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EFFECT OF TEMPERATURE ON THE BINDING OF SALICYLATE BY HUMAN SERUM ALBUMIN*

JOHN F. ZAROSLINSKI, STEVEN KERESZTES-NAGY, ROLAND F. MAIS and YVO T. OESTER

Research Service, Veterans Administration Hospital, Hines, Ill. and Departments of Pharmacology and Biochemistry, Loyola University Stritch School of Medicine, Maywood, Ill., U.S.A.

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Abstract—The thermodynamic properties of the binding of salicylate to human serum albumin were determined at pH 7·4. Frontal analysis gel filtration, which previously has been shown to provide superior reproducibility, critical temperature control and improved accuracy for two-site resolution, was used. Consideration of the nonlinear binding data led us to examine heterogeneity of binding, electrostatic effects and discrete multiple binding sites. We found that our data were most appropriately described by a two-site model constrained to mean values of $\bar{n}_1 = 1{\cdot}46$ and $\bar{n}_2 = 4{\cdot}10$. This resulted in the following thermodynamic values: primary site $\Delta H_1 = -41{\cdot}05$ kJ mole^{-1} and $\Delta S_1 = -47{\cdot}0$ J °K^{-1} mole^{-1} ($\Delta F_1 = -27{\cdot}02$ kJ mole^{-1} at 25°), and secondary site $\Delta H_2 = -19{\cdot}61$ kJ mole^{-1} and $\Delta S_2 = 0{\cdot}5$ J °K^{-1} mole^{-1} ($\Delta F_2 = -19{\cdot}75$ kJ mole^{-1} at 25°). Thus the binding to both sites was temperature dependent and increased with decreasing temperature. Nevertheless, the thermodynamic parameters for the two sites are significantly different. The properties of the primary site resemble those for the binding of tryptophan and its derivatives, while the secondary binding of salicylate is like that for the long-chain fatty acids and anionic azo dyes. Calculations based on the observed parameters draw attention to the major contribution of the secondary site to the binding of salicylate at higher therapeutic (30 mg/100 ml) concentrations. It is postulated that the binding of salicylate involves both hydrophobic and ionic contributions. The former is disrupted by chlorpromazine, while the latter is affected by warfarin and chloride.

THE STUDY of interaction between salicylate and plasma proteins began a century ago with Feser and Friedberger.[1] Subsequently van Leeuwen[2] showed that the binding of this drug is fairly extensive and reversible. Since then, a large number of reports have appeared describing salicylate binding under various conditions, studied by a variety of experimental techniques.[3–12] Unfortunately, most of these studies dealt with the problem pragmatically, characterizing the binding process in terms of fraction of drug bound. This value, of course, varies with the drug and protein concentrations as well as with the various experimental conditions; thus, it has little significance in predicting the extent of binding in a given circumstance or in appreciating the nature of binding forces involved.

We previously reported [13] gel frontal analysis chromatographic studies of salicylate and human serum albumin (HSA), and interpreted the data in terms of primary and secondary binding sites, characterized by n_1, K_1, n_2, K_2 values. This investigation has now been extended to determine the enthalpy and entropy of the binding process derived from the variation of association constants with temperature. Such

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information is essential to characterize the nature of the interaction, to assess the reality of the model used (i.e. primary and secondary sites), and to interpret binding data for competing ligands. This latter aspect is especially important, as we have reported[14] mutual interference with albumin binding of warfarin and salicylate. The observed displacement did not conform to the simple competitive model in which these two drugs would bind to identical sites. Therefore, data presented in this paper could be useful not only in estimating the fractions of free and bound drugs, and the contribution of the primary and secondary sites to binding under various therapeutic drug levels and temperatures, but also in delineating the mechanism of displacement reaction between warfarin and salicylate.

MATERIALS AND METHODS

In prior studies of protein binding of drugs conducted in our laboratory, we have found that Sephadex gel frontal analysis chromatography provided greater precision and accuracy in the determination of binding parameters than did equilibrium dialysis.[13] Therefore, we used this technique in preference to the more widely employed equilibrium dialysis method.

Solutions were prepared in pH 7·4, 0·067 M phosphate buffer dissolved in distilled water. Protein solutions (0·3 per cent) were prepared daily by addition of crystalline human serum albumin (Pentex) to the phosphate buffer. Salicylate solutions were prepared by addition of ^{14}COOH-labeled salicylic acid (Amersham–Searle Corp.) to sufficient unlabeled carrier, previously dissolved in buffer to provide the desired initial drug concentration. The radiochemical purity of the labeled salicylate was 99 per cent and its specific activity was 31 mCi/m-mole. The amount of labeled drug was adjusted as necessary to provide at least 15,000 cpm/ml in the initial sample.

