Effect of Temperature on Binding of Warfarin by Human Serum Albumin

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Abstract □ The *in vitro* binding of warfarin by human serum albumin was studied at various temperatures and at pH 7.4 by a frontal gel filtration technique. The results can be best described in terms of a two class-of-binding site model, in which the numbers of primary and secondary sites are constrained to the average values for all experiments (n_1 = 1.38 and n_2 = 3.73). Analysis of the temperature dependence of the binding yielded the following thermodynamic parameters: $\Delta H_1 = -2.55$ kcal/mole, $\Delta S_1 = 16.1$ eu, and $\Delta F_1 = -7.34$ kcal/mole for the primary binding and $\Delta H_2 = -5.08$ kcal/mole, $\Delta S_2 = -1.10$ eu, and $\Delta F_2 = 4.72$ kcal/mole for the secondary binding. Calculations based on these results showed that, for the therapeutic concentration range, warfarin was over 99% bound to albumin present in physiological concentration. These findings are compared and contrasted to binding data in the literature for warfarin and salicylate.

Keyphrases □ Warfarin—binding to human serum albumin, effect of temperature □ Albumin, human serum—binding of warfarin, effect of temperature □ Binding, drug–protein—warfarin to human serum albumin, effect of temperature □ Anticoagulants—warfarin, binding to human serum albumin, effect of temperature

Drug binding studies carried out *in vivo* provide practical information about the fraction bound and free drug concentrations. The unbound drug in circulation affects the degree of pharmacological response *via* tissue distribution and clearance, while the bound fraction influences drug absorption and transport. Unfortunately, the interpretation of *in vivo* studies is complicated by many factors, such as the existence of specific transport proteins, binding by red cells, and competition for binding sites by endogenous and exogenous substances.

An example of displacement of drug bound to albumin by other drugs is the potentiation of the anticoagulant effect of warfarin by phenylbutazone (1); chloral hydrate (2); mefenamic, ethacrynic, and nalidixic acids (3); and salicylate (4). However, the exact mechanism of displacement of warfarin by these drugs has not been settled. To clarify the nature of binding and the type of inhibition involved, a detailed study of the interaction of warfarin and salicylate with human serum albumin was begun.

As the first step, new binding methodology was developed, combining the sensitivity and precision of radioisotope dilution with the rapidity and reproducibility of the frontal gel filtration technique. Subsequently, this methodology was employed in the study of salicylate binding to human serum albumin at various temperatures (5). Analyses of the data were carried out by computer programs corresponding to the various binding models considered.

As a further development, this paper describes a study of the binding of warfarin to human serum albumin. It compares the results with similar data already available. The mathematical basis for analysis and comparison of binding data presented in various forms was published recently (6). The detailed description of salicylate and warfarin binding to human serum albumin in terms of the two class-of-site model paves the way for the quantitative analysis of the mutual displacement of these two drugs from albumin. Some aspects of these interactions were discussed previously (7).

EXPERIMENTAL

Materials and Methods—Gel filtration frontal analysis chromatography, which was successfully employed in previous drug binding studies (5–8), was used for the determination of bound warfarin. Various concentrations of drug were prepared in 0.067 *M* phosphate buffer, pH 7.4, containing 0.3 g/100 ml of crystalline human serum albumin[1]. Labeled ^{14}C-warfarin[2], of 99% radiochemical purity and a specific activity of 23 mCi/mmole, was added to the drug solutions at a constant level to provide about 20,000 cpm/ml. Initial studies were conducted with ^{14}C-warfarin[3], 0.2 mCi/mmole, which was found to be identical to the higher activity labeled material.

The gel filtration technique used was that described by Cooper and Wood (9). A cross-linked dextran gel[4] was allowed to swell in 0.067 *M* phosphate buffer, pH 7.4, at the temperature prescribed (6–37°). Precision bore columns[5] were modified for more rapid flow by replacing the fritted polyethylene disk with nylon mesh[6]. The gel was packed into the column to a depth of 25 cm, providing a bed volume of about 20 ml. The columns and system were equilibrated for 1 hr prior to making the runs. The system was maintained at the desired temperature with a constant-temperature regulator[7].

