Interpretation of Protein–Drug Interaction through Fraction Bound and Relative Contribution of Secondary Sites

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Abstract □ Three mathematical procedures were devised which allowed for the interconversion and interpretation of protein–drug binding data in the literature. A minicomputer program enabled the calculation of the fraction of drug bound for a specified total drug and protein concentration, given the protein binding constants and the number of sites. The corollary problem of finding the total drug concentration for a particular fraction drug bound was included. In addition, the concept of partial fraction bound for binding systems with multiple classes of sites was introduced and a formula was derived which made possible the calculation of this value from the usual binding constants. Finally, an equation was derived which allowed the total drug concentration and the fraction bound to be obtained from the binding information for selected r values. These were obtained from the Scatchard plot and the associated binding constants. Examples of the utility of these procedures are supplied based on data for human serum albumin binding of four drugs of clinical interest. Evidence is given to show the prominence of the contribution by the secondary sites to the total binding at therapeutic levels of the drugs.

Keyphrases □ Protein–drug interaction—interpretation through fraction bound and relative contribution of secondary sites, interconversion of binding data, multiple classes of sites □ Drug–protein binding—multiple classes of sites, determination of partial fraction bound, secondary sites, interconversion of data □ Binding, drug–protein—fraction bound calculated from total drug, data interconversion and comparison, multiple classes of sites

Considerable information has been amassed in the last 2 decades on protein–drug interactions. The simplest way to describe such data is by the concept of fraction bound. This value is directly obtained from experimental free and total drug levels. Conversely, the calculation of the free and total drug levels for a specified fraction bound is also possible, but only for simple systems involving a single class of binding sites. For such a system, the Goldstein (1) equation relates the fraction bound to the binding parameters for a specified free drug level:

$$\beta = \frac{nP}{nP + K_d + c} \qquad \text{(Eq. 1)}$$

where n is the number of binding sites characterized by a common dissociation constant, K_d. The fraction bound, β, is described for each free drug concentration, c, for a specific protein level, P. However, this equation depends upon free drug concentration and cannot be used to calculate fraction bound for a designated total drug concentration, the most commonly known variable. This relationship does not provide for the interpretation of discrete multiple-site binding (*i.e.*, classes of binding sites differing significantly in their association constants).

Clearly, the calculation of fraction of drug bound from binding constants and concentration of protein and ligand will depend upon selection of a model that most closely approximates the true state of binding. Aside from the simplest case of a single class of sites, the model may include discrete multiple sites, which from a practical standpoint rarely exceed four classes. Alternative models that may be considered are one where there is heterogeneity of the binding sites on the protein molecules and one where prominent electrostatic effects of the ligand may exist. Combinations of these models are possible, and it is conceivable to have a system of multiple binding sites in which one or more classes display electrostatic interaction (2). Each of the models can be described mathematically. Selection of the proper model is based on the conformity of the values (such as fraction bound) predicted by the expression to the experimentally obtained data. Studies have shown that the simple two classes-of-sites model conforms most closely to our data for the binding of salicylate and warfarin to albumin (3, 4).

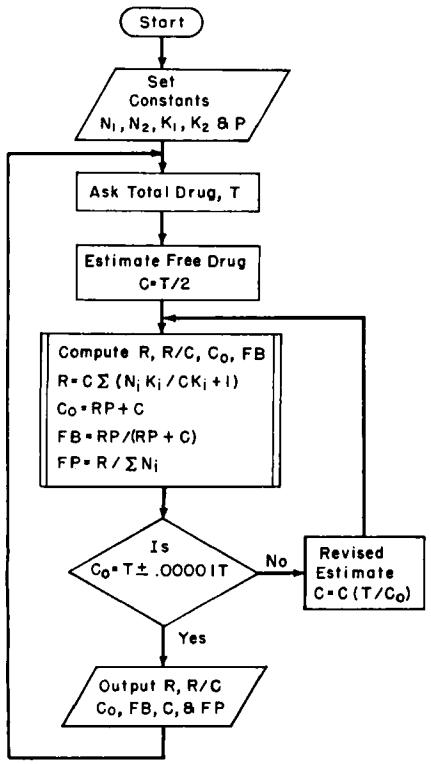
This paper expands upon the concept of fraction bound by extending the interpretation to the contribution of the individual classes of sites to total binding. This interpretation, called partial fraction bound, is analogous to partial pressures in gasometry. In addition, a computer program is presented which allows for the calculation of fraction bound and free drug concentration from protein and total drug concentrations when the binding parameters are known for a given binding system. Other equations are derived which are useful for the interconversion of binding parameters and readily allow comparison of literature values.