The gel filtration frontal analysis technique described by Cooper and Wood[15] was employed. G-25 Sephadex (coarse grade, Pharmacia) was allowed to swell in 0·067 M phosphate buffer, pH 7·4, at the prescribed temperature for several hr. Precision-bore jacketed columns were used (Adjusto-chrome, Ace Glass, Inc.; I.D., 1·0 cm) with the following modification. The Teflon discs were removed from the adjustable plungers and replaced with No. 35 Nitex nylon mesh (Kontes Glass Co.) cemented in place with Silastic. In this way a substantial increase in column flow rate was achieved. The gel was packed into the column to a depth of 25 cm providing a bed volume of about 20 ml. The columns were equilibrated for 1 hr at the prescribed temperature with buffer flowing. The temperature of the system (sample, buffer and columns) was maintained at the desired value by a Haake constant temperature circulator. Samples of known albumin and salicylate concentration were equilibrated at the prescribed temperature for several hr before use. All samples were prepared to contain approximately the same level of radioactivity regardless of actual drug concentration. A 45-ml sample of each preparation was applied to the column from a reservoir by gravity flow at a rate of approximately 4 ml/min. The column was subsequently eluted with 45 ml phosphate buffer.

In pilot studies, serial aliquots were assayed for both protein and salicylate to determine elution volumes for the beta or central plateau (protein-bound drug in equilibrium with free drug) and the gamma plateau (free drug). In subsequent experiments, we collected three 1-ml aliquots at elution volumes corresponding to the beta plateau and four 1-ml aliquots corresponding to the gamma plateau. Total and free

drug concentrations were then determined as described below. Derived values for duplicate samples, which were reasonably coincident, were averaged to yield a single datum.

Salicylate determinations. One-ml aliquots of fractions obtained from the Sephadex column were added to 10 ml InstaGel scintillator (Packard) in a conventional glass counting vial. The ^{14}C was measured with a Packard model 3380 liquid scintillation counter. Each sample was counted to less than 1 per cent standard deviation. Instrument efficiency was monitored by use of an appropriate standard. Quenching as evaluated by channels ratio was negligible.[16] The salicylate content of each sample was arrived at by use of appropriate isotope dilution technique computations. Recovery of labeled drug in all instances was greater than 98 per cent.

Protein was determined by the microbiuret method of Goa.[17] Irreversible binding of protein to the column was not encountered, and protein recoveries in our experiments were greater than 98 per cent. Sodium was determined by flame photometry using lithium as the internal standard.

Calculations. The extent of binding was calculated on the basis of the following equation:

$$\text{(salicylate bound)} = \text{(salicylate total)} - \text{(salicylate free)}.$$

Concentrations of total and free salicylate were determined by radioassay of aliquots from the beta and gamma plateaus. A molecular weight of 69,000 was used for human serum albumin in these calculations. Concentrations were corrected for solute space and the free salicylate concentrations were adjusted for the Donnan distrubution according to the procedure described by Keen.[18]

The data so obtained were subjected to curve fitting by means of an IBM 1620 computer. Two binding site resolution was accomplished by a nonlinear least-square fit to the following equation in which c is the independent variable:

$$r = \frac{n_1 K_1 c}{1 + K_1 c} + \frac{n_2 K_2 c}{1 + K_2 c} \tag{1}$$

where K_1 and K_2 are the association constants corresponding to n_1 and n_2, the number of primary and secondary binding sites, c is the free drug concentration, and r is the molar ratio of bound drug to binding protein. The n_1 and n_2 values obtained for various replicate experiments as well as at various temperatures were averaged and a revised set of K_1 and K_2 values were derived corresponding to these constrained average $\bar{n}_1$ and $\bar{n}_2$ values. (Specific details of the computer program employed are available upon request from the authors.)