The samples were maintained for several hours at the prescribed temperature in the water bath that comprised part of the thermoregulator system. Because of a slight retention of the warfarin by the gel, the sample volume had to be increased to 60 ml from the usual 45 ml reported previously for salicylate (8). The absorption was inconsequential in the frontal analysis system, as reported previously (9). Preliminary studies indicated that satisfactory plateaus in the total and free drug regions were obtainable for this volume of sample. Three 1-ml aliquots were taken in the β-plateau region and four in the γ-region. The results were averaged from the duplicate samples.

Warfarin Determinations—The test solutions, containing warfarin in varying concentrations, were prepared accurately by weight from standardized stock solutions, which were verified for exact concentration by UV absorption spectrophotometry. One-milliliter aliquots of the fractions obtained on a fraction collector[8] from the gel column were added to 10 ml of scintillation solution[9] in a conventional low K-I glass counting vial[10].

The carbon-14 activity was measured in a three-channel beta liquid scintillation spectrometer[11]. Each sample was counted to less than

[1] Miles Laboratories, Kankakee, Ill.
[2] CFA. 499, Amersham/Searle, Arlington Heights, Ill.
[3] Wisconsin Alumni Research Foundation Institute, Madison, Wis.
[4] Sephadex G-25, coarse grade, Pharmacia Fine Chemicals, Piscataway, N.J.
[5] Adjusto-Chrom 5919, 1 × 60-cm set for 25-cm column, Ace Glass Inc., Vineland, N.J.
[6] Kontes Glass Co., Vineland, N.J.
[7] Haake Series FE constant-temperature circulator with KR refrigerated chiller, Polyscience Corp., Evanston, Ill.
[8] Brinkmann-Serva Linear II, Brinkmann Instruments, Westbury, N.Y.
[9] Insta-Gel, Packard Instrument Co., Downers Grove, Ill.
[10] Packard Instrument Co., Downers Grove, Ill.
[11] Model 3380, Packard Instrument Co., Downers Grove, Ill.

Table I—Calculated Fraction Bound (*FB*) of Warfarin to 4 g/100 ml of Human Serum Albumin at 37°[a]

Plasma Warfarin Concentration, mg/liter	*FB*, %	FB_1, %
2	99	96
5	99	96
55	99	96
500	88	55

[a] Data obtained by iterative calculation with the PDP-8e computer.

1% *SD*. Instrument efficiency was monitored by including matched standards for the counting. Quenching, which was evaluated by the channels ratio method of Baillie (10), was insignificant for the protein level encountered in these solutions.

Warfarin content of the samples was determined by comparison of sample counts against the counts of the standard for the total drug level, and recovery was greater than 98%. Protein was determined by the microbiuret method of Goa (11), and recoveries exceeded 98%. Sodium, as a measure of the Donnan effect, was determined by flame photometry[12], using lithium as an internal standard.

Calculations—The extent of binding was calculated as follows:

$$\text{warfarin bound} = \text{warfarin total} - \text{warfarin free} \quad \text{(Eq. 1)}$$

The concentrations of total and free warfarin were determined by radioassay of aliquots from the β- and γ-plateaus. The molecular weight of albumin was taken as 69,000 for these calculations. The concentrations were corrected for solute space, and the free warfarin concentration was adjusted for the Donnan equilibrium using the sodium distribution according to the procedure of Keen (12).

The molar ratio of the bound warfarin (r) and the free warfarin concentrations (c) was used to obtain a two class-of-site solution by means of an iterative least-squares program[13]. The data were also tested for aptness of fit to a three class-of-site model using a much faster computer[14] for the program execution; this approach represents an improvement over the procedure previously described (8)[15].

The resulting values for n_1 and n_2, the primary and secondary binding sites, were averaged for the entire set of duplicate runs encompassing the full range of temperatures studied. These values were then used, in a modification of the two-site computer program, to evaluate the original data. This method resulted in new primary and secondary association constants constrained to average values for the number of primary and secondary sites. These values of the association constants were then used in the least-squares linear regression for the van't Hoff plot. The thermodynamic parameters were derived from the van't Hoff regressions.