EXPERIMENTAL

Determination of binding parameters was made on the following drugs: salicylate, warfarin, chlorpromazine, and quinidine. All compounds except quinidine were studied with gel filtration chromatography using radioisotope dilution analysis. The quinidine data were obtained from equilibrium dialysis experiments in which quinidine was determined spectrophotometrically after extraction from the dialysis solutions. The frontal technique was used for the gel filtration, and the conditions employed were described previously (3).

Materials—All drug–human serum albumin solutions were made up to 0.3 g % albumin[1] in 0.067 M phosphate buffer, pH 7.4. The following ^{14}C-labeled drugs were used: salicylate-^{14}COOH, 31 mCi/mmole, with radiochemical purity of 98%[2]; warfarin-^{14}C, 23 mCi/mmole, with radiochemical purity of 99%[3]; and chlorpromazine-(ring-^{14}C), 31 mCi/mmole, with radiochemical purity of 99%[4]. Quinidine was employed as the hydrochloride[5]. For the quinidine study, the albumin concentration was approximately 2 g % to simulate literature data (5). The ^{14}C-labeled drugs were added to the

[1] Miles Laboratories, Inc., Kankakee, Ill.
[2] CFA. 185, Amersham/Searle, Arlington Heights, Ill.
[3] CFA. 449, Amersham/Searle, Arlington Heights, Ill.
[4] Applied Science Laboratories, State College, Pa.
[5] 14,592-0, Aldrich Chemical Co., Milwaukee, Wis.



Scheme I—Flowsheet for the PDP-8 computer program to derive the fraction bound from the total drug concentration and the binding constants

working drug solutions to provide at least 15,000 cpm/ml. The thixotropic phosphore[6] used as the scintillator for the β-ray spectrometry performed well with protein solutions.

Analytical Methods—The gel filtration frontal analysis technique of Cooper and Wood was used (6), and all samples were run in duplicate. A cross-linked dextran gel[7] was conditioned with the 0.067 *M* phosphate buffer, pH 7.4, at 25° for several hours prior to use. The polyacrylamide gel[8] used for the chlorpromazine study was similarly treated. Jacketed columns[9] and buffer and sample reservoirs were serviced by a thermoregulator system[10] which maintained the temperature at 25 ± 0.2°. Satisfactory elution profiles were obtained for the different drugs under the following conditions.

Salicylate—Forty-five-milliliter samples were applied at a rate of 4 ml/min to 1 × 25-cm columns[7]. Three aliquots for total drug were taken in plateau at 47–53 ml and four aliquots for free drug were taken at 60–64 ml.

Warfarin—Sixty-milliliter samples were applied at a rate of 4 ml/min to 1 × 25-cm columns[7]. Three aliquots for total drug were taken in plateau at 62–68 ml and four aliquots for free drug were taken at 77–81 ml.

Chlorpromazine—Sixty-milliliter samples were applied at a rate of 2.3 ml/min to 1 × 25-cm columns[11]. Three aliquots for total drug were taken in plateau at 62–69 ml and four aliquots for free drug were taken at 77–81 ml.

A discard sample, duplicating the highest concentration of drug, was run through the columns initially for each drug system to condition the column to offset any adsorption on the column that would upset the equilibrium of the system.

Equilibrium Dialysis—The dialysis was performed in 5-ml Karush cells[12], using regenerated cellulose[13] as the membrane. The dialysis, as previously described (3), required 16 hr of shaking at 60 strokes/min. The dialyzed solutions were extracted with ethylene dichloride under controlled pH conditions, and the extracts were measured for quinidine by UV spectrophotometry[14] (8).

