

of a 5-ng sample. Retained material was eluted from the column with 0.01 *N* hydrochloric acid. Under identical conditions, the column composed of coupled bovine serum albumin alone retained less than 1% of the radioactive material. Essentially identical results were obtained when ³H-lysergide was added initially to the columns in pooled human plasma or human urine rather than buffer. Columns appeared to be equally effective upon at least one reuse after washing thoroughly with 0.01 *N* hydrochloric acid and then buffer.

Upon further quantitation and development, and perhaps with the use of a more specific antibody of higher titer, the described techniques might be utilized to advantage in conjunction with quantitative radioimmunoassay determinations of lysergide, either to isolate selectively very small quantities of lysergide far below the current sensitivity capabilities or to separate lysergide from tissue homogenates which may contain materials that render present assay methods inexact. Such methodology may be of importance in the study of lysergide levels in the brain and other organs.

(1) H. Van Vunakis, J. T. Farrow, H. B. Gjika, and L. Levine, *Proc. Nat. Acad. Sci. USA*, **68**, 1483(1971).

(2) A. Taunton-Rigby, S. E. Sher, and P. R. Kelley, *Science*, **181**, 165(1973).

(3) L. J. Loeffler and J. V. Pierce, *J. Pharm. Sci.*, **62**, 1817(1973).

(4) P. Cuatrecasas and C. P. Anfinsen, in "Methods in Enzymology," vol. XXII, W. B. Jacoby, Ed., Academic, New York, N.Y., 1971, pp. 345-378.

(5) L. J. Loeffler and J. V. Pierce, *Biochim. Biophys. Acta*, **317**, 20(1973).

Larry J. Loeffler *

Carla J. Hinds

Division of Medicinal Chemistry
School of Pharmacy
University of North Carolina
Chapel Hill, NC 27514

Received July 11, 1975.

Accepted for publication August 4, 1975.

* To whom inquiries should be directed.

Determination of Protein Binding Parameters in Systems Involving Interaction between Sites

Keyphrases □ Protein binding—determination of parameters in systems involving interaction between sites, evaluation of method □ Binding, protein—parameters, interaction between sites, evaluation of method □ 1-Anilino-naphthalene-8-sulfonate—discussion of binding parameters

To the Editor:

A recent report by Ma *et al.* (1) utilized a log *Q* versus \bar{v} plot to determine the protein binding parameters of 1-anilino-naphthalene-8-sulfonate in systems where interactions between binding sites were assumed to be occurring. As a result of this study, it is our feeling that the basic concepts underlying such

systems and the application of the log *Q* versus \bar{v} plot (2) to them should be reviewed. The intent of this communication is to ensure the proper use of the log *Q* versus \bar{v} treatment by investigators encountering binding behavior similar to that reported by Ma *et al.* (1) for the interaction of 1-anilino-naphthalene-8-sulfonate with serum albumin.

The log *Q* versus \bar{v} method applies to systems involving interactions between binding sites when *n* (number of) binding sites are initially all equivalent insofar as the binding of a drug molecule is concerned. As soon as one such molecule is bound, however, the affinity constant for a subsequent molecule's binding to a neighboring site is altered. The binding of successive molecules further alters the affinity constant for a remaining unoccupied site. Thus, in the case of *n* sites, the intrinsic binding constants K_1, K_2, \dots, K_n are not equal but may progressively increase or decrease. To calculate affinity constants under such conditions, a function *Q* is used:

$$Q = \bar{v}/(n - \bar{v})[D] \quad (\text{Eq. 1})$$

where \bar{v} is the number of moles of drug bound per mole of protein at a free drug concentration [*D*], with *n* representing the number of binding sites possible (2). A plot of log *Q* versus \bar{v} is made, and the limiting values at [*D*] → 0 and [*D*] → ∞ give K_1 and K_n , respectively. Also, the limiting slopes, $d \ln Q/d\bar{v}$, at $\bar{v} \rightarrow 0$ and $\bar{v} \rightarrow n$ give $(n - 1)(K_2 - K_1)/nK_1$ and $(n - 1)(K_n - K_{n-1})/nK_{n-1}$, respectively (2). The log *Q* versus \bar{v} plot is generally appropriate when *K* values are close (ratio of largest to smallest much less than 1000).