The partial and total fractions bound, FB_1 and *FB*, were calculated from the total warfarin concentration by means of an iterative procedure employing a digital computer[16] (13). The data of Table I were derived in this way. Garten and Wosilait (14) described a solution to this problem, employing a computer[17], when there are two classes of sites. The advantage of our solution is that it can be done with a programmable calculator and is applicable to more than two classes of sites.

RESULTS AND DISCUSSION

Figure 1 compares typical elution patterns for warfarin and salicylate for a level of albumin of 0.3 g/100 ml. The β- and γ-plateaus correspond to the total and free drug concentrations, respectively. The column size and sample volume necessary to achieve the proper plateaus are determined experimentally, the main factor being absorption to the gel with some drugs. Presumably, because of interaction of warfarin with the gel, a larger sample volume is required with this drug than with salicylate to establish a satisfactory elution pattern. However, as long as flat β- and γ-plateaus are present, the binding equilibrium between warfarin and albumin is not altered by the retardation of the drug in the column. In contrast to large volume frontal analysis, small volume zonal chromatography would provide erroneous data as to the degree of binding of warfarin to albumin.

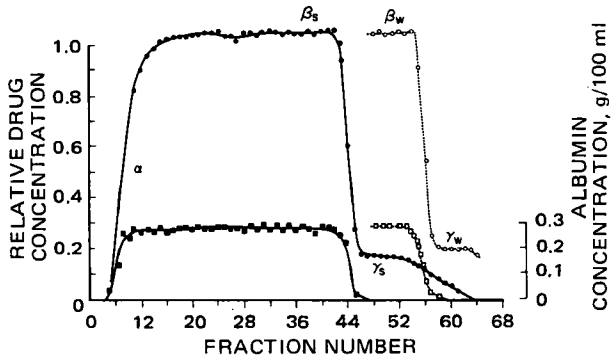

Figure 1—*Comparison of elution profiles for 45 ml of salicylate–human serum albumin (●) and human serum albumin (■) and 60 ml of warfarin–human serum albumin (○) and human serum albumin (□). Frontal gel chromatographic separation was on 1 × 25-cm columns of Sephadex G-25 coarse. The fractions were 1.3 ml.*

Figure 2 is a Scatchard display of the binding data for duplicate runs of eight points each. The sample concentrations are selected to provide optimal spacing of the points along the curve. The plot is curvilinear, an invariable finding with warfarin–albumin binding

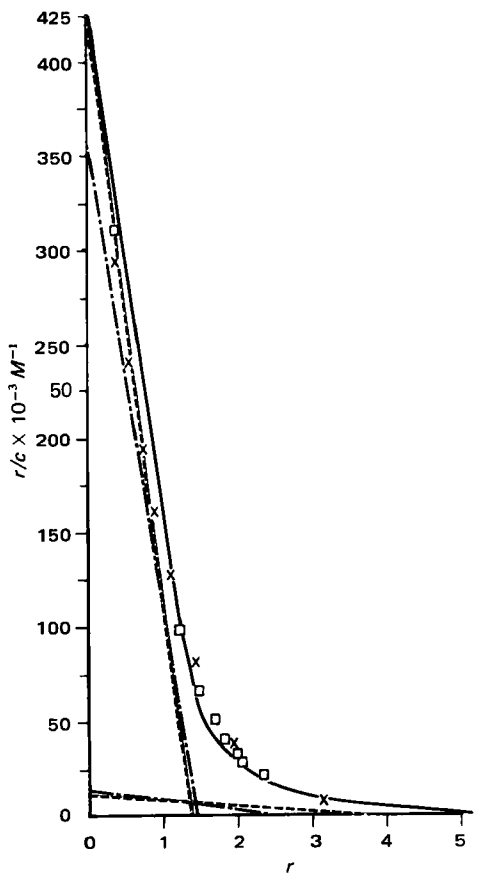

Figure 2—*Binding of warfarin to albumin in 0.067 M phosphate buffer, pH 7.4, at 25°. Two sets of data are displayed, each representing separate eight-point trials (×,□). Each point is a duplicate determination. The smooth curve is the best fit to these data resulting from the computer solution based on predetermined values for the number of sites ($n_1 = 1.38$, and $n_2 = 3.73$). Key: – · –, unconstrained solution; and - - - -, constrained solution.*

