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ANALYSIS OF LIGAND BINDING CURVES IN TERMS OF SPECIES FRACTIONS

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The ligand binding curve for a macromolecular system presents the average number of ligand molecules bound per macromolecule as a function of the chemical potential or the logarithm of the ligand concentration. We show that various observable properties of this curve, for example its asymptotes and derivatives, are expressible in terms of linear combinations of the mole fractions α_i of macromolecules binding i molecules of ligand. Whenever enough such properties of the binding curve are known, the linear equations in α_i can be solved to give the mole fractions of each of the various macromolecular species. An application of these results is that a Hill plot for hemoglobin-ligand equilibrium where the asymptotes approach unit slope can be made to yield the four Adair constants by a simple algebraic method. A second use is that a knowledge of the first and second derivatives of the binding curve at points along the curve can yield the species fractions as functions of the degree of saturation without direct knowledge of the ligand binding constants. These methods are illustrated by some numerical examples.

1. Introduction

A ligand binding curve embodies the complex functional relation between the average number of ligand molecules bound to a macromolecule and the chemical potential (or more commonly the logarithm of the concentration) of the free ligand. The shape of the curve and its dependence on temperature, pressure, and concentrations of components, contain the basic thermodynamic information describing the binding reaction [1]. Underlying the general thermodynamic properties of the binding curve is the set of complex reactions that occur as binding takes place. When the binding macromolecule undergoes association or dissociation which depends on its state of ligation, the situation is particularly complex. Levitski and Schessinger [2] and Colosimo et al. [3], who describe this case as "polysteric linkage", have shown such aggregation effects essentially to require computer-calculated descriptions.

However, when the macromolecule neither associates nor disassociates during ligation, Wyman [1,4,5] points out that the binding curve can be simply described in terms of a binding potential proportional to the logarithm of a polynomial in the activity of the ligand – the so called binding polynomial – of which the leading (constant) term is unity; and Schellman [6] has shown how this binding polynomial is simply related to the free energy of binding. The degree of the polynomial is equal to the number of ligand binding sites contained in the macromolecule [‡1] and the various coefficients are known as the Adair constants. A knowledge of these constants as a function of the various physical and chemical variables which determine the state of the system provides a complete macroscopic description of the behavior of the macromolecule in relation to the ligand in question. In this paper

[‡1] This is true whether or not the constants are dependent on one another, as they are when the sites are all alike and independent, or when the binding curve is symmetrical or in the case of an allosteric system.

we describe a practical method for determining these constants based on the possibility of expressing various observable features of the binding curve, such as its first and higher derivatives or the asymptotes of the corresponding Hill plot, in terms of linear combinations of the mole fractions of the several ligated forms.

2. Theory

Consider a macromolecule M which contains t sites for ligand X. The stepwise ligand-binding reactions for adding ligand X to a species MX_{i-1} containing $i-1$ ligands are

$$MX_{i-1} + X \rightarrow MX_i, \quad \text{where } i = 1 \text{ to } t. \tag{1}$$

The mole fraction of the species containing i ligands, which we denote by α_i, is defined in terms of the concentrations $[MX_i]$ of species MX_i ($i = 0$ to t) as

$$\alpha_i \equiv [MX_i] / \sum [MX_i]$$

(summation $\sum$ is from $i = 0$ to t) (2)

The equilibrium constants for the reactions given by eq. (1) are expressible in these terms as

$$K_i = [MX_i] / [MX_{i-1}][X], \tag{3}$$

or in terms of mole fractions, as

$$K_i = \alpha_i / \alpha_{i-1} x, \tag{4}$$

where $[X] = x$ is the free ligand activity (concentration). Eq. (4) may be arranged as a linear equation in α_i and α_{i-1}

$$(K_i x)\, \alpha_{i-1} - \alpha_i = 0. \tag{5}$$

We can follow the same procedure for the overall reaction

$$M + iX \rightarrow MX_i \quad (i = 1 \text{ to } t), \tag{6}$$

in terms of its equilibrium constant β_i:

$$\beta_i = \alpha_i / \alpha_0 x^i \quad (i = 0 \text{ to } t). \tag{7}$$

Here we have defined β_i and the stepwise K_i's as

$$\beta_i = K_1 K_2 \ldots K_i. \tag{8}$$

Eq. (7) can also be arranged in linear form

$$(\beta_i x^i)\, \alpha_0 - \alpha_i = 0. \tag{9}$$

There are t equations of this form that define the interrelationships of fractional concentrations as binding takes place.

