in acid solution (6), lyophilized, and stored at 4°. Fatty acid content in several batches of human serum albumin, defatted by the present procedure, was below 0.1 mol/mol albumin. The defatted protein was characterized with respect to binding of warfarin, as follows. The binding isotherm at 37° was obtained by equilibrium dialysis (Fig. 1). Albumin kept over silica gel for 2 years showed an increased affinity for warfarin, giving lower concentrations of free warfarin by a factor of 0.7. Fresh preparations were used for all measurements reported in Results. Comparison with results published by other investigators indicates that our albumin has a similar affinity for warfarin as preparations used by some (7–12), while others (13–15) have reported lower equilibrium concentrations of

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free warfarin, about 2- to 4-fold, when compared at a low ratio of warfarin to albumin and at pH 7.4, ionic strength 0.15 M and 37°.

Further binding isotherms were recorded at 25.2°, 30.6°, 35.0°, and 40.0°, and a linear van't Hoff plot was drawn, giving the following thermodynamic parameters at low warfarin/albumin-binding ratios: $\Delta G^\circ = -7.4$ kcal/mol at 37°; $\Delta H^\circ = -5.3$ kcal/mol; and $\Delta S^\circ = 7$ eu/mol. It finally was found, as also shown by Wilding *et al.* (12), that binding of warfarin at low ligand/protein ratios is enhanced by oleate, up to about 4 mol oleate per albumin, where free warfarin equilibrium concentrations were measured to about half of those present in the absence of fatty acid. High oleate concentrations decreased warfarin binding.

Racemic warfarin sodium salt was a gift from Nyco MED, Rødovre, Denmark. The enantiomers *R*– and *S*-warfarin were obtained as a gift from Endo Laboratories, Garden City, NY. Specific optical rotations at 589 nm and 25° were +128° and −134°, respectively, for the two substances dissolved in 0.5 M NaOH. West *et al.* (16) report ±149°. This is in agreement with the finding of Sellers and Koch-Weser (7) who, using materials from the same source, found that separation of the enantiomers is incomplete. Our binding isotherms for the enantiomers at warfarin/albumin ratios from 0.8 to 2 could not be distinguished. This is consistent with the report of Sellers and Koch-Weser (7) who found that the affinity for *S*-warfarin is nearly the same, although slightly higher than that for *R*-warfarin at ligand/protein ratios less than 0.6. Our investigations consequently were carried out with racemic warfarin.

[^{14}C]Warfarin (3α-acetonyl[α-^{14}C]benzyl-4-hydroxycoumarin) was obtained from The Radiochemical Centre (Amersham, England) and was purified by TLC in toluene:dioxane (9:1) on silica gel plates and checked as follows. In a microdialysis chamber (17), 25 μl of a buffered solution containing 1 mM human serum albumin and 0.4 μCi of [^{14}C] warfarin (283 μM) was dialyzed against 25 μl of buffer at 37°. The buffer was changed 20 times at 20-min intervals, and radioactivities were measured. The logarithm of the radioactivity was plotted as a function of time. A straight line is expected if the labeled warfarin is radiochemically pure. An observed curvature indicated impurity. It was found by computer simulation that the commercial preparation before chromatography contained 0.8–1.0% of a loosely bound or unbound impurity. After chromatography, the impurity accounted for 0.1–0.2% of the total radioactivity.

Oleic acid (ultrapure) was obtained from Fluka AG, Buchs, Switzerland.

Binding of warfarin was studied by equilibrium dialysis using cellophane membranes cut from dialysis tubing (Union Carbide Corp., type 32/36). Dialysis chambers had a volume of 1.2 ml and contained 1 ml of solution. The chambers were rotated in an air thermostat at 37°. The time taken to obtain equilibrium was 4–5 hr; the time allowed was 6 hr. Binding of warfarin to membranes and chamber walls could not be detected. Other sources of error, such as leakage of albumin through the membrane, albumin decay, and osmosis of water, were negligible. Warfarin was measured in the protein-free solution by spectrophotometry at 307 nm. In some experiments, studying variation of binding with pH and temperature, warfarin concentration was measured by high pressure liquid chromatography with a liquid chromatograph (Waters Associates, model 6000 A) equipped with a U6K injector and a model 440 absorbance detector with a 313-nm filter. The column [25 cm × 4.6 mm stainless steel (Knauer)] was slurry packed with 5-μm Spherisorb ODS particles. The mobile phase for the elution of warfarin was 50% acetonitrile in 0.25 M acetate buffer (pH 4.75). The flow rate was 2.0 ml/min (2000 p.s.i.; retention volume, 5.0 ml). Minimum detectable concentration for warfarin was 0.2 μM after injection of 25 μl of sample. Good agreement was obtained with the methods.