Scintillation Spectrometry—One-milliliter aliquots of the samples were added to 10 ml of the scintillator solution. The samples were counted to less than 1% *SD* in a scintillation spectrometer[15]. Quenching of the solutions was evaluated by the channels ratios method of Baillie (9) and found to be insignificant at this protein level. The counts were converted to drug concentration by comparison with standards of similar composition and of known concentration. The concentrations were corrected for solute space and for the Donnan effect according to the procedure employed by Keen (10).

Calculations—Data from the scintillation (counts) and UV spectrometry (absorbance) were converted to the concentrations of bound and free drug. A minimum of eight points was used for derivation of the binding parameters for each system. The data were fit to the two classes-of-sites model described in Eq. 2 by means of a nonlinear least-squares technique using the computer program previously described (3).

ANALYSIS AND TREATMENT OF DATA

Derivation of Fraction Bound from Total Drug Concentration—The mass law expression for binding of a ligand to a macromolecule in which two classes of sites are involved is (11):

$$r/c = \frac{n_1K_1}{1 + K_1c} + \frac{n_2K_2}{1 + K_2c} \qquad \text{(Eq. 2)}$$

where n_1 and n_2 are the number of primary and secondary sites with corresponding association constants of K_1 and K_2. The free ligand concentration is c. By definition, r is the number of moles of ligand bound per mole of binding macromolecule, B/P, and the moles of ligand bound, B, is the difference between the total ligand and the free, $T - c$. From this follows:

$$r = \frac{T - c}{P} \qquad \text{(Eq. 3)}$$

Substitution of this expression for r in Eq. 2 and replacement of the association constants with their reciprocals, the dissociation constants ($1/K = k$), result in the following:

$$\frac{T - c}{Pc} = \frac{n_1}{c + k_1} + \frac{n_2}{c + k_2} \qquad \text{(Eq. 4)}$$

Clearing this expression of fractions yields:

$$c^3 + c^2[P(n_1 + n_2) + k_1 + k_2 - T] + c[P(n_1k_2 + n_2k_1) - T(k_1 + k_2) + k_1k_2] - Tk_1k_2 = 0 \qquad \text{(Eq. 5)}$$

The root of this complicated cubic equation defines c, the free ligand concentration, as a function of the total ligand, T. The fraction bound (FB) can then be written:

$$FB = \frac{T - c}{T} = \frac{T - f(T)}{T} \qquad \text{(Eq. 6)}$$

An extension of the above would show that a system of three classes of sites would result in a complicated fourth degree polynomial, *etc.*

For practical purposes, these equations are unmanageable. Therefore, an iterative procedure was devised for use with a digital computer[16]. This procedure not only solves the problem but gives additional information in a few seconds. The primary and secon-

[6] Insta-Gel, Packard Instrument Co., Downers Grove, Ill.
[7] Sephadex G25, coarse grade, Pharmacia Fine Chemicals, Piscataway, N.J.
[8] Bio-Gel P-4, 50–100 mesh, Bio-Rad Laboratories, Richmond, Calif.
[9] Adjusto-Chrom 5919, 1 × 60-cm set for 25-cm column, Ace Glass Inc., Vineland, N.J.
[10] Haake series FE constant-temperature circulator with KR refrigerated chiller, Polyscience Corp., Evanston, Ill.
[11] Bio-Gel P-4 was used in place of Sephadex G-25, which did not give a satisfactory free drug plateau for this size column with sample volumes less than 100 ml.
[12] No. 3221, Bellco Glass Inc., Vineland, N.J.
[13] Cuprophan, J. P. Bemberg Co., Wuppertal, West Germany.
[14] Beckman DB-G spectrometer.
[15] Model 3380, Packard Instrument Co., Downers Grove, Ill.
[16] PDP-8, Digital Equipment Corp., Maynard, Mass. The program was written in Focal 8 for the PDP-8 minicomputer; however, a similar approach should be possible with any of the more sophisticated programmable calculators that have decision-making capabilities.