The set of t linear equations from either eq. (5) or (9) involve $t + 1$ unknown fractions $\alpha_0, \ldots, \alpha_t$. Another independent equation must be used if the α_i's are to be solved. The simplest is the condition that the sum of mole fractions must equal unity:

$$\sum \alpha_i = 1. \tag{10}$$

Then from eq. (9) we obtain

$$\alpha_0 = 1 / \sum \beta_i x^i, \tag{11}$$

and

$$\alpha_i = \beta_i x^i / \sum \beta_i x^i. \tag{12}$$

Next consider how the fractions α_i are related to various properties that describe the shape of the binding curve. The average number $\bar{\nu}$ of ligands bound is given as

$$\bar{\nu} = \sum i \alpha_i, \tag{13}$$

and from eq. (9) and (11) we write

$$\bar{\nu} = \sum i \beta_i x^i / \sum \beta_i x^i. \tag{14}$$

This quantity may be identified as the first moment of the x's about zero and the identification can be extended to higher order as follows:

$$\overline{\nu^2} = \sum i^2 \alpha_i, \tag{15}$$

or

$$\overline{\nu^2} = \sum i^2 \beta_i x^i / \sum \beta_i x^i, \tag{16}$$

$$\overline{\nu^3} = \sum i^3 \alpha_i, \tag{17}$$

or

$$\overline{\nu^3} = \sum i^3 \beta_i x^i / \sum \beta_i x^i, \tag{18}$$

etc. These higher moments can themselves be related to derivatives of the binding curve ($\bar{\nu}$ versus ln x) by

taking the derivative of eq. (14) and multiplying by x; thus

$$x\frac{d\bar{\nu}}{dx} = \frac{\Sigma i^2\beta_i x^i}{\Sigma\beta_i x^i} - \left(\frac{\Sigma i\beta_i x^i}{\Sigma\beta_i x^i}\right)^2, \tag{19}$$

or with eqs. (14) and (16),

$$\frac{d\bar{\nu}}{d\ln x} = \overline{\nu^2} - (\bar{\nu})^2 = \overline{(\nu-\bar{\nu})^2}, \tag{20}$$

where $\overline{(\nu-\bar{\nu})^2}$ is the second moment of the ν's now taken about the mean value of ν. This result was given by Wyman [1] as a generalization of an earlier one due to Lindstrom-Lang. In the same way

$$\frac{d^2\bar{\nu}}{d(\ln x)^2} = \overline{\nu^3} - 3\bar{\nu}\,\overline{\nu^2} + 2(\bar{\nu})^3 = \overline{(\nu-\bar{\nu})^3} \tag{21}$$

$$\frac{d^3\bar{\nu}}{d(\ln x)^3} = \overline{\nu^4} - 4\bar{\nu}\,\overline{\nu^3} - 3(\overline{\nu^2})^2 + 12(\bar{\nu})^2\,\overline{\nu^2} - 6(\bar{\nu})^4$$

$$= \overline{(\nu-\bar{\nu})^4} - 3\overline{(\nu-\bar{\nu})^2}\;\overline{(\nu-\bar{\nu})^2} \tag{22}$$

$$\frac{d^4\bar{\nu}}{d(\ln x)^4} = \overline{(\nu-\bar{\nu})^5} - 10\overline{(\nu-\bar{\nu})^3}\;\overline{(\nu-\bar{\nu})^2} \tag{23}$$

and it is evident that further derivatives will likewise be expressible in terms of still higher moments either about zero or about $\bar{\nu}$ [‡2]. In fact from a knowledge of $\bar{\nu}$, $d\bar{\nu}/d(\ln x)$, ..., $d^r\bar{\nu}/d(\ln x)^r$ all such moments from 1 to $r+1$ can be obtained [‡3].