Binding of warfarin to human serum albumin as a function of salt concentrations and of added oleate was studied by measuring the rate of dialysis of [^{14}C]warfarin from the equilibrium mixture, containing 10 μM warfarin and 600 μM albumin, through a cellophane membrane into another compartment containing an identical solution without warfarin, as previously described (17). Radioactivity on both sides was measured after 20-min dialysis time in a Packard Tricarb scintillation spectrometer.

RESULTS

Binding isotherm for racemic warfarin. Bound and free equilibrium concentrations of racemic warfarin are plotted in Fig. 1, showing bound warfarin relative to the concentration of albumin as a function of free warfarin concentration on a logarithmic scale, and in a Scatchard graph, bound/free versus bound (Fig. 2). The former plot indicates that binding increases continuously with rising concentration of free warfarin; a level of saturation is apparently not approached at the highest warfarin concentrations applied where 3 molecules of the ligand are bound per molecule of albumin. The binding isotherm (Fig. 1) fails to indicate a step in the curve which could point towards a model with one or two high affinity sites. Such a step indeed would not be expected unless the affinity of binding to one or two sites is very markedly higher than that of the secondary binding. At a first glance, the Scatchard plot (Fig. 2) suggests high affinity binding of two warfarin molecules and low affinity binding of two additional molecules. Such interpretations of the Scatchard plot often have been seen in the literature but are clearly erroneous (18, 19). A more detailed analysis, as follows, is needed in order to test various binding models.

Measurements presented in Fig. 1 were carried out with a constant concentration of albumin, 30 μM. A few experiments with 100 μM albumin gave points located on the same curve, as expected if albumin-to-albumin interaction is absent. Binding equilibria could thus be analyzed in terms of stepwise binding according to the

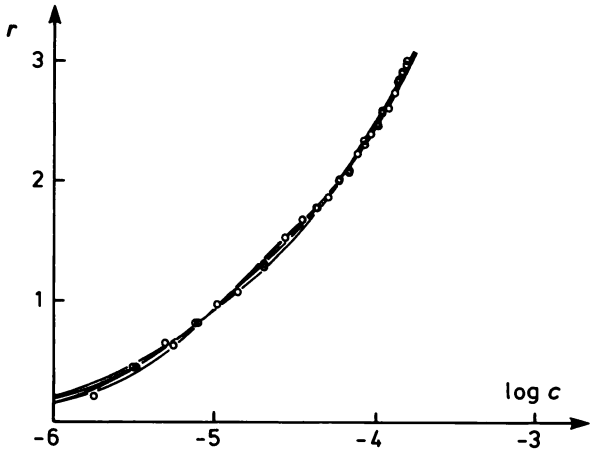

FIG. 1. *Binding equilibria for racemic warfarin to defatted human serum albumin, pH 7.4, 66 mM sodium phosphate, 37°*

Observed points are plotted as *r*, moles of warfarin bound per mole of albumin versus log free warfarin concentration. The four curves, from bottom to top as seen at the extreme left of the graph, represent solution 3 (Table 1) with minimal K_1, solution 4 with two equal and independent high affinity sites and two weaker sites, and solution 2 with maximal K_1. These three solutions all represent acceptable fits to the data, $p > 0.25$. A somewhat inferior fit is given by the last curve, uppermost at the left extreme, solution 6 in Table 1 corresponding to one high affinity and four weaker sites.

