On the Analysis of Competitive Binding of Various Ligands to Cooperative and Independent Binding Sites of Macromolecules

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The paper deals with the practical evaluation of multiple binding equilibria of macromolecules and different ligands competing for the same binding sites. The necessary formalism is reviewed and set up for the equilibria involving a macromolecule with various classes of independent binding sites and/or a class of cooperative sites and up to three different ligands in competition for them. In particular, it was necessary to extend the Hill approximation to treat simultaneous competition for cooperative as well as independent binding sites, while earlier attempts are shown to be inadequate.

Criteria are developed for a qualitative analysis of complex binding patterns using the Scatchard-plot of the experimental data in order to obtain a model of the binding structure and an adequate set of input parameters for the numerical analysis. Numerical examples refer to the binding of calcium and magnesium to the sarcoplasmic reticulum as studied by competitive replacement of manganese ions [3].

Introduction

Important biological problems are often connected with the investigation of multiple equilibria and the determination of their chemical kinetic parameters involved in the binding of macromolecules with a variety of ligands competing for the different types and numbers of binding sites. In order to evaluate the relevant kinetic parameters, ideally, the binding of each ligand should be studied separately, followed by investigation of the mutual competition for each class of binding sites. However, in many cases this procedure cannot be applied, for instance when removal of tight binding ligands from a macromolecule of interest turns out to be impossible. As an example, we refer to the complicated ionic binding pattern of the calcium and magnesium dependent ATPase of the sarcoplasmic reticulum [1, 2] for which we reported earlier [3] the results of an analysis of the competitive binding of manganese, calcium and magnesium ions to at least four classes of binding sites.

The purpose of this paper is a more detailed description of the numerical and analytical means employed in the evaluation and their critical discussion.

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The particular problems concerning the sarcoplasmic vesicles arose because

1. the naturally occurring calcium and magnesium cannot be removed fully from the tight binding sites;
2. the binding sites are not independent of each other but include a class of cooperative binding sites.

Although general mathematical descriptions for multiple binding patterns are available and will be referred to, the analysis of simultaneous competition for cooperative and independent binding sites has not been carried through to an applicable numerical procedure. This represents the principal aim of the present paper together with a critical analysis of the cooperative model used.

I. Representation of experimental data

In order to obtain binding constants from experimental data in kinetic equilibrium studies it is most common to represent the data in form of the Scatchard-plot [4]. When $\bar{X}$ is the number of ligands bound per macromolecular unit and x the concentration of free ligands, $\bar{X}/x$ is plotted as a function of $\bar{X}$.

It seems well established and will also become obvious from the specific example discussed in this paper that the Scatchard plot is most suited for a qualitative evaluation of the experimental informa-

tion. This applies in particular to the determination of the number of relevant binding classes and the distinction of independent and cooperative binding sites. Alternative representations are usually less sensitive to these particular features. With the same argument the Scatchard-plot renders most efficiently an initial set of input parameters for quantitative evaluation of the data. In addition, usually the qualitative analysis is a necessary prerequisite to choose the suitable formalism and numerical strategy for an optimization procedure.

On the other hand we would like to stress the problems connected with a quantitative determination of binding parameters from Scatchard plots, which have often been overlooked.

As pointed out by many authors [*e.g.* ref. 5] the error in the plotted quantities $\bar{X}/x$ and $\bar{X}$ is not proportional to the error in the experimental quantities. For instance, when the total ligand concentration X_T, the concentration of free ligands x and the concentration of macromolecular units M_T are the experimental variables as it is the case in typical EPR binding studies, the relative mean square error becomes

$$\frac{\Delta\frac{\bar{X}}{x}}{\frac{\bar{X}}{x}} = \pm \sqrt{\left(1+\frac{1}{M_T\frac{\bar{X}}{x}}\right)^2\left(\frac{\Delta x}{x}\right)^2+\left(1+\frac{1}{M_T\frac{\bar{X}}{x}}\right)^2\left(\frac{\Delta X_T}{X_T}\right)^2+\left(\frac{\Delta M_T}{M_T}\right)^2}. \tag{1}$$

Therefore, when $M_T\cdot\frac{\bar{X}}{x} \gg 1$ is not fulfilled anymore, the relative error of $\bar{X}/x$ depends also on $1/\ M_T\cdot\frac{\bar{X}}{x}$ and increases with it. Hence, the data points in a Scatchard-plot cannot be considered with equal statistical weight as done in a graphical analysis. A typical example is given by Klotz [19].