Table I—Drug Binding Constants for Human Serum Albumin at 25° in 0.067 *M* Phosphate Buffer (pH 7.4)

Drug	n_1	K_1, M^{-1}	n_2	K_2, M^{-1}	Albumin, $\times 10^5$ *M*
Warfarin	1.46	241,000	2.42	5600	4.01
Salicylate	1.28	70,700	3.80	3300	4.21
	0.76	70,500	2.96	8600	42.8
Chlorpromazine	0.69	14,000	52.3	140	4.29
Quinidine	0.39	20,600	3.15	910	27.5

dary sites, n_1 and n_2, along with their corresponding association constants, K_1 and K_2, and the protein concentration are supplied to the computer. The program (Scheme I) calls for a total drug value, T. One-half of this value is taken as a first estimate of the free drug concentration, c. The total drug concentration for this estimate is calculated from $C_0 = rP + c$ and is then compared with the given value of T; a corrected estimate of c is then derived from $c(T/C_0)$. The iteration proceeds until the absolute difference is less than a small preset value based on the total drug. The program then prints out the usual Scatchard plot parameters r and r/c, along with the fraction bound, FB, and the fraction of protein sites occupied, FP, which is equal to $r/\Sigma n_i$. The final free drug concentration, c, which is printed out, is an exact value. This program is available (12) along with the corollary program for determining r, r/c, C_0, FP, and c from a selected fraction bound drug, FB. Unlike the computer program for the resolution of binding data (3), which is limited to two classes of sites, the present programs accommodate at least four classes. They also provide for changes in the level of protein, assuming that such changes do not influence the binding parameters. This was verified for the salicylate–albumin system in the range of 0.3–3.0 g % albumin (13).

Partial Fraction Bound—Many drug–protein systems are appropriately resolved into two or more classes of binding sites (10, 11, 14, 15). No expression relating the fraction bound to the observed variables is available for this situation. Analysis of the binding of salicylate (3) and warfarin (4) to human serum albumin at pH 7.4 requires such an expression. The experimental term used to describe the amount of bound ligand is fraction bound. This is commonly used in the literature and is related to the clinically important term fraction free by $FB = 1 - FF$. An expression will now be developed for fraction bound relevant to the individual classes of sites. Intuitively, fraction bound for a system of two classes of sites can be defined as:

$$FB = FB_1 + FB_2 \qquad \text{(Eq. 7)}$$

An equivalent expression, in terms of the binding constants n_1, n_2, K_1, and K_2, arises naturally from the relation of FB to r. By definition, the expression for fraction bound is:

$$FB = \frac{T - c}{T} = \frac{B}{T} \qquad \text{(Eq. 8)}$$

where T, B, and c are the total, bound, and free ligand concentrations, respectively. Since r is the moles of ligand bound per mole of binding macromolecule ($r = B/P$), substitution in Eq. 8 yields:

$$FB = \frac{rP}{T} \qquad \text{(Eq. 9)}$$

The mass action law expression for the binding system having i classes of sites is (16):

$$r = c\sum_{i=1}^{i}\frac{n_i k_i}{1 + K_i c} \qquad (i = 1, 2, \ldots, n) \qquad \text{(Eq. 10)}$$

Exchanging the value of r in Eq. 10 for that in Eq. 9 yields:

$$FB = \frac{Pc}{T}\sum_{i=1}^{i}\frac{n_i K_i}{1 + K_i c} \qquad \text{(Eq. 11)}$$

For two classes of sites, this is:

$$FB = \frac{Pc}{T}\left(\frac{n_1 K_1}{1 + K_1 c}\right) + \frac{Pc}{T}\left(\frac{n_2 K_2}{1 + K_2 c}\right) \qquad \text{(Eq. 12)}$$