RESULTS

Figure 1 displays the binding of salicylate to HSA at 25° in the form of a Scatchard plot. The 16 experimental points represent two separate eight-point trials, each performed in duplicate. The solid straight lines correspond to the constrained computer resolution of the data. The unconstrained resolution of these same data is represented by dashed lines. As can be seen, there is little change in the slopes and intercepts between the constrained and unconstrained solutions. Also the residual sum of squares, which serves as an indicator of the goodness of fit, is not significantly different in the two cases. A similar situation has been found to exist at other temper-

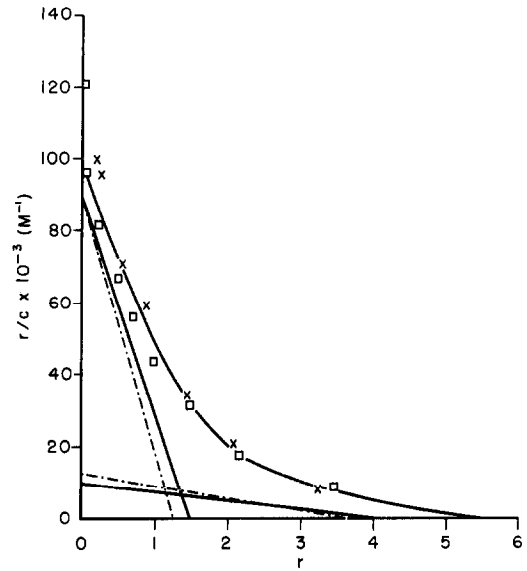

FIG. 1. Binding of salicylate to albumin in 0·067 M phosphate buffer, pH 7·4 at 25°. Two sets of data are displayed, each representing separate eight-point trials (×, □). Each point is a duplicate determination. The smooth curve is the best fit to these data as a result of the computer solution based on predetermined values for the number of sites ($\bar{n}_1 = 1{\cdot}46$, and $\bar{n}_2 = 4{\cdot}10$). The two solid lines are this resolution. The alternate unconstrained resolution is shown as dashed lines for comparison.

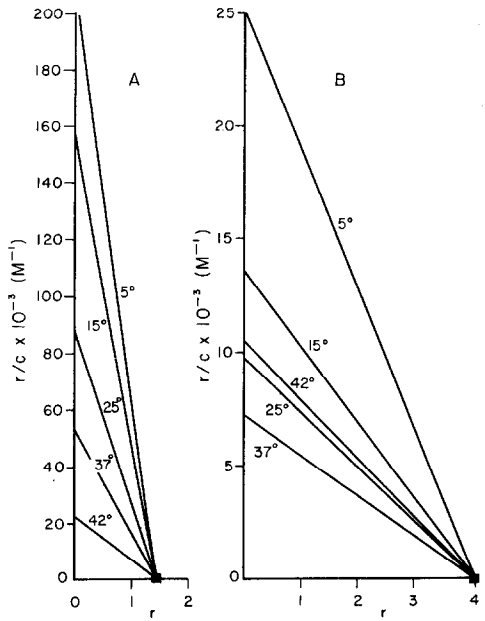

FIG. 2. Temperature dependence of the binding of salicylate to HSA, as shown by the constrained two-site resolution of the binding data. Panel A represents the variation of the binding with temperature at the primary binding site, and panel B at the secondary sites. In all experiments, the pH (7·4) and the protein concentrations (0·3 g/100 ml) were maintained constant.

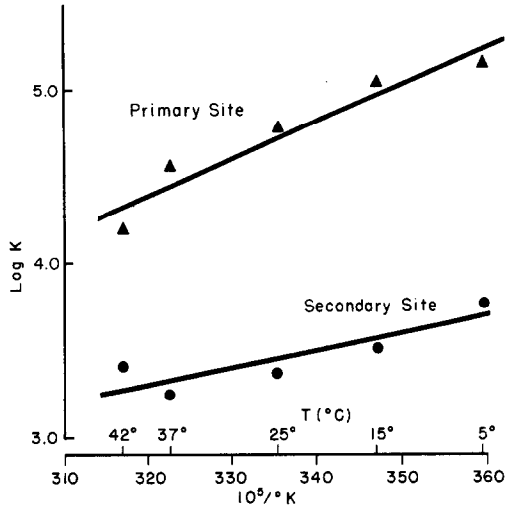

FIG.3. Van't Hoff plot of the data for the primary (△) and secondary (○) sites. The correlation coefficient for the regression line A is 0·96; that for B is 0·87.

atures studied. The smooth curve through the points is the resultant of the two linear components summed along vectors of constant free drug concentration ($1/c$). The curves obtained in this way for the constrained and unconstrained data are practically indistinguishable.