[12] Flame photometer 343, Instrumentation Laboratories, Lexington, Mass.
[13] IBM 1620, IBM Data Processing Division, White Plains, N.Y.
[14] IBM 360.
[15] Specific details of the computer programs employed are available upon request from the authors.
[16] PDP-8e, Digital Equipment Corp., Maynard, Mass.
[17] IBM 360/65.

Table II—Comparison of the Number of Primary and Secondary Binding Sites on Human Serum Albumin Reported for Warfarin and Salicylate

n_1	n_2	Drug	Reference
1.38	3.73	Warfarin	This paper
1.46	4.10	Salicylate	5
2	4	Warfarin	14
1 (1.3)[a]	6 (5.7)[a]	Warfarin	13
2 (2.38)[b]	(9.03)[b]	Warfarin	22
(2.13)[c]	(21.75)[c]		
3.5[d]	—	Warfarin	2
1.4[e]	—	Warfarin	2

[a]Our computer evaluation of the published data. [b]Our computer evaluation of the published data at 3°. [c]Our computer evaluation of the published data at 27°. [d]Reported value at 3°. [e]Reported value at 37°.

studies. A possible explanation for this result is that electrostatic interactions of the bound and incoming charged drug molecules significantly influence the binding process. However, appropriate electrostatic corrections for this effect, when applied to the present data, failed to produce a linear plot as was mentioned.

Similarly, electrostatic considerations were inadequate to explain the curvilinear Scatchard plots reported with an azo dye (15), various fatty acid anions (16), penicillin (12), and salicylate (5) binding to albumin. In these studies, the data were interpreted in terms of multiple binding sites and the electrostatic corrections were not employed. Generally, analysis based on two independent classes of sites adequately fits the binding data. Such a model is characterized for four parameters, n_1, K_1, n_2, and K_2; these are the number of sites and their respective association constants. The computer program derived values for these constants corresponding to the best fit to the experimental r and c values.

The unconstrained solution of the experimental data is illustrated in Fig. 2, where $n_1 = 1.46$ and $n_2 = 2.42$. These best-fit n values from all experiments were averaged, and the means were used in a computer program accepting fixed numbers of binding sites. The resulting constrained solution is also portrayed in the graph. There is no appreciable difference between the constrained and unconstrained evaluation, as reflected by the single smooth curve through the experimental points. This curve represents the vectorial summation of the linear components. The residual sum of squares, an additional indicator of the goodness of fit, is not significantly different in the two cases. This constrained model is in accord with the concept that the number of binding sites is invariate under the same experimental conditions for a particular system. Indeed, no trend was noted in the variation of n values relative to temperature and to warfarin or albumin concentration that would upset this idea.

A three class-of-site resolution of the experimental data was also attempted. This model resulted in low fractional primary sites and extraordinarily high tertiary sites. Additionally, an F test comparison of the residual sum of squares for the two and three class-of-site models revealed that the latter fits the data no better than the former. Similar findings for the binding of salicylate led to the adoption of the two class-of-site interpretation as the most appropriate.

Table II lists the various reported n values for the primary and secondary sites. The 95% confidence limits for n_1 are 1.08 and 1.59; for n_2, they are 0.22 and 7.14. For comparison, the number of binding sites for salicylate is also shown. Clearly, the number of primary and secondary binding sites for warfarin and salicylate are nearly identical. This finding suggests that these two drugs may displace one another from albumin by competing for identical sites on the albumin molecule.

As is evident from Table II, many investigators prefer to report integral number of binding sites. These numbers are most likely adjusted values, based on the reasonable assumption that, with homogeneous albumin, the number of sites should be integers. To estimate the degree of adjustment, published data from several studies (17–19) were analyzed by our computer program. The results are shown in Table II in parentheses. On the basis of the information collected, it is not possible to determine conclusively whether n_1 is 1 or 2; the degree of uncertainty is even larger with n_2. Thus, in all calculations, the fractional number of sites was employed, ensuring the best agreement of calculated and experimental information.