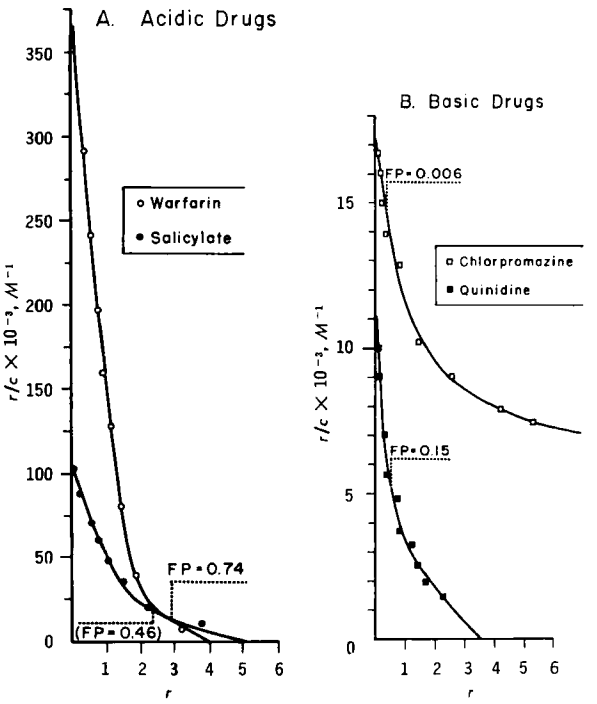


Figure 1—*Scatchard plots of four representative drugs illustrating the point (dotted line) at which the relative contributions of the primary and secondary sites to the binding are equal.* FP *is the calculated fraction of the protein sites occupied at the point of equal binding by primary and secondary sites.*

This operation confirms Eq. 7 and suggests the concept of partial fraction bound, which is the relative contribution of the independent classes of sites to the total bound fraction. Equation 11 and Goldstein's (1) Eq. 1 can be shown to be equivalent by process of identity when $i = 1$.

Determination and Interconversion of *r/c* Values from Specified Values of *r*—Frequently, it is necessary to determine equivalent total drug concentrations when comparing literature values or reproducing the work of others in the laboratory. The minimum data available in the literature usually consist of a Scatchard plot denoting a smoothed fit to experimental data (r values) and the binding parameters n and K for a constant protein level of P. The key here is to derive an expression relating the total drug to the desired r value. The free drug concentration, c, is very crucial for the comparison and, unfortunately, it cannot be obtained directly from the plot.

A solution to the problem of obtaining the total drug concentration for a given r value and the binding constants restricted to a two classes-of-sites system will now be given[17]. The following is obtained from the analytical geometry of the Scatchard plot for such a system:

$$r/c = \frac{y_1 + y_2 \pm \sqrt{(y_1 - y_2)^2 + 4a_1a_2}}{2} \qquad \text{(Eq. 13)}$$

where:

$$y_1 = a_1 - rK_1 \qquad (a_1 = n_1K_1) \qquad \text{(Eq. 14)}$$

$$y_2 = a_2 - rK_2 \qquad (a_2 = n_2K_2) \qquad \text{(Eq. 15)}$$

Equation 13 allows for the calculation of an exact r/c value from n_1, n_2, K_1, and K_2 for any given r value. The latter is reasonably specified from the plot. Consequently, the free drug concentration, c, can now be obtained:

$$c = \frac{r}{r/c} \qquad \text{(Eq. 16)}$$

[17]The derivation is involved and will not be given here. This approach is applicable to three or more classes of sites. The derivation is available from the authors on request.

Table II—Comparison of Fraction Bound Observed for Albumin Level of .3 g % with Fraction Bound Calculated from Constants Obtained for Binding of Salicylate to Albumin at 0.3-g % Level

(a) Total Drug $\times 10^5$ M	(b) FB Observed	(c) FB Calculated	(d) Percent Difference, $\frac{c-b}{b} \times 100$
2.39	0.970	0.977	0.78
10.22	0.966	0.974	0.88
25.57	0.956	0.976	1.17
40.00	0.953	0.958	0.50
59.70	0.931	0.940	0.97
102.56	0.891	0.893	0.22
184.39	0.728	0.785	7.77
		Average	1.75

Since $r = B/P$ or $B = rP$ and by definition $T = B + c$:

$$T = rP + c \qquad \text{(Eq. 17)}$$

Substituting this value for T in Eq. 9:

$$FB = \frac{rP}{rP + c} \qquad \text{(Eq. 18)}$$

The preceding relationships and their combinations permit a complete evaluation of most binding data from the literature and allow for satisfactory comparative use of such information as will be shown.