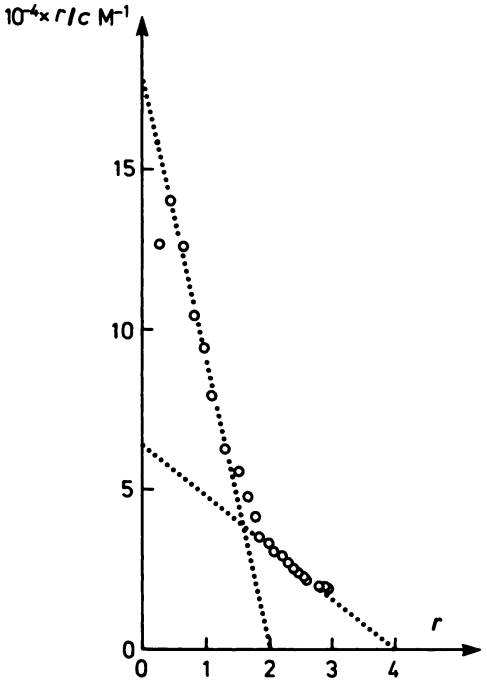

FIG. 2. *Scatchard graph for binding of warfarin, same data as in Fig. 1*

Each point represents the average from two experiments. A common, although incorrect, interpretation of this graph seems to indicate a unique solution with two high affinity and two secondary sites.

stoichiometric binding Eq. 1 (20).

$$r = \frac{K_1c + 2K_1K_2c^2 + \cdots + nK_1K_2 \cdots K_nc^n}{1 + K_1c + K_1K_2c^2 + \cdots + K_1K_2 \cdots K_nc^n} \quad (1)$$

where r is bound ligand (mol/mol albumin), c is the free ligand concentration, and K_1, K_2 $\cdots$ K_n are stoichiometric binding constants. Computer fitting of the K values gave a best fit with binding of maximally four warfarin molecules and the stoichiometric binding constants shown in Table 1. The corresponding curve is drawn in Fig. 1. Weighted deviations of the observed points from the curves were evaluated by calculating the residual standard error.

$$s = \sqrt{\frac{\sum_{i=1}^{N} \frac{(r_{i(\text{obs})} - r_{i(\text{calc})})^2}{r_{i(\text{obs})}^{\beta}}}{N - 1}} \quad (2)$$

It was found that an almost even distribution of weighted residuals was produced if the weighting exponent β was given a zero value, i.e., when all observations were weighted equally.

The best fit is not the only acceptable solution. Sixty solutions were computer generated, all having residual standard errors within narrow probability limits, 0.25 by the F test (21). All of these 60 solutions thus represented acceptable fits to the observed data. Two of these are given in Table 1, solution 2 having the highest value found for K_1, and solution 3 with the lowest K_1. Neighbor K values are negatively correlated so that a high K_1 produces a low K_2, a high K_3, etc. The binding isotherms produced when these sets of constants are entered in Eq. 1 are nearly indistinguishable from the best fit curve, as shown in Fig. 1. It is noted from Table 1 that the variation of K_1 and K_2 is considerable and that K_3 and K_4 are practically indeterminate. The product, $K_3 \times K_4$, is rather well defined, about 4.7×10^7 M^{-2}.

The ratio of K_1/K_2 in the best fit solution is 3.5. If in a first approximation we disregard the weak binding of the third and fourth warfarin molecules, it is seen that this solution is close to a site model with two equal and independent primary sites, which would give $K_1/K_2 = 4$, besides two secondary sites with lower affinity. Solution 4 with two equal and independent high affinity sites is included in Table 1. It gives a slightly inferior fit, as seen from the higher value of s, but is still acceptable with good probability, when judged by the F test. In this solution, secondary binding of two warfarin molecules is strongly cooperative, as seen from the low ratio $K_3/K_4 = 0.1$.

It has further been examined whether cooperativity of the first two molecules could be consistent with the observations. If cooperativity is absolute in a stoichiometric binding description, it would mean that a complex containing one warfarin and one albumin molecule would not exist; binding of one ligand molecule would be followed immediately by binding of the second. Cooperative solutions in this sense are not compatible with the present data for binding of warfarin. However, solutions with moderate cooperativity among the first two warfarin molecules are acceptable. In this case, binding of one warfarin molecule increases the affinity for binding of the second to a limited extent; complexes with only one bound warfarin can still exist. The mechanism probably involves an allosteric change of one site, induced through a conformational change by binding of warfarin to another site. This is exemplified by solution 5 in Table 1. This is the same as solution 3, since maximal cooperativity among the first and second warfarin molecules is found in the solution which has a minimal K_1 and a maximal K_2.

