*Biochemical Pharmacology*, Vol. 34, No. 11, pp. 1999–2005, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

# SPECIFIC AND NON-SPECIFIC LIGAND BINDING TO SERUM ALBUMIN

ZENEI TAIRA* and HIROSHI TERADA†‡

*Faculty of Pharmaceutical Sciences, Tokushima University of Arts and Science, Yamashiro-cho, Tokushima 770, Japan; and †Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi-1, Tokushima 770, Japan

(*Received* 14 *March* 1984; *accepted* 13 *August* 1984)

**Abstract**—The applicability of a model of specific and non-specific binding sites for the binding of organic ligands to serum albumin was examined statistically. It is proposed in this model that there are a limited number of a single class of high affinity sites and an unlimited number of low affinity sites. Ligands bind to the former sites independently, as in the Scatchard model for a single class of binding sites, and to the latter sites in a partition-like manner, the amounts bound increasing linearly with the concentration of free ligands. In this study, data on the binding of warfarin to human serum albumin reported by Wilting *et al.* [J. Wilting *et al.*, *J. biol. Chem.* **255**, 3032 (1980)] and on the bindings of indomethacin and salicylic acid to human serum albumin reported by Hultmark *et al.* [D. Hultmark *et al.*, *Acta pharm. suecica* **12**, 259 (1975)] were used. These binding data were analyzed according to the model of specific and non-specific sites as well as the Scatchard model of two classes of binding sites. Statistical analyses showed that the model of specific and non-specific binding sites fitted the data for binding of all compounds very well, indicating the applicability of this model. An index of the upper limit of ligand concentrations necessary for accurate analysis of the binding data was also discussed.

The interaction of drugs and endogenous small organic molecules, such as fatty acids and bile acids, with serum albumin has been studied extensively, because serum albumin acts as a carrier of these compounds in the blood, and their biological potencies and stabilities depend on their affinities for serum albumin [1–3]. Since serum albumin binds many kinds of compounds, its interactions with these compounds have been regarded as typical examples of non-specific interactions. However, the number of the primary binding sites on albumin for most ligands is very small, being far less than 10 per mole of albumin in the absence of cooperative binding [1–3]. Furthermore, ligands of different chemical classes bind to geometrically different sites of albumin: binding to a certain site requires specific chemical and conformational features [3–5]. Since these results indicate the existence of specific binding sites, characterization of the binding sites has been attempted [3, 4].

Most binding data have been analyzed according to the Scatchard model [6], assuming that binding sites of a similar nature are of the same class and interact with ligands independently without any mutual interaction either when they are free or are occupied by ligands. However, Scatchard plots, where the ratio of the degree of binding to the free ligand concentration, $R/F$, is plotted against the degree of binding $R$, sometimes show that the $R/F$ value is constant in the high $R$ region, in contrast to the Scatchard model, where a definite number of binding sites is assumed and thus the curve of binding should cross the $R$-axis. Namely, increase of $R$ takes place continuously with increase in $F$, though in the Scatchard model $R$ should reach a definite value in the high $F$ region. Thus, another model should be considered.

From the above results, it is reasonable to assume that, besides binding to a definite number of specific sites, ligands also interact nonspecifically with the hydrophobic surface of serum albumin. This idea is supported by the fact that the affinities of a wide variety of ligands are directly dependent on their hydrophobicities irrespective of their chemical structures [7–9]. In this case, the number of binding sites is very great, and thus the binding of ligands to these sites increases linearly with the equilibrium concentration of the free ligand $F$, as if the ligand were partitioned between an organic solvent and water.

In this study, we analyzed binding data based on a model of two types of ligand binding sites: specific and non-specific sites, as described above. This type of model has been proposed for ligand interaction with heterogeneous biopolymers, especially for the interaction of hormones with their receptors [10, 11]. However, it does not seem to have been considered for the interactions of small organic molecules with serum albumin. No detailed examinations have thus far been carried out on such a model, although in some cases the existence of non-specific binding sites on albumin has been suggested [12, 13]. Examination of the applicability of this model is useful for clarifying the mechanism of binding of ligands to serum albumin. In this paper, an index of the upper limit of binding data for accurate analysis of binding results is also discussed.

## BINDING MODEL AND ANALYSIS OF BINDING DATA

Let us suppose that a ligand binds to serum albumin in two ways, by specific and non-specific binding.

‡ To whom correspondence should be addressed.