RESULTS AND DISCUSSION

Figure 1 shows the binding data in the form of Scatchard plots for the four drugs studied. The two-site computer resolution gives rise to the binding parameters for the acidic and basic drugs shown in Table I.

Quinidine has been reported (5) to bind to a single class of sites with $n = 1$ and $K = 7700$. Two classes-of-sites resolution of the data (Table I) results in considerably different values for fraction bound when calculated from parameters in this study using the concentrations employed by them. Comparison values are derived through the use of Eqs. 2 and 16–18. For their lowest concentration of free quinidine (3.0×10^{-6} M), these workers found the fraction bound to be 0.62 compared to the value of 0.74 in this study. The highest concentration they employed (2.2×10^{-3} M) resulted in 0.09 fraction bound compared to this study's value of 0.27. This disparity may reflect the variability of equilibrium dialysis as previously cited in the study of salicylate binding (3).

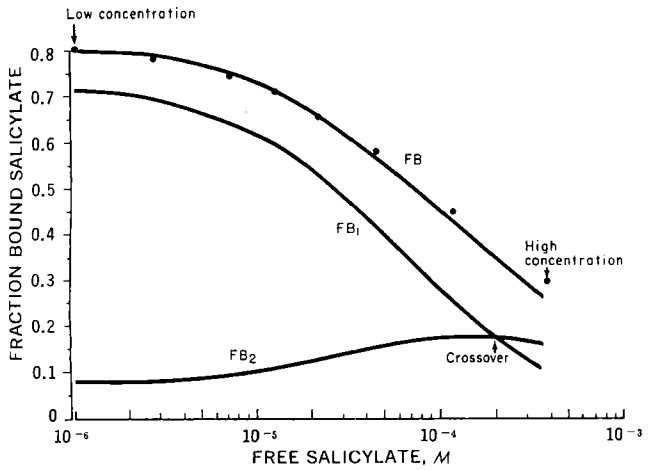


Figure 2—*Contribution of the partial fraction bound due to the primary and secondary sites,* FB_1 *and* FB_2, *to the total fraction of drug bound,* FB. *The curves were derived from the binding parameters obtained by computerized smoothing of the original binding data (3). Values for the curves were calculated with the aid of the computer program referred to in the text. The experimentally observed values for fraction bound are shown as points.*

Table III—Partial Fraction Bound Profiles for Selected Drugs

Drug Parameter	Salicylate	Warfarin	Chlorpromazine	Quinidine
Low concentration ($\times 10^5$ M)	0.2	1.6	0.4	1.6
FB[a]	0.79	0.92	0.42	0.74
Percent FR_1[b]	87	95	56	72
Crossover ($\times 10^5$ M)	27.7	37.5	3.7	24.0
FB	0.41	0.31	0.38	0.59
Percent FR_1	50	50	50	50
High concentration ($\times 10^5$ M)	50.0	60.0	100.0	235.0
FB	0.30	0.21	0.24	0.27
Percent FR_1	40	45	11	16

[a] FB = fraction bound. [b] Percent $FR_1 = (FB_1/FB) \times 100$, the percent contribution of the primary sites to the total binding.

The fraction bound–total drug interconversion program (Scheme I) was used to test the value of experimental binding constants in predicting binding information for protein levels other than those used to determine the constants (13). The binding constants from the 0.3 g % ($P = 4.21 \times 10^{-5}$ M) albumin study for salicylate binding (Table I) were employed in the computer to determine the total drug concentrations for a given range of fraction bound at the 3.0 g % level of albumin. Salicylate samples were then prepared at concentrations dictated by the computer, and the fraction bound was determined from these by gel filtration as described (Table II, columns a and b). The constants from this study are listed in Table I for $P = 42.8 \times 10^{-5}$ M. The same computer program was then used to verify the equivalence of the data from the two studies. This was done by computing the fraction bound for the total drug levels used in the 3.0 g % albumin study, but based on the constants determined in the 0.3 g % study (Table II, column c). The calculated values deviated by less than 2% from the observed fraction bound for the entire range of drug concentrations studied (Table II, column d). The large deviation reflects the poorer fit of the smoothed curve at very high concentrations.