The binding data obtained at the various temperatures are shown in Fig. 2, in the form of a Scatchard-type display of the constrained two-line resolution. Clearly, the binding of salicylate to HSA is strongly temperature dependent and increases with decreasing temperature both at the primary and secondary binding sites. The slopes of these lines, corresponding to the experimental binding constants, were used as points for the construction of the Van't Hoff plots shown in Fig. 3. Binding constants as well as other thermodynamic parameters were calculated from the linear regressions arising from these plots. The results are summarized in Table 1. The enthalpy, which is twice as large for the primary as for the secondary sites, is primarily responsible for the large negative free energy of binding. This difference between ΔH_1 and ΔH_2 reflects the 8-fold variation of K_1 between 5° and 42° as opposed

TABLE 1. THERMODYNAMIC PROPERTIES OF THE SALICYLATE–HSA BINDING SYSTEM*

Temp. (°)	K_1 ($M^{-1} \times 10^{-3}$)	ΔF_1 (kJ $mole^{-1}$)†	K_2 ($M^{-1} \times 10^{-3}$)	ΔF_2 (kJ $mole^{-1}$)
5°	177·1	−27·96	5·1	−19·75
15°	95·7	−27·49	3·8	−19·75
25°	53·9	−27·02	2·9	−19·76
37°	28·4	−26·46	2·1	−19·76
42°	22·1	−26·22	1·9	−19·77

* $\bar{n}_1 = 1{\cdot}46$; $\Delta H_1 = -41{\cdot}05$ kJ $mole^{-1}$; $\Delta S_1 = -47{\cdot}0$ J $°K^{-1}$ $mole^{-1}$; $\bar{n}_2 = 4{\cdot}10$; $\Delta H_2 = -19{\cdot}61$ kJ $mole^{-1}$; $\Delta S_2 = +0{\cdot}5$ J $°K^{-1}$ $mole^{-1}$.

† The units are those of the Système International. The calorie = 4·186 joules (J).

to the 2·5-fold variation of K_2 in the same temperature range. Also, ΔS_1 for the primary sites is relatively large and negative, while for the secondary sites it is practically zero, resulting in a temperature-independent ΔF_2 within the temperature limits studied.

In order to appreciate the applicability of these binding parameters under a different set of conditions, we compared projected fraction-bound salicylate at approximately 3·0 g/100 ml ($4{\cdot}28 \times 10^{-4}$M) HSA concentrations with experimentally observed data at the same HSA concentration and four separate salicylate concentrations at 25°. This comparison is shown in Table 2.

It appears that the pharmacologically important parameter, the fraction-bound (or fraction free) drug level, can be predicted rather well on the basis of binding constants obtained at a much lower HSA concentration. Such a finding indicates that the binding constants are largely independent of the protein concentrations, at least in the 10-fold range which we have studied. A similar conclusion was reached by Karush[19] with bovine serum albumin and azo dye binding.

We have also calculated the temperature dependence of fraction-bound drug as well as the contribution of the primary and secondary sites to the total binding, both at low and high therapeutic salicylate levels and physiological albumin concentration. The results of these calculations are shown in Table 3. There is no simple direct procedure for calculating fraction-bound drug from a given total drug concentration. The data of Table 3 and its comparison with literature values, obtained under different conditions, depend on the interchangeability of fraction bound and total drug. An iterative computer program was devised to accomplish the interconversion of these values. This program, which is written in Focal 69 for the PDP 8 digital computer, is available from the DECUS Program Library.[20] A description of the other calculations which we have employed, including the concept of partial fraction bound for multiple site systems, is being written and will be published at an early date.

As can be seen from Table 3, at low (3 mg/100 ml) concentrations, salicylate is almost completely bound to HSA at all temperatures, the major contributor being the primary site. At high (30 mg/100 ml) therapeutic drug concentrations, the total fraction bound is significantly reduced, while the free drug concentration varies with temperature and the secondary binding sites contribute over 50 per cent to the total binding.

TABLE 2. COMPARISON OF OBSERVED AND CALCULATED FRACTION-BOUND SALICYLATE AT 25°

Total HSA concn. ($M \times 10^4$)	Total salicylate concn. ($M \times 10^5$)	Fraction bound* (%)	
		Observed	Calculated†
4·28	184·3	72·8	78·5
4·28	129·9	84·7	85·9
4·28	102·5	89·1	89·3
4·28	40·0	95·3	95·8

* Three g/100 ml of HSA.
† Calculated from 0·3 g/100 ml data.