We finally consider an integral property of the binding curve. The median ligand concentration x_m [1] satisfies the integral equation

$$\int_0^t \ln(x/x_m)\,d\bar{\nu} = 0. \tag{24}$$

This equation follows from the equivalence of the areas defined (1) by the ordinate and the binding curve and (2) the ordinate and $\ln x_m$. The free energy of saturating the macromolecule is given by $tRT\ln x_m$. It is also equivalent to the standard state free energy change for the total binding reaction $t\,X + M \rightarrow MX_t$ given by $-RT\ln\beta_t$. Thus we obtain

$$\beta_t = (1/x_m)^t. \tag{25}$$

From the median property and the asymptotic behavior of the binding curve as observed in the Hill plot three linear equations may be obtained with the aid of eqs. (5) and (9):

$$K_1 x\alpha_0 - \alpha_1 = 0, \tag{26}$$

$$(x/x_m)^t\,\alpha_0 - \alpha_t = 0, \tag{27}$$

$$K_t x\alpha_{t-1} - \alpha_t = 0. \tag{28}$$

The distribution moments, along with the condition that the sum of fractions equals unity, give the additional set of linear equations:

$$\alpha_0 + \alpha_1 + \alpha_2 + \alpha_3 + \ldots + \alpha_t = 1, \tag{29}$$

$$\alpha_1 + 2\alpha_2 + 3\alpha_3 + \ldots + t\alpha_t = \bar{\nu}. \tag{30}$$

$$\alpha_1 + 2^2\alpha_2 + 3^2\alpha_3 + \ldots + t^2\alpha_t = \overline{\nu^2}, \tag{31}$$

$$\alpha_1 + 2^3\alpha_2 + 3^3\alpha_3 + \ldots + t^3\alpha_t = \overline{\nu^3}, \tag{32}$$

$$\vdots$$

$$\alpha_1 + 2^i\alpha_2 + 3^i\alpha_3 + \ldots + t^i\alpha_t = \overline{\nu^i}. \tag{33}$$

If the shape of the binding curve is symmetrical about its midpoint ($x = x_{50}$, $\bar{\nu} = t/2$) then additional linear equations also apply [‡4].

The well-known Hill plot [7] shows $\ln[\bar{\nu}/(t-\bar{\nu})]$ versus $\ln x$. The slope n of this plot at any point is

$$n \equiv \frac{d\ln[\bar{\nu}/(t-\bar{\nu})]}{d\ln x}, \tag{34}$$

which along with eq. (20) can be written as

$$n \equiv \frac{t}{\bar{\nu}(t-\bar{\nu})}\left[\overline{\nu^2} - (\bar{\nu})^2\right]. \tag{35}$$

By extension one sees that the higher derivatives of n will be related to the higher distribution moments.

[‡2] This follows from the fact that $d\overline{\nu^r}/d\ln x = \overline{\nu^{r+1}} - \overline{\nu^r}\,\bar{\nu}$.

[‡3] It will be noted that the first derivative, and consequently the second moment $\overline{(\nu-\bar{\nu})^2}$, may be interpreted as a generalized buffering power; thus all derivatives of this buffering power with respect to $\ln x$ can be expressed in terms of such higher moments.

[‡4] These equations, which result from the symmetry constraints $\beta_i/\beta_t^{i/t} = \beta_{t-i}/\beta_t^{(t-i)/t}$ given by Wyman [4] are the following: $x_m = x_{50} = 0$, $(x/x_{50})^t\alpha_0 - \alpha_t = 0$, $(x/x_{50})^{-1}\alpha_1 - \alpha_{t-1} = 0, \ldots\ldots, (x/x_{50})^{-i}\alpha_i - \alpha_{t-i} = 0$.