A summary of the binding data obtained at various temperatures

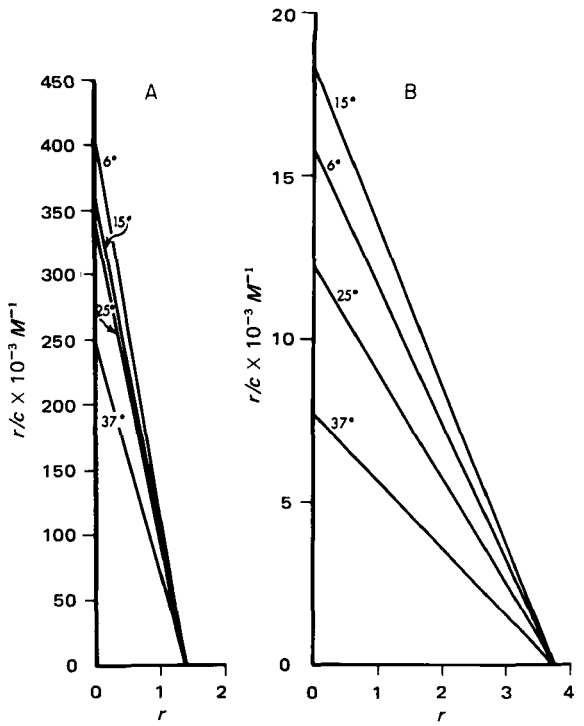

Figure 3—*Temperature dependence of the binding of warfarin to human serum albumin, as shown by the constrained two-site resolution of the binding data. The variation of binding with temperature at the primary and secondary binding sites is represented in sections A and B, respectively. In all experiments, the pH (7.4) and protein concentrations (0.3 g/100 ml) were maintained constant.*

is shown in Fig. 3 in the form of a Scatchard display of the constrained two class-of-site resolutions. The slopes of the lines correspond to the experimental primary and secondary association constants. Decreasing temperature results in an increase of the binding constants for both classes of sites. The van't Hoff plot, log K *versus* the reciprocal of the absolute temperature, is shown in Fig. 4. The straight lines were derived by regression analysis and serve to calculate corrected binding constants as well as standard enthalpies for the primary and secondary binding.

A summary of the association constants for the binding of warfarin to albumin is assembled in Table III. In general, the agreement among the reported K_1 and K_2 values can be considered good, especially since the experimental techniques used were different and the interpretation of the data, as reflected in the calculation of binding parameters, was not uniform. An apparent exception is the unusually large K_1 value reported by Meyer and Guttman (19). In an attempt to analyze the reasons for this discrepancy, the data from their curvilinear Scatchard plot were resolved in our computer program. Corresponding

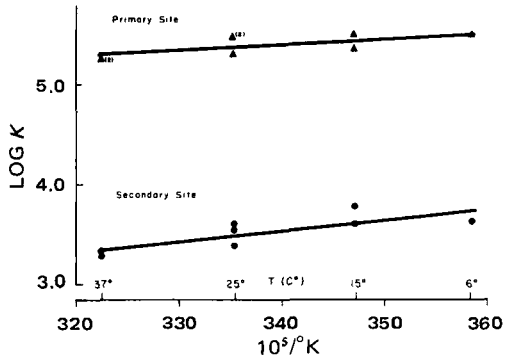

Figure 4—*Van't Hoff plot of the data for primary (▲) and secondary (●) sites. The regressions are: log* $K_1 = 0.00557\ (10^5/T) + 3.51201$, *and log* $K_2 = 0.01110\ (10^5/T) - 0.23081$. *The correlation coefficients are 0.67 and 0.81, respectively.*

Table III—Comparison of Primary and Secondary Binding Constants Reported for Warfarin and Human Serum Albumin