A ligand binds to specific sites (S sites) according to the law of mass action as in the Scatchard model with a single set of binding sites, and to non-specific sites (NS sites) as in the partition between an organic solvent and water, i.e. specific binding is characterized by a high affinity and a small binding capacity, whereas non-specific binding is characterized by a low affinity and an unlimited binding capacity for the ligand. There is no cooperativity in either case. Thus, the moles of ligand bound per mole of serum albumin, $R$, is the sum of the moles of ligand bound to these two kinds of sites ($R_s$ and $R_{ns}$) as shown in equation 1.

$$R = R_s + R_{ns}. \quad (1)$$

The binding to S sites obeys the law of mass action, in which the number of binding sites ($N_s$) and the association constant ($K_s$) govern the binding, as shown in equation 2.

$$R_s = N_s K_s F/(1 + K_s F) \quad (2)$$

where $F$ is the concentration of free ligand at equilibrium.

The binding to NS sites is partition-like. Thus the ligand binding, $R_{ns}$, is linearly dependent on $F$ as expressed by equation 3.

$$R_{ns} = K_{ns} F \quad (3)$$

where $K_{ns}$ is the association constant of the binding to NS sites, which is similar to a partition coefficient.

The total amounts of a ligand bound per mole of serum albumin, $R$, is expressed by equation 4.

$$R = N_s K_s F/(1 + K_s F) + K_{ns} F. \quad (4)$$

This model is referred to as the "specific and non-specific binding model (S and NS model)". A plot of $R$ against $F$ according to equation 4 yields Fig. 1A, in which $R$ was computed with values of $N_s$, $K_s$ and $K_{ns}$ of 1, $10 \times 10^5$ and $0.5 \times 10^5$, respectively, as an example.

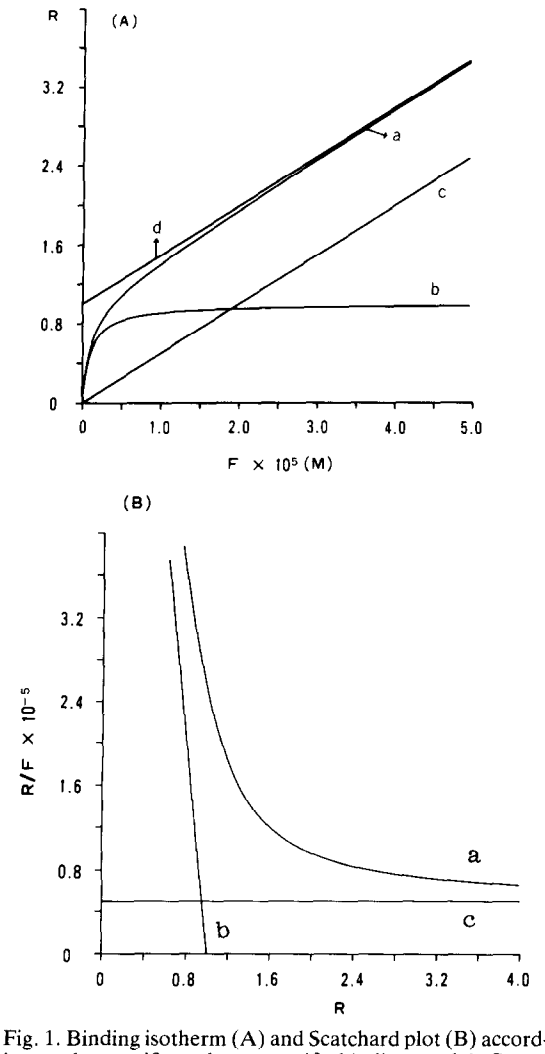


Fig. 1. Binding isotherm (A) and Scatchard plot (B) according to the specific and non-specific binding model. $R$ represents the moles of ligand bound per mole of serum albumin, and $F$, the concentration of free ligand at equilibrium. The model binding system with $N_s = 1.0$, $K_s = 10 \times 10^5$ and $K_{ns} = 0.5 \times 10^5$ was used to depict the curves. Key: curve (a) total binding; (b) specific binding; (c) non-specific binding; and (d) limiting slope of curve (a).

In Fig. 1A, curve (a) represents the dependence of the total amount of bound $R$ on $F$, and curve (d) is the limiting line of curve (a) at the extreme of $F$ with a slope of $5.0 \times 10^4$ and with an intercept on the Y-axis of 1.0, corresponding to the value of $N_s$. The specific binding ($R_s$) shown by curve (b) reaches a saturation value of $R$ at higher $F$ values, which corresponds to $N_s$ (= 1). Curve (c) represents the non-specific binding, being the straight line for $R_{ns} = 5.0 \times 10^4 F$. It is noteworthy that, in curve (a), $R$ increases first rapidly, and then linearly with increase in $F$, and $R$ does not attain a saturation level, which should be equal to the total number of binding sites. This type of curve has sometimes been observed for the bindings of organic compounds to serum albumin, even when the binding was measured over a very wide range of $F$ [14–16]. This is the reason why the binding data satisfy the Freundlich adsorption isotherm [17, 18].