3. Applications to hemoglobin

In order to illustrate how use can be made of this formalism, we shall consider the case of hemoglobin with its four ligand binding sites.

Case I

First we examine the features of a Hill plot as shown in fig. 1. Such a plot is most often characterized by the asymptotes which determine K_1 and K_4, the Hill coefficient n_{50} at fifty percent saturation where $\bar{\nu}=2$, and the free ligand concentration or partial pressure at half saturation (x_{50}). The five linear equations in α_i's that contain this information are derived from eqs. (29), (30), (31), (35), (26) and (28).

$$\alpha_0 + \alpha_1 + \alpha_3 + \alpha_4 = 1$$

$$\alpha_1 + 2\alpha_2 + 3\alpha_3 + 4\alpha_4 = \bar{\nu} = 2$$

$$\alpha_1 + 4\alpha_2 + 9\alpha_3 + 16\alpha_4 = \overline{\nu^2} = n_{50} + 2$$

$$K_1 x_{50}\alpha_0 - \alpha_1 = 0$$

$$K_4 x_{50}\alpha_3 - \alpha_4 = 0. \qquad (36)$$

Solving for α_1, α_2, and α_3 then gives

$$\alpha_1 = \frac{(\frac{1}{2} + K_4 x_{50})n_{50}}{D},$$

$$\alpha_2 = \frac{(1+3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1)}{D} - \frac{(1+3/2K_1 x_{50} + \frac{3}{2} K_4 x_{50} + 2K_4/K_1)n_{50}}{D},$$

$$\alpha_3 = \frac{\frac{1}{2} + 1/K_1 x_{50}}{D}. \qquad (37)$$

where

$$D = 1+3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1.$$

We see immediately that since α_2 cannot be less than zero, only certain values of n_{50} are consistent with particular values of K_1, K_4, and x_{50}. The allowed values of n_{50} are [*5]

[*5] Thus in the limiting case where $K_4/K_1 \to \infty$, $n_{50} \leq 4$. Similarly in the limiting case $K_4/K_1 \to 0$, $n_{50} \leq 1$.

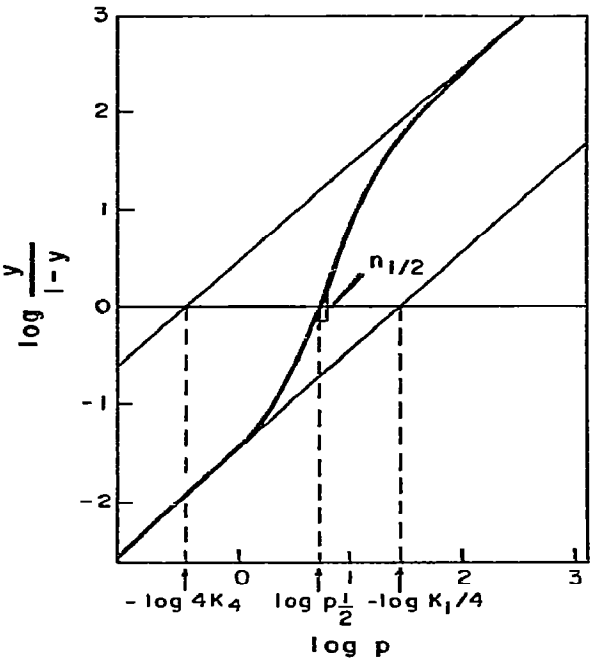

Fig. 1. Hill plot showing: (a) limiting asymptotes at low and high degrees of saturation for evaluation of K_1 and (b) defining position of slope $n_{1/2}$ at half saturation pressure $p_{1/2}$.

$$n_{50} > \frac{1+3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1}{1+3/2K_1 x_{50} + \frac{3}{2} K_4 x_{50} + 2K_4/K_1}. \qquad (38)$$