In this paper the Scatchard-plot is used throughout for the representation and description of both experimental and numerical data; it is also used for a first stage qualitative evaluation rendering the binding pattern and initial parameter set of the numerical fitting procedure using the formalism described in the following.

We want to emphasize that the Scatchard-plot is often used in biochemical research and that therefore the present paper is not the only one dealing with this topic (see *e.g.* [20, 21]). However, it seems not reasonable to revue all publications in which data are evaluated simply by using Scatchard-plots or those in which special features of Scatchard-plots are derived.

II. General formalism

For the mathematical description of multiple equilibria we use the formalism given by Wyman [6] which renders compact relations that can be easily extended to treat the situation when various ligands compete for the same binding sites.

In the following we consider a macromolecular unit M with

q binding sites for ligand X,
r binding sites for ligand Y,
s binding sites for ligand Z.

When the interaction among binding sites on different macromolecules can be neglected the numbers $\bar{X}$, $\bar{Y}$, $\bar{Z}$ of bound ligands X, Y, Z per macromolecule can be written (see ref. 6) as

$$\bar{X} = \frac{\partial}{\partial \ln x}\left(\ln\frac{M_T}{M_0}\right); \tag{2a}$$

$$\bar{Y} = \frac{\partial}{\partial \ln y}\left(\ln\frac{M_T}{M_0}\right); \tag{2b}$$

$$\bar{Z} = \frac{\partial}{\partial \ln z}\left(\ln\frac{M_T}{M_0}\right). \tag{2c}$$

M_0 is the concentration of ligandfree macromolecules, M_T the total concentration of M and x, y, z are the activities of the ligands X, Y, Z.

Now we consider the macromolecule to have different but independent binding classes, *i.e.* the binding of the ligands X, Y, Z to one binding class is not affected by their binding to another class.

The probability w_i of finding macromolecules for which the binding sites of the i^{th} binding class are unoccupied equals their concentration M_{0i} normalized to the total concentration M_T, *i.e.* $w_i = \frac{M_{0i}}{M_T} = f_i(x, y, z)$. Correspondingly, the probability w of

finding totally ligandfree macromolecules is given by $w = \frac{M_0}{M_T}$. Because of the postulated statistical independence the probability w is given by the product of the individual probabilities w_i, namely $w = \prod_{i=1}^{n} w_i$. Therefore, the relative concentration of totally ligandfree macromolecules relates to the individual M_{0i} by

$$\frac{M_0}{M_T} = \prod_{i=1}^{p} \frac{M_{0i}}{M_T} = \prod_{i=1}^{p} f_i(x, y, z). \tag{3}$$

In the following it is advantageous to treat specific binding patterns which were chosen here with the specific applications [3] in mind.

III. Examples of binding patterns

III.1. Independent binding sites

k, l, m may denote the association constants for the equilibrium of binding between a macromolecule with a single binding site and the ligands X, Y, Z. For the relative concentration of ligand free macromolecules we have [7, 8]:

$$\frac{M_T}{M_0} = 1 + k\,x + l\,y + m\,z. \tag{4}$$

This is easily extended to the case of different classes of independent binding sites, independent within the class as well as among the classes. With the intrinsic constants k_i, l_i, m_i within the i^{th} class successive application of Eqn (3) renders

$$\begin{aligned} \frac{M_T}{M_0} &= \prod_{i=1}^{p} \prod_{j=1}^{N_i} (1 + k_i\,x + l_i\,y + m_i\,z)_j \\ &= \prod_{i=1}^{p} (1 + k_i\,x + l_i\,y + m_i\,z)^{N_i}. \end{aligned} \tag{5}$$

N_i is the number of binding sites in the i^{th} class while p gives the number of classes.