A Scatchard plot of these binding data is shown in Fig. 1B, indicating that the relation between $R/F$ and $R$ for the total binding is downward convex. This relationship can be expressed by equation 5 [19].

$$R/F = N_s K_s - K_s R_s + K_{ns} \quad (5)$$

The first two terms on the right-hand side of equation (5) represent the binding to S sites, while the last term represents that to NS sites. Thus, curve (a) in Fig. 1B is composed of two straight lines for specific and non-specific interactions [curves (b) and (c) in Fig. 1B respectively]. The Scatchard plot for the specific binding gives a straight line with the slope $-K_s$ and an intercept on the abscissa of $R = N_s$, and that of the non-specific binding gives the horizontal straight line of $R/F = K_{ns}$. The Scatchard plot with regard to total binding [curve (a) in Fig. 1B] gives a constant value at higher $R$ values, since only non-specific binding occurs at these values. A similar horizontal part has often been observed in the Scatchard plot for ligand binding to serum albumin in the high $R$ region [20–24].

In the present study we analyzed binding data according to the $S$ and $NS$ model and compared the results statistically with those analyzed by the

Scatchard model based on the presence of two sets of independent "specific" binding sites 1 and 2. When the numbers of binding sites and association constants of these sites are denoted as $N_1$ and $N_2$, and $K_1$ and $K_2$, respectively, this relation is expressed by equation 6.

$$R = N_1K_1F/(1 + K_1F) + N_2K_2F/(1 + K_2F). \quad (6)$$

The binding data were analyzed by the unbiased least-squares parameter-fitting method reported by Perrin *et al.* [25] with a Panafacom U1400. The results were evaluated statistically by determining the correlation coefficient, $r$, and the standard deviation, S.D., and by Student's $t$-test ($t$-test).

## RESULTS

*Availability of the specific and non-specific binding model.* To determine the availability of the specific and non-specific binding model (S and NS model), we analyzed the reported data on the bindings of some drugs to albumin, by the least-squares parameter-fitting method according to the S and NS model and the Scatchard model, in which two classes of binding sites were assumed. The data used were those on binding to human serum albumin of warfarin at pH 6.1 and 25°, reported by Wilting *et al.* [26], and of salicylic acid and indomethacin at pH 7.4 and 20°, reported by Hultmark *et al.* [27], since these binding data were obtained under rigorous conditions and were easily readable from the literature.

Figure 2A illustrates the relation of $R$ and $F$ for the binding of warfarin based on the S and NS model. The results of this analysis, in which total binding $R$ [curve (a), with curve (d) showing its limiting slope] is considered to consist of specific [curve (b)] and non-specific [curve (c)] binding, coincides well with the original binding data (circles). Figure 2B shows results in the lower $F$ region on an enlarged scale. The values of $N_s$, $K_s$ and $K_{ns}$ were determined as 1.28, $3.17 \times 10^5$ and $2.12 \times 10^4$ respectively. The triangles and squares in Fig. 2 indicate binding data calculated from these binding parameters. Results with indomethacin and salicylic acid are shown in Figs. 3 and 4 respectively.

The binding parameters of these drugs with human serum albumin calculated according to the S and NS model and the Scatchard model are summarized in Table 1. The numbers of data points used in the analyses, the correlation coefficients and the standard deviations from the regressions are shown in Table 1 under n, $r$, and S.D. respectively. The figures in parentheses are values for the 95% confidence intervals.

Table 1 shows that the regressions of analyses of binding data for the three drugs based on the S and NS model are very good with large $r$ and small S.D. values. The small values of the 95% confidence intervals also indicate that the data points for the analyzed binding relations fall in very narrow ranges.

All the results with the Scatchard model also show good correlations, except that with salicylic acid. With salicylic acid, the binding appears to conform to a model consisting of only one class of binding sites, because $K_2$ was computed to be null with great fluctuation, although the Scatchard plot is curvilinear (cf. Fig. 8). Thus, the binding does not conform to the present Scatchard model. The values of S.D. for warfarin and indomethacin are smaller according to the Scatchard model than according to the S and NS model, suggesting better correlations with the Scatchard model. However, this is because there are more binding parameters (= 4) in this model than in the S and NS model (= 3). It is noteworthy that the values of the 95% confidence intervals with the S and NS model are in general smaller than those with the Scatchard model, suggesting better correlations with the former model. Thus, the above results clearly indicate the participation of non-specific