Since the values of α_1, α_2 and α_3 can be computed at x_{50} from the chosen properties of the Hill plot at x_{50}. K_2 and K_3 are given simply by

$$K_2 = \alpha_2/\alpha_1 x_{50},$$

$$K_2 = [(1+3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1) - (1+3/2K_1 x_{50} + \tfrac{3}{2} K_4 x_{50} + 2K_4/K_1)] \times [\tfrac{1}{2} + K_4 x_{50})n_{50} x_{50}]^{-1},$$

$$K_3 = \alpha_3/\alpha_2 x_{50}. \qquad (39)$$

$$K_3 = (\tfrac{1}{2} + 1/K_1 x_{50})/[(1+3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1) - (1+3/2K_1 x_{50} + \tfrac{3}{2} K_4 x_{50} + 2K_4/K_1)]\, x_{50}.$$

Table 1
Comparison of K_2 and K_3 values from Hill plot data with literature values [c]

Parameters	HbA (pH 7.4, 25°C) [a]			HbA (pH 6.96, 19°C) [b]			
	Literature	Hill plot		Literature	Hill plot		
n_{50}	(2.530)	2.6	2.5	(2.967)	3.0	3.0	2.9
x_{50}(mmHg O_2)	(1.929)	1.8	1.8	(9.158)	9.2	9.5	9.2
K_1(mmHg O_2)$^{-1}$	0.316	0.32	0.32	0.0493 ± 7%	0.047	0.047	0.047
K_2(mmHg O_2)$^{-1}$	0.443	(0.37)	(0.50)	0.0427 ± 7%	(0.037)	(0.035)	(0.063)
K_3(mmHg O_2)$^{-1}$	0.500	(0.81)	(0.60)	0.221 ± 78%	(0.27)	(0.25)	(0.16)
K_4(mmHg O_2)$^{-1}$	1.088	1.00	1.00	0.320 ± 21%	0.31	0.31	0.31

a) Tyuma et al. [10], note values were evaluated from reported intrinsic k_i values i.e. $K_1 = 4k_1$, $K_2 = \frac{3}{2}k_2$, $K_3 = \frac{2}{3}k_3$, $K_4 = \frac{1}{4}k_4$.
b) Roughton and Lyster [9].
c) The various data colums listed under "Hill plot" result from reading from the original Hill plots alternative values for one or more of the graph parameters. In any column calculated parameters are enclosed in parentheses.

These equations permit all four Adair constants for hemoglobin ligation to be evaluated directly from a Hill plot whenever K_1 and K_4 either are known beforehand or can be obtained by extrapolating the Hill plot to its unity slope asymptotes. Table 1 shows two examples from hemoglobin literature. In these cases x_{50}, n_{50}, K_1 and K_4 were obtained directly from Hill plots such as given by Tyuma et al. [8] and Roughton and Lyster [9]. We compare our calculated values of K_2 and K_3 with values for these constants obtained elsewhere by the more complex method of least-squares fitting of constants to the hemoglobin saturation isotherm.

The effect of taking different n_{50} values is shown to have a marked effect on values of K_2 and K_3, largely through the influence on the fraction α_2. This same effect is, of course, also noted in the error estimate for K_2 and K_3 given by Roughton and Lyster [9]. The influence of the choice of x_{50} is less marked.

It is clear that K_2 and K_3 as calculated by our technique agree well with values obtained from other sources. Thus this method is a useful and convenient way of obtaining a full set of Adair constants for hemoglobin-ligand systems directly from available Hill plots.