Temperature	$K_1 \times 10^3, M^{-1}$					$K_2 \times 10^3, M^{-1}$				
	This Study	Ref. 23	Ref. 19	Ref. 20	Ref. 2	This Study	Ref. 23	Ref. 19	Ref. 20	Ref. 2
3°	—	385 (114)[a]	—	—	160	—	— (0.6)[a]	—	—	—
6°	321	—	—	—	—	5.6	—	—	—	—
15°	278	350	—	—	—	4.2	—	—	—	—
25°	240	—	6240 (174)[a]	—	—	3.1	—	2.6 (2.2)[a]	—	—
27°	—	231 (97)[a]	—	89	—	—	— (0.3)[a]	—	6.7	—
37°	203	217	—	—	180	2.2	—	—	—	—
42°	190	—	—	—	—	2.0	—	—	—	—

[a] Our computer evaluation of the published data.

r and r/c values from the Scatchard plot least-squares fit of Meyer and Guttman were read by means of a microcomparator. The best fit to these readings was obtained with a K_1 value of 174×10^3 ($n_1 = 1.3$) and a K_2 value of 2.2×10^3 ($n_2 = 5.7$). This solution differs substantially from that of Meyer and Guttman only in the value for K_1.

Clearly, very minor variations or errors in r/c at low r values have a large influence on the derived value for K_1, especially when steep curves are extrapolated to zero r/c values. The spectrophotometric method used by these authors (19) for the determination of warfarin did not allow measurement of experimental r values much below 1.2. This limitation is contrasted with the ability of the radioisotope technique, which allowed routine determinations at r values as low as 0.2. Thus, while the generally much lower K_1 values shown in Table III are consistent with the experimental data of Meyer and Guttman, their reported K_1 value is out of line with the experimental binding data observed at r values below 1.

O'Reilly's extensive binding studies with warfarin largely parallels the present work. This author, while recognizing multiple binding sites, did not attempt to resolve his data in these terms. His K_1 association constant, shown in Table III, represents the limiting slope of the curvilinear Scatchard plot. For a better comparison of the reported binding data as well as the binding parameters, the data of O'Reilly and Kowitz (17) were subjected to our computer analysis. The results at two temperatures are also included in Table III. The derived K_1 values are two- to threefold lower than the association constants reported previously (17). This difference is an example of another interpretation of binding data. The value reported previously (17) is the association constant for the first anion bound, while the present value is the association constant for the binding by the primary class of sites. The four parameters derived from the data of O'Reilly and Kowitz are in good agreement with their data over the entire range of experimental measurements.

The binding constant reported by Garten and Wosilait (20) (Table III) is somewhat lower than would be expected from interpolation of our K values to 27°. These workers employed Hart's procedure (21), which produces a least-squares fit to a linearized form of the mass action expression for binding. This procedure considers r the "independent" and r/c the "dependent" variables and minimizes the sum of squared deviations in r/c. Such a comparison is not entirely valid, however, since the n_1, n_2, and K_2 values are different and the evaluation of K is interdependent with them.

A better comparison of the binding data is on the basis of fraction bound and free drug concentrations at identical drug and protein levels. This type of comparison shows the present data to be essentially the same as those of Garten and Wosilait (20). The binding constants at 37° given by Sellers and Koch-Weser (2) are in excellent agreement with the present data. Notably, their n_1 value at this temperature is identical with the average n_1 value obtained in this study. The agreement no longer exists for the comparison of association constants at 3°, mainly for the reason that they reported an increase in the number of binding sites with a decrease in temperature. This increase was not evident in the present work.

Comparison of binding studies on the basis of fraction of bound drug of identical drug and albumin concentrations is highly desirable. A computer program for this purpose was developed (6). Calculations show that at therapeutic plasma warfarin levels, warfarin is more than 99% bound to albumin at a concentration of 4 g/100 ml, regardless of which published set of binding parameters is used. The secondary sites contribute less than 4% of the binding at a warfarin concentration of 55 mg/liter. A 10-fold increase in the total plasma warfarin level would reduce the fraction bound to 88% and increase the contribution of the secondary binding to 45% of the total. A summary of these calculations is given in Table I.