Solutions with one high affinity site and considerably weaker binding of several warfarin molecules to equal and independent sites do not fit the experimental points so well. In order to find the best possible solution of this type, we fitted the points by Scatchard's equation, formulated for one high-affinity site and several weaker, independent, and equal sites,

$$r = \frac{ck_1}{1 + ck_1} + \frac{n_2ck_2}{1 + ck_2} \quad (3)$$

where k_1 and k_2 are the site-binding constants for the high affinity site and the weak sites, respectively, and n_2 is the number of weak sites. We stipulate that one high affinity site is present if at least 90% of the first, small amount of warfarin is bound to one site and no more than 10% is bound to weaker sites. This criterion is fulfilled if $k_1 = 9 \times n_2k_2$ which was entered in Eq. 3. The best solution we could find was for $n_2 = 4$, i.e., for a total of five sites and with the site-binding constants shown in Table 1, solution 6. Stoichiometric binding constants for this solution were calculated from the site-binding constants as described by Fletcher *et al.* (22) and given in the table. The fit to the experimental points is poor,

TABLE 1

Analysis of warfarin-human serum albumin-binding data (Fig. 1)

Solution	Stoichiometric binding constants					s^a	$F = s^2/s_0^2$	Probability limit[b]
	K_1	K_2	K_3	K_4	K_5			
			M^{-1}					
1. Best fit	167,000	48,300	890	55,000		0.0291 = s_0	1	
2. Highest K_1	192,000	39,000	2,600	18,000		0.0323	1.23	>0.25
3. Lowest K_1	141,000	56,900	2.43	19,000,000		0.0322	1.22	>0.25
Site models								
4. Two equal and independent high affinity sites	175,000	43,900	2,160	21,200		0.0320	1.21	>0.25
5. Two equal and moderately cooperative high affinity sites	141,000	56,900	2.43	19,000,000		0.0322	1.22	>0.25
6. One high affinity site	247,000	23,200	9,060	4,073	1,530	0.0469	2.60	<0.01

	Site-binding constants					
	k_1	k_2	k_3	k_4	k_5	s^a
			M^{-1}			
6.[c]	222,000	6,180	6,180	6,180	6,180	0.0469
7.	206,000	21,000	5,000	5,000	5,000	0.0452
8.	170,000	10,000	10,000	10,000	0	0.0651

[a] Residual variance, from Eq. 2; $\beta = 0$, i.e., all deviations are weighted equally.
[b] Probability limit for $N = 45$.
[c] Solutions 6–8 are one high affinity site as in solution 6 above.

as shown by an unacceptably high value of F ($p < 0.01$). It is recognized, however, that a systematic error in the determination of free and bound warfarin concentrations in the equilibrium dialysis experiments could occur at low ligand concentrations and would then render this solution acceptable. One of the curves in Fig. 1 shows the solution with one high affinity site and four weak sites. Considering the possibility of a small systematic error, this curve, in our judgement, could not be rejected.

Allowing for three binding classes, using a three-term Scatchard equation analogous to the two-term Eq. 3 does not produce essentially better fits. The best solution found is shown in Table 1, solution 7, and has $n_1 = 1$, $n_2 = 1$, and $n_3 = 3$. Attempts to fit a model with only four, independent sites to the present data were not successful; the best fit is given in solution 8 which is unacceptable with a high value of s.

Fig. 2 shows a Scatchard plot of the same binding data as given in Fig. 1. Conventional, albeit erroneous (19), extrapolations to intercept with both axes appear to indicate the presence of two equal and independent high affinity sites and two weaker sites and this solution seems here to present itself as the only possible result. A more realistic Scatchard graph is drawn in Fig. 3, where the 60 computer-generated, stoichiometric solutions with a maximal four bound warfarin molecules are shown as 60 partly confluent curves. Solutions 2 and 3 in Table 1, giving maximal and minimal K_1, are represented by the upper and lower of the curves at the intercept with the ordinate axis. All other solutions give intermediate curves.

Klotz' affinity profiles (25) shown in Fig. 4, picturing stoichiometric binding constants K_i as iK_i vs. i, illustrate the binding mechanisms involved in some of the solutions. A possibility with cooperative binding of two warfarin molecules, solution 3 in Table 1, another solution