TABLE 3. SALICYLATE BINDING—EFFECTS OF TEMPERATURE AND CONCENTRATION*

	Salicylate				
	3 mg/100 ml		30 mg/100 ml		
Temp.	FB	FB_1	FB	FB_1	% Free
5°	0·99	0·90	0·91	0·38	9
15°	0·99	0·86	0·89	0·37	11
25°	0·98	0·82	0·87	0·37	13
37°	0·96	0·76	0·83	0·36	17
42°	0·95	0·74	0·82	0·35	18

* FB = total fraction bound; FB_1 = fraction bound to primary site; % Free = free drug as % of total.

DISCUSSION

Most albumin binding studies involving other ligands, such as penicillin, barbiturates and tryptophan,[18,21,22] result in curved Scatchard plots. Such non-ideal behavior, in principle, could be attributed to electrostatic repulsive forces. Like other investigators in this field,[18,19,22–24] we attempted to apply corrections to rectify our data data.

We used the relation employed by Keen,[18] which is:

$$(r/c) \exp 2w(Z_p + r) = K(n-r) \tag{2}$$

where r, c and n have the same meaning as in equation 1. Z_p is the charge on the protein molecule (12 for albumin at pH 7·4),[25] K is the association constant, while w and κ, the Debye-Hückel electrostatic binding parameters, are defined as follows:

$$w = \frac{e^2z^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \tag{3}$$

and

$$\kappa = 3{\cdot}2866 \times 10^7 \times (\mu)^{1/2} \times (298{\cdot}15/T)^{1/2} \tag{4}$$

where e is the electronic charge, z is the charge on the small ion, D is the dielectric constant of the solvent, k is the Boltzmann constant, T is the absolute temperature, b is the equivalent radius of the albumin molecule (30 Å) and a is the radius of exclusion (32·5 Å),[26] μ is the ionic strength. The value (298·15/T) adjusts κ for temperatures other than 25°.

The results of these corrections are shown in Fig. 4. Clearly the plots remain curvilinear, indicating that electrostatic repulsion is not the reason for the nonlinear behavior. Similar conclusions were reached by others.[18,19,27] In general, such corrections in the case of albumin are thought to be inapplicable due to displacement of already bound buffer ions by the anions under study.[28,29]

It is possible that the curvilinear Scatchard plots are due to a statistical distribution of the free energy of binding, as was observed by Longsworth and Jacobsen.[30] The curvilinear plots observed with antigen–antibody reactions are accounted for in this way.[31] The adaptation of the Sips distribution, originally proposed by Klotz,[32]

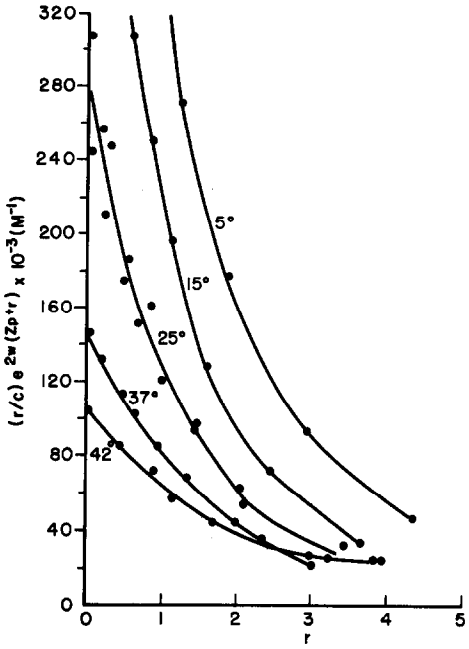

FIG. 4. Binding of salicylate to HSA at various temperatures corrected for electrostatic interaction of the binding molecules according to the equation $(r/c)\exp 2w(Z_p + r) = K(n-r)$.[1]The assumption of interaction is not supported by the result, since the transformation failed to straighten the lines.

was used to resolve the nonlinear Scatchard display of our data. The relation used is:

$$\frac{r}{n} = \frac{c^{\alpha}K_0}{c^{\alpha}K_0 + 1} (0 < \alpha < 1) \tag{5}$$

where α is an index of the heterogeneity of the free energy of binding, c is the molar free drug concentration, K_0 is the intrinsic association constant, n is the number of binding sites, and r is the molar ratio of bound drug to binding protein.