In using the log *Q* versus \bar{v} plot, it should be noted that Eq. 1 implies that the value of *n* must be known or at least inferred with confidence in order to make a determination of the intrinsic binding constants. Ma *et al.* (1) suggested that *n* can be determined as that assumed value that best provides a linear log *Q* versus \bar{v} plot. This usage is not proper, because the semilogarithmic plot need not approach linearity even when the value for *n* is known. As indicated by Eq. 1, the relationship between log *Q* and \bar{v} is strictly linear only when the *n* binding constants are independent and equivalent. In such a situation, *Q* obviously is independent of free drug concentration and, indeed, is equal to the intrinsic binding constant. Thus, with independent and equivalent sites, a plot of log *Q* versus \bar{v} is linear with a slope of zero.

When the binding sites are not independent, *i.e.*, when there is enhanced or inhibited binding of a small molecule to an available site, two situations may arise. In one case, the ratio of intrinsic binding constants, $K_{(s+1)}/K_{(s)}$, is a constant independent of *s* for all values of *s* from 1 to *n* (2). The constant ratio may be greater or less than unity, depending on whether interactions result in facilitated or inhibited binding. In the other system, the ratio of binding constants, $K_{(s+1)}/K_{(s)}$, is not constant for all values of *s* from 1 to *n*. Both systems will be considered.

In the first case, where a constant ratio of binding constants is present, a log *Q* versus \bar{v} plot may have a region of linearity but the plot would not be expected

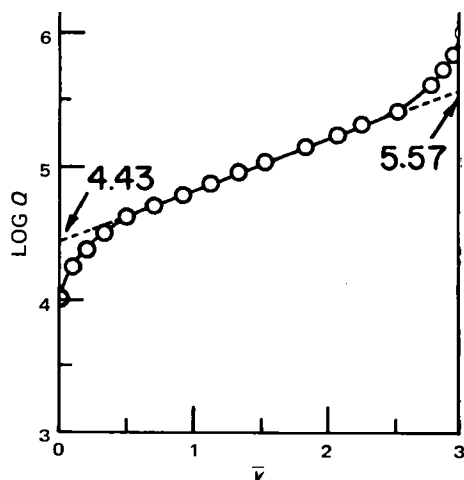


Figure 1—Relationship between $\log Q$ and \bar{v} for the binding of a hypothetical drug where $n = 3$ with increasing affinity constants of $K_1 = 10^4$, $K_2 = 10^5$, and $K_3 = 10^6$.

to be linear over the entire \bar{v} range. As discussed by Edsall *et al.* (2) and as depicted by the hypothetical data in Fig. 1, Eq. 1 does not predict a linear semilogarithmic relationship.

The data in Fig. 1 were generated by assuming three dependent sites where a constant ratio of binding constants of 10 occurs. The constants used were $K_1 = 10^4$, $K_2 = 10^5$, and $K_3 = 10^6$. Figure 1 does show, however, that linearity may be found over a large range of \bar{v} (0.5–2.5 in this hypothetical case). It is evident that, in accordance with Eq. 1, curvature in a $\log Q$ versus \bar{v} plot should not be used to indicate that an erroneous selection of the n value was made. When linearity is found over a large range of \bar{v} values, the interpretation should be that the linearity is a consequence of the unique and constant ratio among the intrinsic binding constants.

In a system where the ratio of affinity constants, $K_{(s+1)}/K_{(s)}$, is not constant for all values of s from 1 to n , curvature and even breaks in a $\log Q$ versus \bar{v} plot are to be expected, depending on the relationship and magnitude of the differences between the intrinsic binding constants. As an example, Fig. 2 shows the relationship between $\log Q$ and \bar{v} for three hypothetical systems. In these systems, $n = 3$ and, as opposed to Fig. 1, inhibited binding is assumed upon successive site occupation. For curve B of Fig. 2, a constant ratio of affinity constants was used ($K_1 = 10^6$, $K_2 = 10^5$, and $K_3 = 10^4$). This curve is included only for comparison. In curve A, where the assumed constants were $K_1 = 10^6$, $K_2 = 5 \times 10^5$, and $K_3 = 10^4$, the ratio K_2/K_1 is 25 times that of K_3/K_2 . An expected marked curvature is found in this case. In curve C, where $K_1 = 10^6$, $K_2 = 5 \times 10^4$, and $K_3 = 10^4$, the ratio K_3/K_2 is four times larger than the ratio K_2/K_1 . Deviations from linearity are apparent in this hypothetical system also, but it might be interpreted as approximately linear for the \bar{v} region between 1 and 2.