Case II

We shall consider that the median concentration x_m and the higher moments $\overline{\nu^2}$ and $\overline{\nu^3}$ are available from the integral and first and second derivatives of the binding curve. Then the set of five equations can be written as

$$\alpha_0 + \alpha_1 + \alpha_2 + \alpha_3 + \alpha_4 = 1$$

$$\alpha_1 + 2\alpha_2 + 3\alpha_3 + 4\alpha_4 = \bar{\nu}$$

$$\alpha_1 + 4\alpha_2 + 9\alpha_3 + 16\alpha_4 = \overline{\nu^2}$$

$$\alpha_1 + 8\alpha_2 + 27\alpha_3 + 64\alpha_4 = \overline{\nu^3}$$

$$Q\alpha_0 - \alpha_4 = 0 \qquad (40)$$

where $Q = (x/x_m)^4$. We solve for the various α_i's to obtain

$$\alpha_0 = \frac{1}{1-Q}M,$$

$$\alpha_1 = -\frac{(3+Q)M}{1-Q} + 3 - \tfrac{5}{2}\bar{\nu} + \tfrac{1}{2}\overline{\nu^2},$$

$$\alpha_2 = \frac{(3+3Q)M}{1-Q} - 3 + 4\bar{\nu} - \overline{\nu^2},$$

$$\alpha_3 = -\frac{(1+3Q)M}{1-Q} + 1 - \tfrac{3}{2}\bar{\nu} + \tfrac{1}{2}\overline{\nu^2},$$

$$\alpha_4 = \frac{QM}{1-Q}, \qquad (41)$$

where

$$M = (1 - \tfrac{11}{6}\bar{\nu} + \overline{\nu^2} - \tfrac{1}{6}\overline{\nu^3}).$$

A singularity occurs at $Q = 1$, i.e. $x = x_m$, but elsewhere the α_i's are determinable. This is due to the

Table 2
Mean disagreement between the fractions of hemoglobin species calculated from Adair equation and those determined without knowledge of binding constants by numerical analysis of the binding curve given by statistical constants [a]

Property	Species					No. Pts.	Method [b]
	M	MX	MX_2	MX_3	MX_4		
$\bar{\alpha}_i$ [c]	0.295	0.147	0.115	0.147	0.295	10 or 20	Direct
σ_i [d]	±0.002	±0.006	±0.007	±0.006	±0.002	20	BC
σ_i	±0.000	±0.000	±0.000	±0.000	±0.000	20	HP
σ_i	±0.017	±0.055	±0.080	±0.055	±0.016	20	BCN
σ_i	±0.011	±0.025	±0.028	±0.025	±0.011	10	BC

[a] ($K_1 = 4, K_2 = \frac{3}{2}, K_3 = \frac{2}{3}, K_4 = \frac{1}{4}$) [b] See text. [c] Average value of the fraction of the indicated species over the interval $\bar{\nu} = 0$ to $\bar{\nu} = 4$. [d] Standard derivation of species fractions determined from binding curve about analytical values. Results averaged over the interval $\bar{\nu} = 0$ to $\bar{\nu} = 4$.

fact that when $Q = 1$, $\alpha_0 = \alpha_4$ with the result that the determinant of the last four equations (in $\alpha_1, \alpha_2, \alpha_3, \alpha_4$) vanishes.

To explore these equations further simulated binding curves were generated from published equilibrium constants [10]. A set of points equally spaced along the abscissa from 1 to 99% saturation were taken to represent the binding curve. The evaluation of $\overline{\nu^2}$ and $\overline{\nu^3}$ required numerical approximation of first and second derivatives at points along the curve. The approximate derivatives were obtained by treating the data points either directly in the form of the binding curve (BC method) or by transforming the points to the form of the Hill plot and taking numerical derivatives of the transformed data (HP method). The α_i's were evaluated for the given set of data points from the set of equations (41). At each point comparison of the estimated α_i values was made with the directly calculated α_i values (eq. (12). The standard deviation σ_i of the estimated α_i values from the directly calculated α_i values were determined for the data set. The average value $\bar{\alpha}_i$ for the data set was also determined. The results expressed in terms of $\bar{\alpha}_i$ and σ_i for some illustrative cases are presented in tables 2 and 3.