These findings may be compared to those for the binding of salicylate published previously (5). In general, salicylate is somewhat less strongly bound than warfarin. Under identical conditions, 96% would be bound at the 20-mg/liter level; at 300 mg/liter, the therapeutic limit for salicylate, 83% would be in the bound form. The major importance of such considerations is to point out that drugs capable of displacing warfarin from its albumin binding sites may raise the effective free drug level manyfold, thereby leading to hemorrhagic complications (22).

The data presented here on the temperature dependence of warfarin binding agree well with those reported by O'Reilly (23). From the variation of the association constant with temperature, he derived a standard enthalpy change of −3.48 kcal/mole and a standard entropy change of +11 eu. Microcalorimetric measurements yielded a ΔH of −3.14 kcal/mole. These values, together with the other selected thermodynamic data for the binding of the various ligands by albumin, are shown in Table IV. Comparison of primary and secondary thermodynamic parameters reveals that the binding to primary sites is associated with significant positive entropy, while the secondary binding is exclusively enthalpy driven.

Furthermore, the temperature dependence of the secondary binding sites is about twice as large as that for the primary sites. Thus, at lower temperatures, the contribution of the secondary binding would increase. Such a shift toward binding to the more numerous secondary sites may explain the increased numbers of binding sites

Table IV—Standard Thermodynamic Parameters for Various Ligands Binding to Human Serum Albumin

Ligand	ΔF_1[a], kcal/mole	ΔH_1, kcal/mole	ΔS_1, eu	ΔF_2, kcal/mole	ΔH_2, kcal/mole	ΔS_2, eu
Warfarin[b]	−7.34	−2.55	+16.1	−4.76	−5.08	−1.10
Warfarin (Ref. 23)	−7.37	−3.50	+11.0	—	—	—
Azo dyes (Ref. 15)	−6.54	−3.93	+8.8	—	—	—
d-Tryptophan (Ref. 25)	−5.40	−11.3	−20.0	—	—	—
Salicylate (Ref. 5)	−6.45	−9.8	−11.2	−4.72	−4.70	+0.10
Thiocyanate (Ref. 26)	−6.20	−8.8	−8.8	—	—	—

[a] In Ref. 5, the thermodynamic parameters were expressed in units promoted by the Systeme International. However, these units have found little usage so far and the comparison with literature values is facilitated by employing the more familiar calorie unit. Conversion from one system to the other is based on the equivalence of 1 cal with 4.1868 J. [b] This paper.

reported by Sellers and Koch-Weser (2, 3) at the lower temperature.

According to Klotz (24), thermodynamic parameters in themselves do not provide a diagnostic criterion for distinguishing the types of forces involved in ligand binding by proteins. Indeed, as shown in Table IV, these parameters are nearly identical for the small thiocyanate ion and the larger organic molecule of salicylate containing the aromatic ring. Despite this anomaly, it is possible to draw relevant conclusions from the strikingly different thermodynamic parameters recorded for warfarin and azo dyes (15) as contrasted with those for *d*-tryptophan (25), salicylate (5), and thiocyanate (26). The binding of the first group is characterized by a low negative enthalpy and a considerable positive entropy; the binding by the second group is characteristically enthalpy driven opposed by the negative entropy.

From these considerations, it seems that hydrophobic forces predominate in the binding of warfarin since electrostatic forces were shown not to contribute to the positive entropy term (18). With salicylate, it appears that van der Waals forces are the most significant in the binding of this drug in albumin. This striking contrast between the binding parameters for warfarin and salicylate raises some serious doubts as to the coincidence of their binding sites on the albumin molecule, despite their nearly identical number of binding sites and their proven displacement interactions with albumin.

In line with this observation is the earlier finding that the inhibitor constants characterizing the displacement abilities of these two drugs are severalfold smaller than the primary association constants (4). Thus, purely competitive inhibition of binding between warfarin and salicylate can be ruled out. In contrast to the primary site parameters, binding to the secondary sites results in similar ΔH and ΔS values for these two drugs (Table IV). In both cases, the entropy term is nearly zero and the negative enthalpy drives the reaction. Therefore, competition at these sites might be expected to conform to a more strictly competitive inhibition model.

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