In each curve in Fig. 2, nonlinearity is again a consequence of the relationship between the intrinsic binding constants and not solely dependent on the value for n . Thus, the use of the $\log Q$ versus \bar{v} treat-

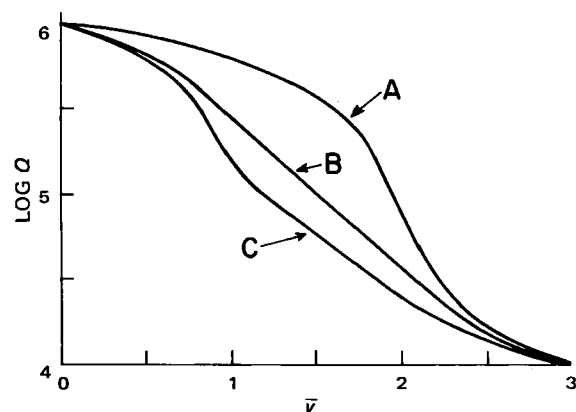


Figure 2—Relationship between $\log Q$ and \bar{v} for the binding of hypothetical drugs where $n = 3$ in all cases. The affinity constants used were: in A, $K_1 = 10^6$, $K_2 = 5 \times 10^5$, and $K_3 = 10^4$; in B, $K_1 = 10^6$, $K_2 = 10^5$, and $K_3 = 10^4$; and in C, $K_1 = 10^6$, $K_2 = 5 \times 10^4$, and $K_3 = 10^4$.

ment of binding data depends on knowing or reasonably estimating the number of binding sites. Figure 2 demonstrates that linearity in a $\log Q$ versus \bar{v} plot should not be used to determine n as was suggested by Ma *et al.* (1).

One other consideration should be pointed out with respect to analyzing a system where a constant ratio of binding constants is found. It is not correct to extrapolate the linear portion of a $\log Q$ versus \bar{v} plot to $\bar{v} = 0$ and $\bar{v} = n$, where $[D] \rightarrow 0$ and $[D] \rightarrow \infty$, to determine K_1 and K_n , respectively, as was done by Ma *et al.* (1). At best, such extrapolated values should be used as gross first estimates of the binding constants. Significant errors in affinity constants may be introduced by simply extrapolating the linear region of the relationship as opposed to extrapolating limiting data. Theory dictates that the extrapolated limiting data determine K_1 and K_n .

To use the method properly, data collected in these extremes must be accurate. Figure 1 illustrates the inaccuracy introduced when an extrapolation of the linear portion of the $\log Q$ versus \bar{v} plot is used to determine K_1 and K_n . Hypothetical data are used in this figure where the ratios K_2/K_1 and K_3/K_2 are equal. The experimental data collected in regions where limiting slopes and intercepts are characterized are subject to large experimental error, regardless of whether or not a constant ratio of affinity constants is operative. Therefore, Edsall and Wyman (3) recommended that extrapolated values using limiting data be used as first estimates of affinity constants which may be used to calculate an expected curve. If the calculated curve significantly differs from the observed, new affinity constants are estimated and the curve fitting is continued until reliable constants are found.

Protein binding systems analogous to those of 1-anilinonaphthalene-8-sulfonate are certainly possible, and it may be proper to interpret and analyze them by assuming that interactions of the type invoked by Ma *et al.* (1) do take place. It is our hope that this communication will clarify the factors that make such an analysis correct and complete.

(1) J. K. H. Ma, H. W. Jun, and L. A. Luzzi, *J. Pharm. Sci.*, **62**, 2038(1973).

(2) J. T. Edsall, G. Felsenfeld, D. S. Goodman, and F. R. N. Gurd, *J. Amer. Chem. Soc.*, **76**, 3054(1954).

(3) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic, New York, N.Y., 1958, p. 633.

Theodore D. Sokoloski *
Betty-ann Hoener

Division of Pharmaceutics and
Pharmaceutical Chemistry
College of Pharmacy
Ohio State University
Columbus, OH 43210

Received February 26, 1974.