In order to obtain the concentration $\bar{X}$, $\bar{Y}$, $\bar{Z}$ of bound ligands per macromolecule M we have to apply operation (2) on Eqn (5). For $\bar{X}$ we obtain for instance

$$\bar{X} = \frac{\partial}{\partial \ln x}\left(\ln \frac{M_T}{M_0}\right) = \sum_{i=1}^{p} \frac{N_i\,k_i\,x}{1 + k_i\,x + l_i\,y + m_i\,z}. \tag{6}$$

a) Independent binding in the absence of competing ligands

In the absence of competing ligands (y, $z = 0$) Eqn (6) renders for the quantity $\bar{X}$

$$\bar{X} = \sum_{i=1}^{p} \frac{N_i\,k_i\,x}{1 + k_i\,x} \tag{7}$$

and for the slope $\frac{\mathrm{d}\frac{\bar{X}}{x}}{\mathrm{d}\bar{X}}$ in the Scatchard-plot

$$\frac{\mathrm{d}\frac{\bar{X}}{x}}{\mathrm{d}\bar{X}} = \frac{1}{x^2}\left(x - \bar{X}\frac{\mathrm{d}x}{\mathrm{d}\bar{X}}\right) = -\frac{\sum_{i=1}^{p} \frac{N_i\,k_i^2}{(1 + k_i\,x)^2}}{\sum_{i=1}^{p} \frac{N_i\,k_i}{(1 + k_i\,x)^2}}. \tag{8}$$

For $x \geqq 0$ the quantity $\frac{\mathrm{d}\frac{\bar{X}}{x}}{\mathrm{d}\bar{X}}$ is less than nought and in the Scatchard-plot we obtain a curve with a negative slope throughout. For a single binding class ($p = 1$) Eqn (7) renders the well known linear relation

$$\frac{\bar{X}}{x} = k\,(N - \bar{X}). \tag{9}$$

From the intercepts of abscissa and of ordinate and from the slope at these points one obtains some relations between the quantities N_i and k_i which are summarized in Table I ($\alpha_1 = 1$). These expressions can be simplified in the following manner. By choosing a suitable permutation of indices it is always possible to arrange the binding constants k_i in such a way that $k_i \geqq k_{i+1}$ holds. If the condition $N_1\,k_1 \gg k_2 \sum_{i=2}^{p} N_i$ is fulfilled, the slope at the intercept on the ordinate is given by

$$\left(\frac{\mathrm{d}\frac{\bar{X}}{x}}{\mathrm{d}\bar{X}}\right)_{\bar{X} \to 0} = -k_1. \tag{10a}$$

The slope at $\bar{X} = 0$ can be used as first estimation of k_1 even when the above condition does not hold strictly. At the intercept on the abscissa the slope is given by

$$\left(\frac{\mathrm{d}\frac{\bar{X}}{x}}{\mathrm{d}\bar{X}}\right)_{\frac{\bar{X}}{x} \to 0} = -\frac{\sum_{i=1}^{p} N_i}{\sum_{i=1}^{p} \frac{N_i}{k_i}}. \tag{10b}$$