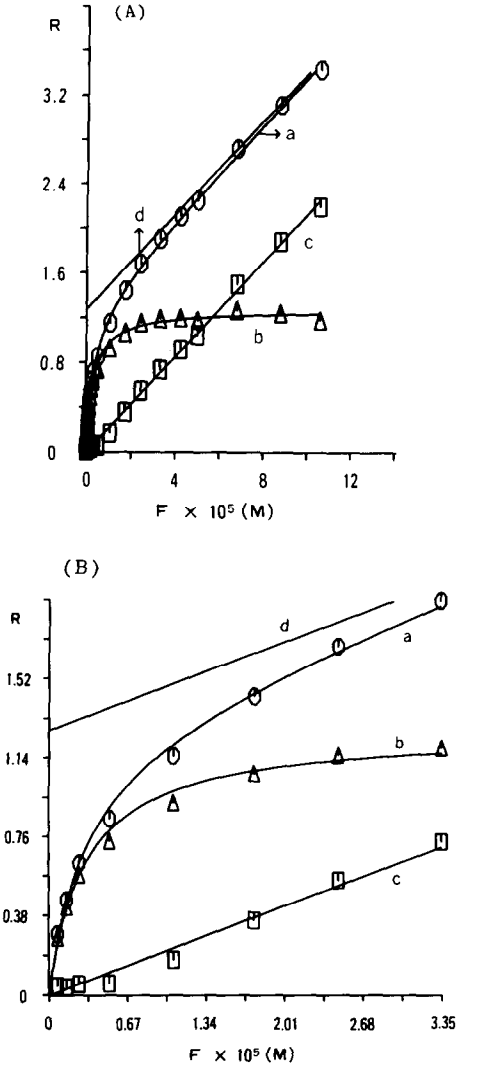


Fig. 2. Binding isotherm of warfarin to human serum albumin at pH 6.1 and 25°, according to the specific and non-specific binding model. Original binding data were taken from the paper of Wilting *et al.* [26]. (A) all data points for binding. (B) Binding in a smaller $F$ region. Circles represent data points. Triangles and squares represent computed values of specific and non-specific binding, respectively, based on the relation showed in Table 1 for the binding of warfarin. Key: curve (a) total binding; (b) specific binding; (c) non-specific binding; and (d) limiting slope of curve (a).

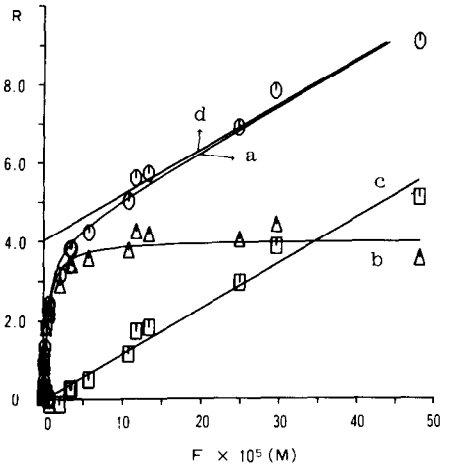


Fig. 3. Binding isotherm of indomethacin to human serum albumin at pH 7.4 and 20°, according to the specific and non-specific binding model. Original binding data were taken from the paper of Hultmark *et al.* [27]. Curves (a) to (d) and symbols are as described in the legend of Fig. 2.

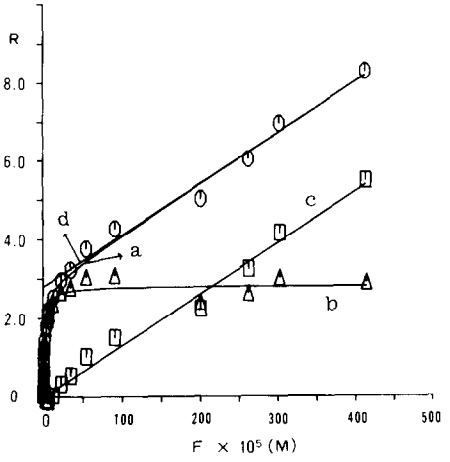


Fig. 4. Binding isotherm of salicylic acid to human serum albumin at pH 7.4 and 20°, according to the specific and non-specific binding model. Original binding data were taken from the paper of Hultmark *et al.* [27]. Curves (a) to (d) and symbols are as described in the legend of Fig. 2.

interaction in the binding of organic compounds with serum albumin.

*Index of binding data for accurate analysis.* Next, to determine how the values of binding parameters changed with the number of binding data, we carried out least-squares analyses based on the two binding models by omitting binding data successively from either the lowest or the highest regions of $F$. The results with warfarin are summarized in Table 2. As can be seen from the values of the 95% confidence intervals of binding parameters with all the binding data (n = 21) for warfarin, the values of $N_s$, $K_s$ and $K_{ns}$ in the S and NS model were in the ranges of 1.22 to 1.34 (i.e. 1.28 ± 0.06), $2.73 \times 10^5$ to $3.61 \times 10^5$ and $2.01 \times 10^4$ to $2.23 \times 10^4$, respectively, and those of $N_1$, $K_1$, $N_2$ and $K_2$ in the Scatchard model were in the ranges of 0.95 to 1.17, $3.54 \times 10^5$ to $5.00 \times 10^5$, 7.03 to 14.23 and $0.15 \times 10^4$ to $0.39 \times 10^4$ respect-