We first examined the non-cooperative or statistical case. Increasing the number of points in the data set from ten to twenty results in a significant improvement as seen by the smaller σ_i values for the BC method. To examine the effect of noise on the data, we introduced random errors in the $\bar{\nu}$ values of the data set such that the standard deviation of the noisy data was 1% of the average $\bar{\nu}$ of the data set. The noisy data

Table 3
Mean disagreement between the fractions of hemoglobin species calculated from Adair equation and those determined without knowledge of binding constants, by numerical analysis of the binding curve: stripped hemoglobin A. [a]

Property	Species					No. Pts.	Method [b]
	M	MX	MX_2	MX_3	MX_4		
$\bar{\alpha}_i$ [c]	0.417	0.109	0.035	0.071	0.369	20 or 40	Direct
σ_i [d]	±0.017	±0.072	±0.110	±0.070	±0.017	20	BC
σ_i	±0.004	±0.022	±0.055	±0.052	±0.016	40	BC
σ_i	±0.002	±0.010	±0.018	±0.010	±0.001	20	HP
σ_i	±0.001	±0.004	±0.009	±0.008	±0.003	40	HP

[a] Tyuma et al [8], $K_1 = 0.456, K_2 = 0.248, K_3 = 0.780, K_4 = 1.01$. [b] See text. [c] Average value of the fraction of the indicated species over the interval $\bar{\nu} = 0$ to $\bar{\nu} = 4$. [d] Standard derivation of species fractions determined from binding curve about analytical values. Results averaged over the interval $\bar{\nu} = 0$ to $\bar{\nu} = 4$.

was then examined by the BC method (BCN) and as seen in table 2 the σ_i values were greatly increased. A comparison of σ_i values with α_i shows that the intermediate species (MX, MX_2, MX_3) are most difficult to evaluate. The use of the HP method leads to perfect recovery of α_i's. This is to be expected since the data for the statistical case when transformed to a Hill plot fall on a straight line and the derivatives then are precisely evaluated.

Next we considered a typical cooperative case as given for stripped hemoglobin A. The results are shown in table 3. The errors in the recovered values of α_i are much larger than for the statistical case. This is presumably due to the much smaller $\bar{\alpha}_i$'s. In fact for the MX_2 species the standard deviation values exceeded the average fraction even when 40 points were used and examined by the BC method. Calculation using the Hill plot transform gives substantial improvement. This reflects the fact that the Hill plot has much less curvature over a wide degree of saturation than does the normal binding curve.

In general the shape of the binding curve involves substantial contribution from higher derivatives with respect to $\ln x$ and numerical differentiation will lead to accurate values of ν^2 and ν^3 only if closely spaced points are taken. These examples underscore the fact that both extremely accurate data and closely spaced observation is needed to estimate α_i values for highly cooperative cases by moment calculations.

The conceptual importance of these equations is that they permit the fractions of species at any point along the binding curve to be calculated using only the *local behavior* of the curve plus the integral of the entire curve. That is, detailed knowledge of the curve at distant points is not required. On the other hand as the examples below make clear, *very* precise knowledge of binding curve shape is necessary if this technique is to be put to practical use.

4. Conclusion

In the examples we have shown, taken from hemoglobin, the number of sites is equal to 4. In principle the method of course is applicable for any number of sites – we have only to make use of progressively higher derivatives of the binding curve. In practice however, given the inevitable limitation to the accuracy of any experimentally obtained binding curve, four is about the limit for which any such analysis is feasible. Beyond this point the value of the higher derivatives of this curve becomes subject to increasingly larger error.

The advantage of the use of linear equations in α_i's is to provide direct estimates of the α_i values from the derivative and asymptotic properties of the binding curve. One can therefore examine whether positive α_i values are obtained throughout the entire span of the binding curve. Values of K_i can be calculated from the derived α_i values and the range of error on the average K_i values estimated. This procedure provides an alternative to the non-linear weighted least-squares methods that are generally used to obtain K_i values from binding data.

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