The variation of the binding profiles of the four drugs is illustrated through the concept of partial fraction bound. Figure 2 illustrates the profile for the binding of salicylate in terms of fraction bound *versus* free drug concentration. The total binding is also resolved into the binding due to the primary and secondary sites using Eq. 12. The points of interest are the values for FB, FB_1, and FB_2 at the low, crossover, and high concentrations indicated in the figure. The crossover point is that concentration where the binding by the primary and secondary classes of sites is equal. It is calculated by a modification of the computer program (Scheme I) for which the conditional statement is changed to read: $FB - 2FB_1 = 0$. Results for the four drugs tested are shown in Table III. It can be seen that the point at which the binding is evenly accounted for by the primary and secondary sites varies considerably with the fraction bound for the various drugs.

Figure 1 shows that this point also occurs with considerable variation between drugs in respect to the molar ratio of bound drugs, r; the value is as small as 0.35 for chlorpromazine and as large as 3.0 for warfarin. This is also reflected in the variation of the fraction of protein sites occupied (FP) at the crossover point for the four drugs in Fig. 2. The basic drug chlorpromazine represents an extreme in this case; the crossover point occurs when only 0.6% of the albumin sites are occupied. Literature data on chlorpromazine are not available for comparison. It is also clear from Fig. 2 and Table III that the secondary sites are responsible for the majority of binding at higher therapeutic levels of the other drugs as well as for salicylate. The smoothed curve (Fig. 2) is calculated with the aid of Eq. 12. The extent to which this curve agrees with the actual data points attests to the suitability of the two-site model for salicylate.

An example of the use of the interconversion formula (Eq. 13) is

the comparison of this warfarin binding study with the study of O'Reilly (17). The data for warfarin in Table I were modified to make them conform to the conditions of temperature and protein concentration employed by O'Reilly. The association constants for warfarin at 27° were calculated from thermodynamic data using the van't Hoff equation; the revised values are K_1 = 238,100 and K_2 = 5200. Equations 13–18 were then used to calculate the value for r/c and FB from the binding parameters corresponding to the r values found by O'Reilly at the albumin concentration he used ($P = 2.32 \times 10^{-5}$ M). For the lowest concentration in his study on warfarin (r = 0.40 and r/c = 178,800), he obtained a value for fraction bound of 0.793. Corresponding values from the present study are r = 0.40, r/c = 259,800, and FB = 0.858. The highest concentration he used (r = 1.90 and r/c = 41,000) resulted in a fraction bound of 0.454. The values in this study are r = 1.90, r/c = 47,100, and FB = 0.522. The values for fraction bound averaged 8.0% higher than those he obtained for the entire concentration range studied. The thermodynamic data used to convert the warfarin binding constants of Table III to 27° resulted in a value for the free energy of binding of −7.29 kcal/mole. This compares favorably with the value O'Reilly obtained of −7.37 kcal/mole.

CONCLUSION

As binding data become more and more reliable due to improved techniques, it becomes apparent that multiple-class binding between serum albumin and various drugs is becoming the rule rather than the exception. This is well illustrated by the four examples presented in this paper. The two classes-of-sites resolution of the binding data provided the impetus to calculate the total fraction bound drug in terms of separate contributions by the two classes of sites. Such analysis revealed that the contributions of the secondary sites to total binding becomes significant at high drug concentrations in all four cases studied.

The computer program described here allows the calculation of protein bound drug at any total drug and protein concentration when the binding parameters are known. The usefulness of this program is illustrated in the case of salicylate, where the contributions of the two classes of sites are calculated at total therapeutic drug levels and physiological protein concentration.

Additional equations derived in this paper facilitate the interconversion of the various types of binding data for comparison and evaluation, as illustrated with warfarin and quinidine.

The concept of partial fraction bound, the computer programs, and the various equations reported here fulfill a need in the interpretation and communication of protein–drug binding information.

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