Table 1. Parameters for the bindings of drugs with human serum albumin according to the specific and non-specific binding model and the Scatchard model*

| | Binding parameter | | | | Specific and non-specific model | | |
|---|---|---|---|---|---|---|---|
| | | | | | Scatchard model | | |
| | $N_s$ | $K_s \times 10^{-5}$ ($M^{-1}$) | | $K_{ns} \times 10^{-4}$ ($M^{-1}$) | | | |
| Drug | $N_1$ | $K_1 \times 10^{-5}$ ($M^{-1}$) | $N_2$ | $K_2 \times 10^{-4}$ ($M^{-1}$) | n | $r$ | S.D. |
| Warfarin | 1.28 (0.06) | 3.17 (0.44) | | 2.12 (0.11) | 21 | 1.000 | 0.032 |
| | 1.06 (0.11) | 4.27 (0.73) | 10.63 (3.60) | 0.27 (0.12) | 21 | 1.000 | 0.022 |
| Indomethacin | 4.04 (0.36) | 2.12 (0.68) | | 1.15 (0.15) | 20 | 0.996 | 0.252 |
| | 3.10 (0.32) | 3.90 (1.14) | 11.96 (2.30) | 0.21 (0.08) | 20 | 0.999 | 0.134 |
| Salicylic acid | 2.82 (0.23) | 0.60 (0.20) | | 0.13 (0.02) | 20 | 0.997 | 0.191 |
| | 2.82 (2.49) | 0.60 (2.09) | 20.36 (∞) | 0.00 (13.34) | 20 | 0.799 | 2.043 |

* Binding data for warfarin at pH 6.1 and 25° reported by Wilting *et al.* [26], and for indomethacin and salicyclic acid at pH 7.4 and 20° reported by Hultmark *et al.* [27], were analyzed. In the table n is the number of data points, $r$ is the correlation coefficient and S.D. is the standard deviation. The values in the parentheses are those of 95% confidence intervals.

Table 2. Dependence of the binding parameters in the specific and non-specific binding model and the Scatchard model on the number of data for the binding of warfarin to human serum albumin used*

| | Specific and non-specific binding model | | | Scatchard model | | | | |
|---|---|---|---|---|---|---|---|---|
| n | $N_s$ | $K_s \times 10^{-5}$ ($M^{-1}$) | $K_{ns} \times 10^{-4}$ ($M^{-1}$) | $N_1$ | $K_1 \times 10^{-5}$ ($M^{-1}$) | $N_2$ | $K_2 \times 10^{-4}$ ($M^{-1}$) | $F \times 10^5$ (M) |
| 21 | 1.22–1.34 | 2.73–3.61 | 2.01–2.23 | 0.95–1.17 | 3.54–5.00 | 7.03–14.23 | 0.15–0.39 | 0.01–10.68 |
| 16 | 1.29 | 3.02 | 2.10 | 1.09 | 4.01 | 11.41 | 0.25 | 0.08–10.68 |
| 14 | 1.29 | 2.89 | 2.09 | 1.10 | 3.85 | 11.94 | 0.23 | 0.15–10.68 |
| 11 | 1.35 | 2.48 | 2.05 | 1.15 | 3.44 | 13.24 | 0.20 | 0.32–10.68 |
| 20 | 1.23 | 3.40 | 2.22 | 1.07 | 4.22 | 11.31 | 0.25 | 0.01–8.86 |
| 18 | 1.19 | 3.60 | 2.31 | 0.81 | 6.01 | 3.51 | 0.14 | 0.01–4.99 |
| 16 | 1.04 | 4.43 | 2.84 | 0.83 | 5.90 | 3.69 | 1.30 | 0.01–3.35 |

* The binding data for warfarin reported by Wilting *et al.* [26] were used. In the table, n is the number of binding data points. Analyses were carried out with binding data points in the range of the free concentration of warfarin at equlibrium $F$ shown in the table, by omitting original data points (n = 21) successively from the lowest or the highest region of $F$ to obtain the numbers of data points n shown in the table. The values with n = 21 are those of the original binding parameters within the range of 95% confidence intervals.

ively. These values are referred to as the original binding parameters in further analyses.

The results in Table 2 show that even deletion of 10 binding data (n = 11) from the region of lowest data points affected both binding models only slightly, whereas deletion of 3 and 5 data points from the highest region of $F$ (n = 18 and 16 respectively) had significant effects on the values of all the binding parameters, resulting in their deviation from the values of the original binding parameters in both models. Similar tendencies were observed with data on the binding of indomethacin and salicylic acid. The results with salicylic acid are shown in Fig. 5. Thus, binding data in the high $F$ region are important for accurate determination of binding parameters, irrespective of the binding model.