Accepted for publication April 14, 1975.

* To whom inquiries should be directed.

Group Contribution Data Obtained by Ion-Pair Extraction of Prostaglandin B₂ with Aliphatic Amines

Keyphrases □ Ion-pair extraction—prostaglandin B₂ with aliphatic amines, methylene group contribution, partition coefficients □ Partition coefficients—ion-pair extraction of prostaglandin B₂ with aliphatic amines, methylene group contribution □ Prostaglandin B₂—ion-pair extraction with aliphatic amines, methylene group contribution, partition coefficients

To the Editor:

The concept of ion-pairs (1) is significant in various areas including chemistry, organic synthesis (2), solvolytic reactions (3), partition chromatography (4–6), pharmaceutical analysis (7–10), and facilitated absorption of quaternary ammonium compounds in the GI lumen (11). Also, systematic studies on the ion-pair extraction successfully yielded functional group contributions to the thermodynamic parameters involved in transferring a given molecule from one phase to another (12, 13). Although our original study (14) was initiated to explore the practical utility of ion-pair formation of prostaglandins in all of these aspects, the present communication is specifically concerned with the free energy of transferring a methylene group from water to chloroform as determined by the ion-pair extraction process.

The importance of the lipophilicity of a drug molecule in the structure-activity relationship has been recognized and, consequently, great effort has been directed toward developing some means by which the partition coefficient of a drug molecule between oil (or lipophilic biological membrane) and aqueous systems can be predicted accurately (13, 15–17). Presently, the most popular way to achieve this goal appears to be the group contribution approach, in which the partition coefficient of a molecule is considered as the sum of the contribution of each functional group present in the molecule. Experimentally, an accurate measurement of the partition coefficient becomes very difficult as the value becomes either too large or too small.

Since the apparent partition coefficient of acidic or basic compounds depends not only upon the pH of the aqueous layer but also upon the type and the concentration of the counterions, by adjusting these factors it is possible to control the partition coefficient so that it is experimentally measurable. For a given charged solute, if the structural changes in a series of counterions are far removed from the electrostatically interacting charged portions of the molecules, it can be assumed that the change in the partition coefficient observed is independent of the electrostatic interaction and that the change solely reflects the structural modification of the counterions.

We determined the ion-pair extraction coefficient (K) of prostaglandin B₂ (I) with 10 simple aliphatic amines ranging from *n*-propyl- to *n*-dodecylamine at 25°:

$$K = \frac{[A^-BH^+]}{[A^-][BH^+]} \quad (\text{Eq. 1})$$

The partition system consisted of chloroform presaturated with water and tromethamine buffer (pH 8 and ionic strength 0.1 *M*) also presaturated with chloroform. Throughout the paper, prostaglandin B₂, amines, and ion-pair are represented as AH, B, and A⁻BH⁺, respectively. The braces {} and brackets [] indicate the molar concentrations of a given species in chloroform and aqueous buffer, respectively. Since, in most cases, the system is very dilute with respect to prostaglandin B₂ and amines, the molar concentrations are assumed to be equal to thermodynamic activities.

Simple algebraic manipulation of Eq. 1 yields:

$$P_{\text{obs}} = K[BH^+] + P_{\text{app}} \quad (\text{Eq. 2})$$

where:

$$P_{\text{obs}} = \frac{\{A^-BH^+\} + \{AH\}}{[A^-] + [AH]} \quad (\text{Eq. 3})$$

and:

$$P_{\text{app}} = \frac{\{AH\}}{[AH] + [A^-]} \quad (\text{Eq. 4})$$

In deriving Eq. 2, it was assumed that ion-pairs do not exist in aqueous solutions, not only because the dielectric constant of water is extremely high but also because the concentrations of prostaglandin B₂ and amines in aqueous solutions are low. The dissociation constant of ion-pairs in an organic solvent of low dielectric constant is usually in the order of from 10⁻⁴ to 10⁻⁷ *M* (12, 18). Therefore, in our study, it was assumed that no significant dissociation takes place in chloroform. Moreover, at pH 8 the concentrations of neutral species of our acid and bases (*i.e.*, AH and B) are in the order of 0.1% of the total concentrations, because the pK_a values of prostaglandin B₂ and