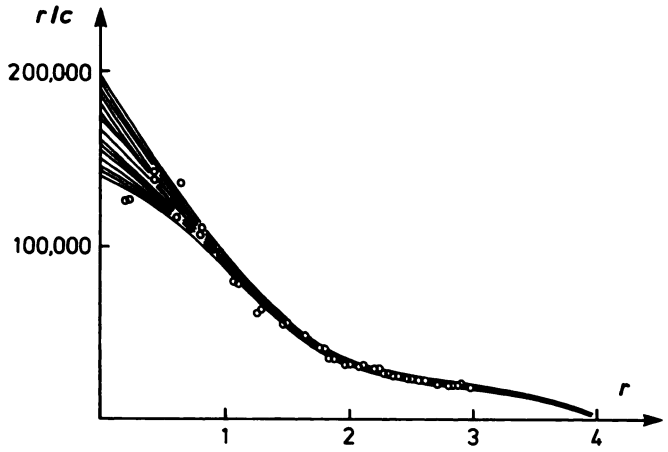

FIG. 3. *Scatchard graph of the same binding data*
Curves are 60 computer-generated stoichiometric solutions for binding of 4 mol of warfarin, all acceptable within the probability limit, $p = 0.25$ by the F test (21).

with homogeneous binding of two molecules, and solution 2 where binding of the first two molecules of warfarin is anticooperative, is drawn in bold lines. The dashed lines indicate solution 6 with high affinity binding of one molecule, giving a more pronounced anticooperativity. The fourth binding constant, K_4, is not included in the graph, due to the very high variation (see also Ref. 26 for further explanation of the affinity profiles).

In summary, human serum albumin binds at least four molecules of warfarin. Stoichiometric binding constants for the first two molecules can be determined although a considerable variation is found. Possible site models include two equal and independent high affinity sites with weaker binding to two additional sites. The two high affinity sites may also be weakly cooperative. So-

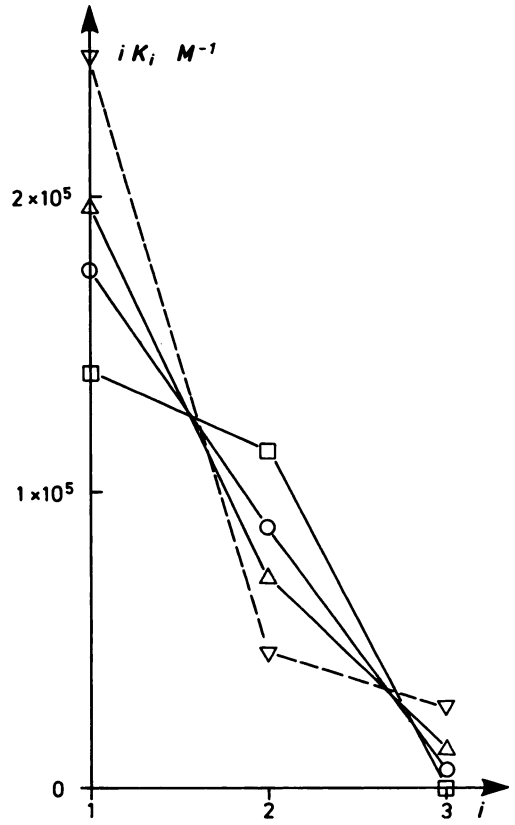

FIG. 4. *Klotz' affinity profiles for binding of warfarin*

The graph shows stoichiometric binding constants, pictured as iK_i vs. i, where i is the number of warfarin molecules successively bound. The four solutions illustrated are the same as shown in Fig. 1: □, cooperative; ○, independent; and △, anticooperative binding of the first two molecules of warfarin. Other solutions among the 60 computer-generated variations are intermediate between these three. A fourth possibility, high affinity binding of one warfarin molecule with weaker binding of a few additional molecules, is shown by the dashed lines.

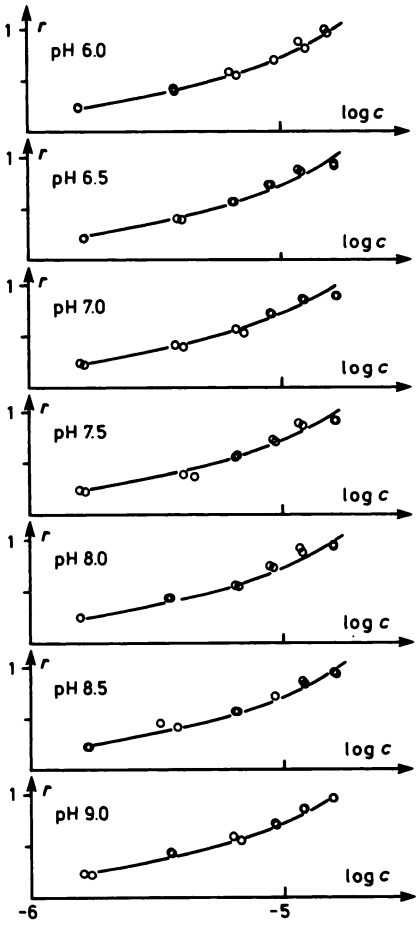

FIG. 5. *Binding isotherms for warfarin to defatted human serum albumin at 37°*

Sodium chloride, 150 mM, was present in all solutions and pH was adjusted with sodium hydroxide without the use of buffers. The seven curves are identical and it is seen that binding of warfarin is independent of pH.

lutions with one high affinity and four weak sites are possible as well.