However, in binding experiments, determination of $R$ in the high $F$ region is generally difficult. Thus, it is useful to know the upper limit for measurement of $R$ in the region of high $F$ (or $R$) for exact analysis of binding. Let us refer to this $R$ value as $R_m$. Figures 6–8 show Scatchard plots of the bindings of warfarin, indomethacin and salicylic acid in the high $R$ region. Numbers beside data points in Figs. 6–8 are numbers of binding data numbered from the lowest to the highest $R$. Let us denote the lowest number of data points from which computed binding parameters fall either closely or completely within the range of 95% confidence intervals of the original binding parameters as $n_m$. Namely, $n_m$ is the minimum number of binding data points from the lowest $R$ for accurate computation. With the S and NS model, the value of $n_m$ was found to be 19 for warfarin, 19 for indomethacin, and 17 for salicylic acid. With the Scatchard model, $n_m$ was 19 for warfarin and 18 for indomethacin. The results in Figs. 6–8 indicate that almost all the data points with numbers of more than $n_m$ are located in the region above the $R$ value denoted as $R_c$. $R_c$ corresponds to the point on the total binding curve crossed by a line from the origin passing through the crossing point of the two straight lines representing the downward convex total binding curve in the Scatchard plot of both binding models (cf. Figs. 6–8). Thus, binding data $R$ should be determined up to at least $R_c (R_m \geq R_c)$ for accurate analysis of binding results.

## DISCUSSION

In this study we examined the availability of the S and NS model for the bindings of the drugs warfarin, indomethacin and salicylic acid to human serum albumin by statistical analysis of published binding data. In their original paper, Wilting *et al.* [26] determined the binding parameters of warfarin according to a Scatchard model consisting of two independent sites by fixing $N_1 : N_1 = 1$, $K_1 = 4.8 \times 10^5$, $N_2 = 10$, and

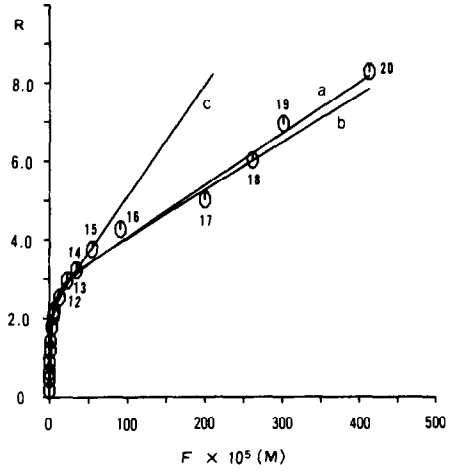


Fig. 5. Effect of the number of data points for the binding of salicylic acid to human serum albumin on values for binding parameters according to the specific and non-specific binding model. Numbers beside data points represent numbers of binding data points numbered in order from the lowest $F$ (or $R$) value. Key: curve (a) analysis of all the data points (numbers 1–20); (b) 18 data points (numbers 1–18); and (c) 15 data points (numbers 1–15).

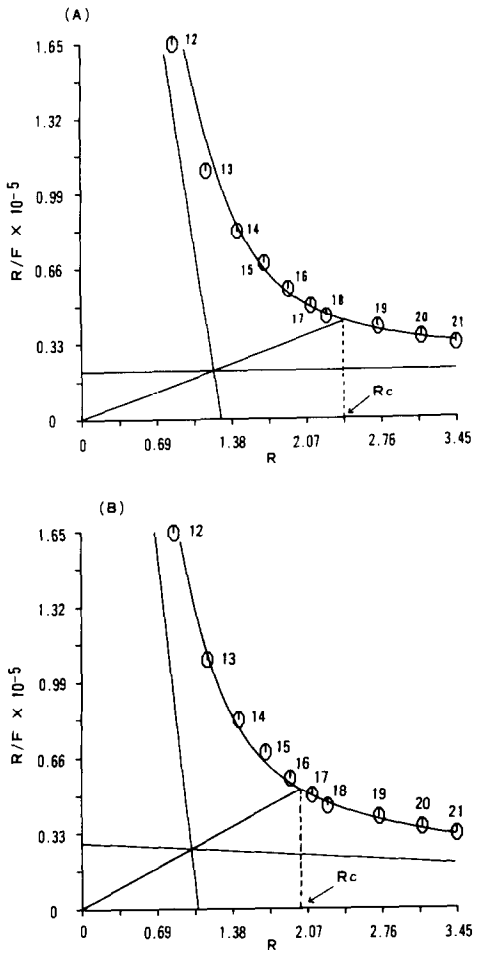


Fig. 6. Scatchard plot of the binding of warfarin to human serum albumin in the higher $R$ region according to the specific and non-specific binding model (A) and the Scatchard model (B). $R_c$ represents the $R$ value at the intersection with the curve of a line from the origin through the crossing point of the two straight lines composing the total binding curve. Numbers beside data points are as in Fig. 5.