The conformity of the experimental data to this model must be compared with that for other equally plausible ones, such as the two-binding site model described by equation 1.[23]

An attempt at three-site resolution has been made by various workers,[18,24,33] based essentially on the method of Scatchard *et al.*,[34] using asymptotic approximations. Three-site resolution with our procedure[13] requires modification and a more elaborate computer. We have used a simpler program based on a least-squares fit to a linearized form of the binding equation. This provides reasonably accurate resolutions when the experimental errors are low and is readily handled by the IBM 1620 computer. It is not statistically well founded, since it does not take account of the proper error structure. For this reason, we will limit our discussion to two groups of sites.

The accumulation of reliable information on a particular binding system compels one to interpret these data in terms of the best estimate (average) of the number of

sites. Thus a new interpretation arises in which the two-site model is constrained to preset values for n_1 and n_2. Such comparisons are rather complicated because the various models have different numbers of parameters. Thus, the unconstrained two-site model has four (n_1, n_2, K_1, K_2). The heterogeneity model has three (n_1 K_1 and α), while the constrained one has only two (K_1 and K_2).

A statistical evaluation of the fit of these models to our data was performed by Mr. Jack Becktel of the Hines VA Cooperative Studies Program Support Center, Veterans Administration Hospital, Hines, Ill. He viewed these models as nested (i.e. one parameterization a subset of another), and found no significant difference in the fit of the constrained and unconstrained models for the 25° data. However, the three-parameter heterogeneity model was inferior in regard to fit. On this basis, we feel justified to use the two-parameter constrained model.

We have attempted to establish clinically useful information through the study of the thermodynamic characteristics of the binding of salicylate to pure HSA. The salicylate range satisfies the requirements for adequate binding appraisal as well as pharmacologic relevance. Admittedly, *in vivo*, factors such as the presence of free fatty acids, chloride ions, bilirubin, etc. may alter the free amount of salicylate. The basic data presented here can be amended to recognize such factors especially significant in disease states. This is equally true of physiologic variations such as metabolic imbalance (viz. diabetic acidosis) and febrile states. Conceivably, alterations in the free salicylate levels corresponding to known physiologic variations (fever to hypothermia) are significant under those conditions. The ΔH_1 of $-41{\cdot}05$ kJ mole^{-1} we obtained at 37° is indicative of the strongly exothermic binding of salicylate to HSA which may have gone unnoticed in the past. As late as 1961, Davison and Smith[35] indicated that changes in temperature have little or no effect on salicylate binding. Temperature dependence of salicylate binding is evident upon examination of the studies published by Ali and Routh,[36] although they did not comment on this effect and reported only the percentage of drug bound with no attempt to evaluate n or K values.

In order to compare the thermodynamic parameters found for salicylate binding to those of similar binding studies involving albumin and other ligands, we have surveyed available literature data. It appears to us that two major categories exist as to the binding parameters: one with relatively large negative enthalpies and negative entropies, and the second with small negative enthalpies and positive entropies. The former class is represented by tryptophan and its derivatives,[22,37] small anions such as I^-[38] and SCN^-[39], as well as salicylate. The second group is represented by long-chain fatty acids,[27] anionic azo dyes[19] and warfarin.[40] Interestingly, warfarin shows significant displacement of salicylate from albumin binding sites.[41] On the other hand, chlorpromazine, a positively charged drug, displaces only salicylate, while Cl^- competes with both salicylate and warfarin. One explanation of these observations is that salicylate has a unique hydrophobic binding site from which it is displaced by chlorpromazine, and an anionic binding site which appears to be common with warfarin, accounting for the partial competition and displacement by Cl^-.[14]

In comparing the salicylate binding parameters for the primary and secondary sites, it appears that the secondary binding resembles more closely the binding of long-chain fatty acids and azo dyes than the primary salicylate binding. Such a difference in the nature of primary and secondary binding may indicate that these two

types of sites indeed have a physical reality attached to them and represent not just a convenient model for description and interpretation of binding data.

As mentioned, extrapolations *in vitro* may overestimate the fraction of drug bound. Still, the calculations presented in Table 3 call attention to the fact that changes in the fraction of salicylate bound with temperature could be sizable. *In vivo*, this raises the possibility of changes in free drug availability, concomitant with hyperthermia as well as with hypothermia. From Table 3, it is also apparent that, although the primary binding site is many times stronger than the secondary, the contribution of the latter cannot be neglected, especially at high doses of salicylate.

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