Warfarin binding at varying pH. Binding isotherms obtained by equilibrium dialysis at seven pH values from 6 to 9 are shown in Fig. 5. The drawn curves are identical and it is seen that variation of binding with pH could not be demonstrated.

These measurements were limited to binding ratios of warfarin/albumin less than 1. Albumin was dissolved in 150 mM sodium chloride without the use of buffers, in order to minimize an effect of varying salt concentrations, and pH was adjusted with sodium hydroxide.

In the pH range 6 to 9, warfarin is present in aqueous solution predominantly as a mono-anion; pK_a is 4.9. It is concluded from the present observations that the stoichiometric 1:1 complex of warfarin:albumin is formed by binding of this anion without involvement of hydrogen ions. An influence of the varying negative charge of the albumin molecule could not be detected at this salt concentration. The N-B conformational transition of albumin (23), which occurs within the present range of pH, did not influence warfarin binding measurably in these experiments.

Warfarin binding at varying salt concentrations. The rate of dialysis of unbound warfarin as a measure of free warfarin concentration was determined in solutions containing 10 μM warfarin and 600 μM albumin, buffered with 5 mM sodium phosphate to pH 7.4 and with varying additions of other salts. The results are plotted in Fig. 6. Sodium sulfate, sodium chloride, and calcium chloride cause an increase of dialysis rate, equal for the three salts when the ionic strenth is used as the independent variable.

Addition of neutral salt to the buffered warfarin-albumin mixture has a dual effect: the pH is changed due to a shift of the primary to secondary phosphate equilibrium, and the warfarin-albumin binding equilibrium is shifted. Since the binding affinity of warfarin to albumin is independent of pH, as shown above, the changes observed can be taken as a result of variation of ionic strength, affecting the binding of warfarin anion to albumin. Specific effects of binding of chloride and calcium ions to albumin were not observed.

Light absorption spectrum of warfarin-albumin. Fig. 7 shows light absorption spectra of warfarin acid (*c*), warfarin anion (*b*), and bound warfarin (*a*). The spectrum of bound warfarin was obtained by placing a buffered so-

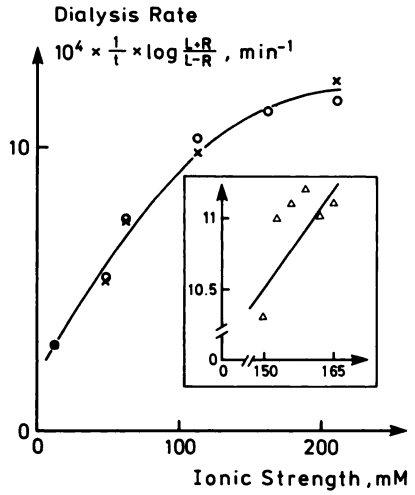

FIG. 6. *Warfarin binding with varying salt concentration*
Free warfarin equilibrium concentration measured by rate of dialysis in solutions containing 300 μM human serum albumin and 10 μM warfarin in dilute sodium phosphate buffer, 5 mM, pH 7.4, 37°, with varying concentrations of added sodium chloride, O; sodium sulfate, ×; calcium chloride, Δ. *L* and *R* are left and right chamber radioactivities measured after the dialysis time *t*.