$K_2 = 0.2 \times 10^4$ [26]. Our results for a Scatchard model are very similar to theirs (cf. Table 1). However, they showed that a model with three independent classes of binding sites also fitted the data points reasonably well. Hultmark *et al.* [27] analyzed the binding data for indomethacin and salicylic acid, according to a modification of the stepwise model [28] and found that these drugs had four or five strong binding sites as well as some weak ones [27]. Several parameters were necessary for analyses of the data points for these drugs.

In the present study, we showed statistically that an S and NS model with only three parameters accommodated the binding data very well in all cases, while a Scatchard model with four parameters fitted the binding data for warfarin and indomethacin equally well, but fitted those for salicylic acid very poorly. Use of fewer parameters is better in statistical analyses, if it results in the same level of statistical significance. Thus, the S and NS model should be examined first for evaluation of the binding data of organic ligands with serum albumin.

The S and NS model examined in this study consisted of a limited number of a single class of high affinity sites and an unlimited number of low affinity sites. The existence of two or more classes of high affinity sites is also a possibility that requires examination. The present study is the first to demonstrate the availability of this model from the statistical point of view, although the existence of non-specific binding has been suggested [12, 13].

Warfarin, indomethacin and salicyclic acid exist in both neutral and anionic forms in the binding system, the relative amounts of these forms depending on the pH of the solution. In partition studies, we found that 2,4-dinitrophenol and indomethacin are transferred from an aqueous to an organic phase in three ways: directly in the neutral and anionic forms, and via an ion-pair complex of the anionic form with counter ions in solution [29, 30]. The extent of trans-

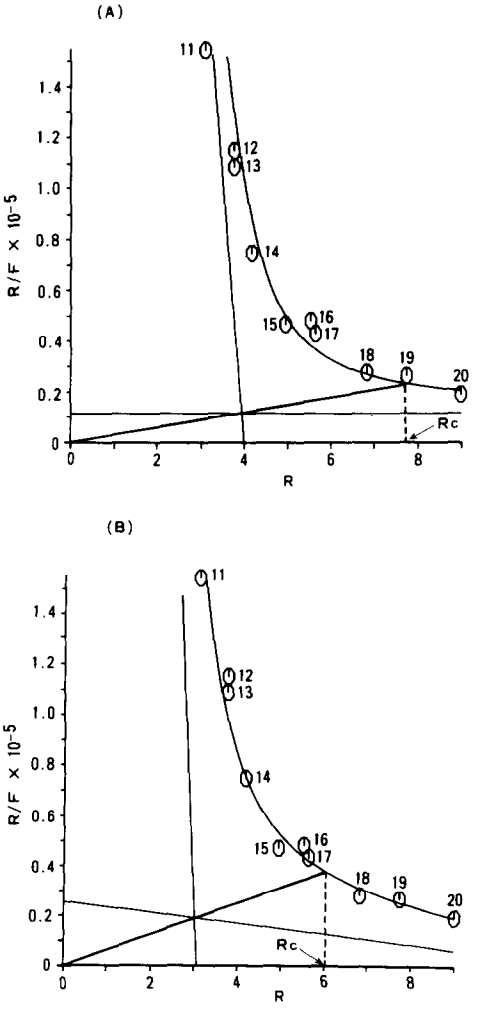


Fig. 7. Scatchard plot of the binding of indomethacin to human serum albumin in the higher $R$ region according to the specific and non-specific binding model (A) and the Scatchard model (B). Numbers beside data points are as described in the legend of Fig. 5.

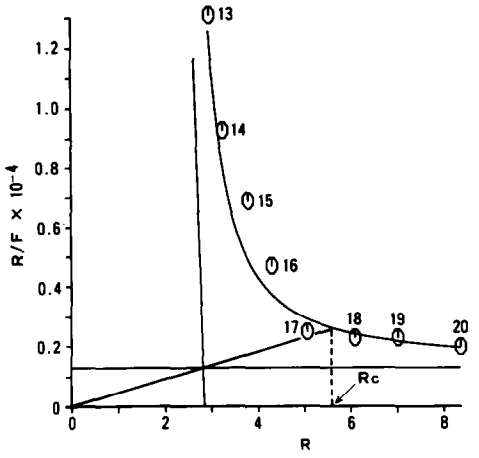


Fig. 8. Scatchard plot of the binding of salicylic acid to human serum albumin according to the specific and non-specific binding model. Numbers beside data points are as in Fig. 5.

fer in these three ways depends on the hydrophobicity of the compounds, and also on the relative amounts of these molecular species in solution. Transfer of the anionic species via the ion-pair complex was, however, always greater than the direct transfer of the anionic species itself [29, 30]. The dependence of the binding ability on the hydrophobicity of organic ligands has been studied extensively, especially with respect to quantitative structure–activity relationships [31, 32].

In view of these results, it is likely that either the neutral species binds directly to specific sites or that the anionic species binds to these sites in the form of an ion-pair with cationic residues of serum albumin. In addition, another species binds to non-specific sites. These sites may be hydrophobic regions at the surface of serum albumin, with which the species partitions as with an organic phase. A study of the mechanisms of specific and non-specific binding is under way.

In this work, we also showed that data points of $R$ in the high $F$ region are very important. As an index of the limiting value of $R$ in the high $F$ region, we showed experimentally that data points of $R$ up to a value of $R_c$ at least were necessary in both binding models. $R_c$ corresponds to the point on the total binding curve intersected by a line from the origin passing through the crossing point of the two straight lines composing the total binding curve in the Scatchard plot. Namely, the lowest possible value of $R$ ($= R_m$) should be about $R_c$ or more. This value could be a useful index in examining the availability of the binding model and the reliability of values of binding parameters. It is noteworthy that at least 5 data points per parameter are necessary in regression analysis [33]. Theoretical consideration of the value of $R_m$ is in progress.

## REFERENCES


1. A. A. Spector, *J. Lipid Res.* **16**, 165 (1975).
2. J. J. Vallner, *J. pharm. Sci.* **66**, 447 (1977).
3. U. Kragh-Hansen, *Pharmac. Rev.* **33**, 17 (1981).
4. K. J. Fehske, U. Schlafer, U. Wollert and W. E. Muller, *Molec. Pharmac.* **21**, 387 (1982).
5. R. Brodersen, T. Sjodin and I. Sjoholm, *J. biol. Chem.* **252**, 5067 (1977).
6. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
7. C. Hansch, K. Kiehs and G. Lawrence, *J. Am. chem. Soc.* **87**, 5770 (1965).
8. F. Helmer, K. Kiehs and C. Hansch, *Biochemistry* **7**, 2858 (1968).
9. J. M. Vandenbelt, C. Hansch and C. Church, *J. med. Chem.* **15**, 787 (1972).
10. H. G. Weder, J. Schildknecht, R. A. Lutz and P. Kesselring, *Eur. J. Biochem.* **42**, 475 (1974).
11. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
12. J. G. Brand and T. Y. Toribara, *Archs Biochem. Biophys.* **167**, 91 (1975).
13. P. Zini, P. D'Athis, J. Barre and J. P. Tillement, *Biochem. Pharmac.* **28**, 2661 (1979).
14. M. Nakagaki and H. Tereda, *J. pharm. Soc. Japan.* **87**, 817 (1967).
15. U. Kragh-Hansen and J. V. Moller, *Biochim. biophys. Acta* **336**, 30 (1974).
16. C. Monot, P. Netter, M. C. Stalars, J. Martin, R. J. Royer and A. Gaucher, *J. pharm. Sci.* **72**, 35 (1983).
17. V. F. W. Garn and K. H. Kimbel, *Arzneimittel-Forsch.* **11**, 701 (1961).
18. W. Scholtan, *Antibiot. Chemother.* **14**, 53 (1968).
19. H. E. Rosenthal, *Analyt. Biochem.* **20**, 525 (1967).
20. F. Karush, *J. Am. chem. Soc.* **76**, 5536 (1954).
21. D. S. Goodman, *J. Am. chem. Soc.* **80**, 3887 (1958).
22. N. Nakano, T. Oshio, Y. Fujimoto and T. Amiya, *J. pharm. Sci.* **67**, 1005 (1978).
23. H. Meisner and K. Neet, *Molec. Pharmac.* **14**, 337 (1978).
24. H. Meisner, *Molec. Pharmac.* **18**, 224 (1980).
25. J. H. Perrin, J. J. Vallner and S. Wold, *Biochim. biophys. Acta* **371**, 482 (1974).
26. J. Wilting, W. F. Van der Giesen, L. H. M. Janssen, M. M. Weideman, M. Otagiri and J. H. Perrin, *J. biol. Chem.* **255**, 3032 (1980).
27. D. Hultmark, K. O. Borg, R. Elofsson and L. Palmer, *Acta pharm. suecica* **12**, 259 (1975).
28. J. E. Fletcher, A. A. Spector and J. D. Ashbrook, *Biochemistry* **9**, 4580 (1978).
29. H. Terada, K. Kitagawa, Y. Yoshikawa and F. Kametani, *Chem. pharm. Bull., Tokyo* **29**, 7 (1981).
30. T. Inagi, T. Muramatsu, N. Nagai and H. Terada, *Chem. pharm. Bull., Tokyo* **29**, 2330 (1981).
31. A. E. Bird and A. C. Marshall, *Biochem. Pharmac.* **16**, 2275 (1967).
32. C. Hansch and W. J. Dunn, III, *J. pharm. Sci.* **61**, 1 (1972).
33. J. G. Topliss and R. J. Costello, *J. med. Chem.* **15**, 1066 (1972).