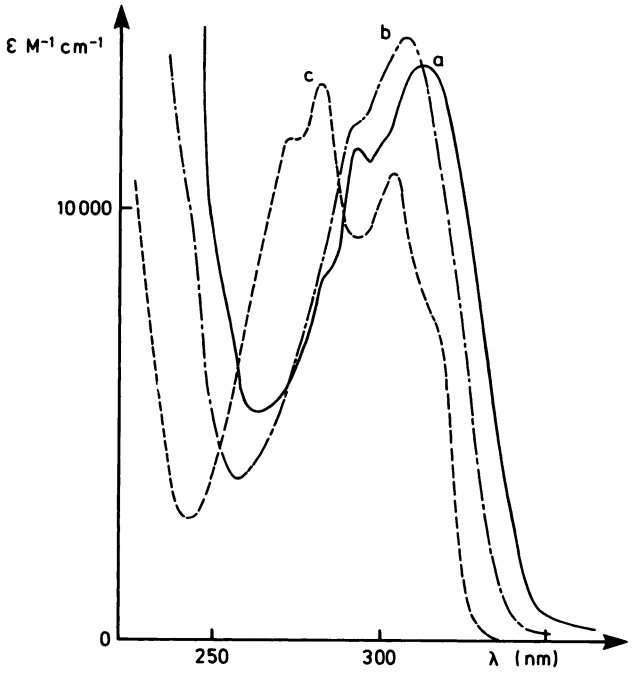

FIG. 7. *Light absorption spectra of warfarin*
a shows a spectrum of albumin-bound warfarin; this was obtained from an albumin-warfarin equilibrium mixture, 300 μM albumin and 100 μM warfarin, pH 7.4, 37°, with an equal concentration of albumin in the reference and further with a reference cell containing warfarin in the same concentration as free warfarin in the mixture. *b* is the spectrum of warfarin anion, pH 7.4; and *c* shows the spectrum of warfarin acid, pH 3.0.

lution of warfarin-albumin in the sample beam and separate cells containing albumin and warfarin in the reference; the concentration of albumin in one reference cell was equal to the albumin concentration in the sample, and the warfarin concentration in the second reference cell was equal to the free equilibrium concentration of warfarin in the sample, as found from the binding isotherm. A cuvette with buffer alone was also placed in the sample beam to compensate for reflection losses. Albumin and warfarin concentrations in the mixture were 300 and 100 μM. This gives 2 μM free ligand. Bound warfarin was present, partly as the 1:1 complex with albumin (about 80%) and partly as the 2:1 complex (about 20%) with less than 0.1% of 3:1 and higher complexes, as calculated from the binding constants in Table 1, Solution 1. The spectrum in Fig. 7 thus shows the light absorption of a mixture of 1:1 and 2:1 warfarin-albumin complexes, predominantly the former. This spectrum is similar to the spectrum of warfarin anion, *b*, red-shifted by 5 nm, and is essentially different from that of warfarin acid, *c*. A small shoulder in the bound warfarin spectrum at 285 nm can be explained by perturbation of the tyrosine absorption in albumin.

It is concluded that the first molecule of warfarin is present in the complex with albumin as a warfarin anion. The red-shift is expected when a π-π^* chromophore is transferred from water to less polar surroundings on binding to the albumin molecule and could possibly also be explained by exciton splitting on fixation of the coumaran and benzene ring planes in a certain dihedral angle (24).

The presence of bound warfarin is an anion within the complex with albumin and the constancy of binding affinity with varying pH shows that the binding of the first warfarin molecule can be written as a simple process: W^- + albumin $\rightleftharpoons$ W^--albumin without participation of hydrogen ions.

DISCUSSION

This paper illustrates that it is possible to describe multiple binding equilibria of warfarin to human serum albumin in stoichiometric terms, using Klotz's stepwise binding Eq. 1. The equilibrium mixture under investigation is considered to contain the stoichiometric complexes of warfarin and albumin, 1:1, 2:1, 3:1, and 4:1, besides free warfarin and unloaded albumin. Small amounts of higher complexes, containing more than four warfarin molecules, may be present as well, but could be ignored since the studies were limited to molar ratios of 3 warfarin or less per mol of albumin. Each stoichiometric complex consists of a mixture of several species with warfarin attached to different sites. If four sites are present, the 1:1 complex consists of a mixture of four different species, each with warfarin attached to one site. The 2:1 complex contains six species, and the 3:1 and 4:1 complexes consist of four and one species. More than four sites may be present and bound warfarin molecules could migrate about, either dissolved in the interior of the protein molecule or adsorbed to its surface, so that each stoichiometric complex could consist of a complicated mixture of various species of unknown structures and in unknown molar proportions; a stoichiometric equilibrium description can still be established and is valid irrespective of our ignorance of site-binding mechanisms and site affinities.

A set of four stoichiometric binding constants is sufficient to describe the warfarin-human serum albumin-

binding isotherm, covering a range of ligand/protein ratios up to 3. Such a set of binding constants, supplemented with information of the concentration range and experimental variance (β and s, Eq. 2), contains all the information available from the equilibrium measurements and can accordingly be taken as a complete account of the experimental observations. On the other hand, a variety of different sets of binding constants may describe the same observed data. This is due to the experimental variation of measured free and bound concentrations and is illustrated in Table 1. The first stoichiometric binding constant, K_1, may assume numerical values from 141,000 to 192,000 M^{-1}, if we allow for the stochastic variation of the observed points, and may be as high as 247,000 if a small, systematic error of the lowest concentrations of free warfarin is included. The variability of K_2 is slightly larger, and K_3 and K_4 are nearly indeterminate, varying by several orders of magnitude. Even so, any set of four binding constants, introduced in binding Eq. 1, gives a good definition of the binding isotherm. This is due to co-variation of the constants; neighbor constants are negatively correlated so that a high value of K_1 goes with a low K_2, a high K_3, and a low K_4. One binding constant, detached from the other members of the set, has a limited significance and is meaningless if it is a K_3 or K_4 value. A full set of stoichiometric binding constants defines the isotherm.

The variation of K values is larger when a stoichiometric binding description is used than it is with the conventional Scatchard binding model having a number of independent sites. Solutions with cooperative binding are disregarded when Scatchard's equation is used. This is illustrated by solution 5, Table 1, which has an extremely low K_1 and a high K_2. Binding of the first two molecules of warfarin is thus cooperative as seen in the affinity profile (Fig. 4) (25). Sets of binding constants with cooperativity tend to contain extreme K values, and disregard of such solutions will lessen the variation of binding constants. Exclusion of cooperativity as a possibility is not justified, however. Binding of 1 mol of warfarin is cooperative with a few mol of oleate (12), indicating that mechanisms for cooperativity are at hand. It is therefore not possible to exclude cooperativity of one molecule of warfarin with another.

The Scatchard graph in Fig. 2 appears to indicate one definitive solution with two equal high affinity sites and two secondary sites. The extrapolation procedure shown in the figure is incorrect (19) and tends to give a false impression of approaching saturation (26), in this case at 4 molecules of warfarin bound per albumin. Fletcher *et al.* (27) limit their use of Scatchard graphs to the determination of primary estimates of binding constants, suitable for further refinement by a computer procedure. In the present analysis of warfarin binding, the observer who would use a Scatchard graph alone would get a false impression of having reached a definitive solution and remain unaware of the variety of possible binding mechanisms and numerical values of binding constants.

Variation of binding with pH in the range from 6 to 9 could not be demonstrated in our experiments (Fig. 5), and we found no specific influence of chloride and calcium ions on the binding of warfarin. Binding decreased with increasing ionic strength. These observations are definitely different from those reported by Wilting and co-workers (14, 28–31) who have studied the effect of pH and calcium and chloride ions in considerable detail and found that binding of warfarin is increased with increasing pH and is influenced by calcium and chloride ions in a manner which indicates that the N-B transition of albumin is important; the B conformation has a higher affinity for warfarin. Their findings have been confirmed by another group (10).. O'Reilly (32) found that variation of ionic strength within a wide range, 0.017–0.34 M, caused no significant change in warfarin binding, but a rise of binding strength was observed as pH was raised from 6 to 9. It does not seem possible to explain these discrepancies by differences of technique or by use of different buffers. A possible explanation is a difference of albumin binding properties. Our albumin preparation does undergo the N-B transition, as demonstrated in the studies of Jacobsen *et al.* (33, 34), who found that changes in the light absorption spectrum of bilirubin-albumin could be related to this conformational change. It seems necessary to conclude that a pH- and calcium-sensitive conformational change of the albumin molecule in the pH range 6 to 9 can take place in one commercial preparation of human serum albumin, after defatting, without a change of warfarin-binding affinity.

The question whether one or two high affinity sites for warfarin are present in human serum albumin should be regarded as an open one until it can be settled by studies other than binding equilibrium measurements.

ACKNOWLEDGMENTS

The authors wish to thank Signe Andersen, Nina Jørgensen, Elin Pedersen, and Karen Busch for skillful technical work, and Frede Nielsen for the drawings. An HP 85 desk computer was obtained from the Danish Medical Research Foundation through Grant 12-2365.

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