Hill coefficient α_1	Intercept of ordinate	Intercept of abscissa	Initial Slope at abscissa origin ($\bar{X}=0$)
$\alpha_1=1$	$\sum_{i=1}^{p} N_i k_i$	$\sum_{i=1}^{p} N_i$	$-\frac{\sum_{i=1}^{p} N_i k_i^2}{\sum_{i=1}^{p} N_i k_i}$
$1<\alpha_1<2$	$\sum_{i=2}^{p} N_i k_i$	$\sum_{i=1}^{p} N_i$	$\to \infty$
$\alpha_1=2$	$\sum_{i=2}^{p} N_i k_i$	$\sum_{i=1}^{p} N_i$	$\frac{N_1 k_1^2 - \sum_{i=2}^{p} N_i k_i^2}{\sum_{i=2}^{p} N_i k_i}$
$\alpha_1>2$	$\sum_{i=2}^{p} N_i k_i$	$\sum_{i=1}^{p} N_i$	$-\frac{\sum_{i=2}^{p} N_i k_i^2}{\sum_{i=2}^{p} N_i k_i}$

Table I: Characteristic features of Scatchard-plots: intercepts of coordinates and initial slope at the ordinate crossing for the binding of the ligand X to a macromolecule with one class of cooperative binding sites and $(p-1)$ classes of independent sites. α_1 is the Hill-coefficient of the cooperative class. N_i is the number of binding sites, k_i the associated binding constant of the i^{th} class.

For $k_p/N_p \ll k_{p-1}/\sum_{i=1}^{p-1} N_i$ Eqn (10b) becomes

$$\left(\frac{\mathrm{d}\dfrac{\bar{X}}{x}}{\mathrm{d}\bar{X}}\right)_{\frac{\bar{X}}{x}\to 0} = -\frac{k_p}{N_p}\cdot\sum_{i=1}^{p} N_i\,. \tag{10c}$$

Even when the above condition is not fulfilled, expression (10c) renders a first approximation for k_p/N_p where N_i is obtained from the intercept on the abscissa (Table I).

When no competing ligands are present, the statement about the negative slope in the Scatchard-plot can be extended such that an experimentally determined positive slope is incompatible with the existence of only independent binding sites.

b) Independent binding in the presence of competing ligands

The question arises as to how these statements are modified by the presence of competing ligands Y and Z. Although no analytical expression is available for $\bar{X}/x$ as a function of $\bar{X}$ in the general case, one finds for the important case of a single binding class of independent sites:

$$\frac{\bar{X}}{x}\left(1+\frac{l_1\dfrac{Y_T}{M_T}}{\dfrac{1}{M_T}+\dfrac{l_1\bar{X}}{k_1 x}}+\frac{m_1\dfrac{Z_T}{M_T}}{\dfrac{1}{M_T}+\dfrac{m_1\bar{X}}{k_1 x}}\right)=k_1(N_1-\bar{X}). \tag{11}$$

(Y_T and Z_T are the total concentration of the ligands Y and Z.) Equation (11) can be obtained easily by combining the appropriate expressions for $\bar{X}$, $\bar{Y}$ and $\bar{Z}$. This is shown in appendix A. Eqn (11) tells us that even when Y_T/M_T and Z_T/M_T are kept constant the function $\bar{X}/x=f(\bar{X})$ is dependent on the macromolecular concentration M_T. Therefore different concentrations M_T result in different curves in the Scatchard-plot.

Even in the presence of competing ligands the slope remains negative in the Scatchard-plot as long as the concentrations Y_T, Z_T and M_T are not changed. Since the proof of this statement is rather lengthy, it is presented in appendix B.

III.2. Cooperative as well as independent binding sites

Cooperative binding has been treated with a variety of formalisms [9–15] originating from different models of cooperativity. If a plausible model for the specific case under consideration does not exist, homotropic cooperativity is best described by the Hill-approximation [9], mainly because it requires only one additional parameter, the Hill-coefficient α.

It turns out that the extension of the Hill-approximation is not trivial when different ligands compete for the same cooperative binding sites. It seems a reasonable simplification when the binding of sterically similar ligands is described by the same Hill coefficient α even if quite different binding constants k, l and m have to be used [14].

Even with this assumption the application of the Hill-approximation is not straightforward. Inconsistent relations have been derived as we will show below explicitly for the result of Danchin [16]. In order to obtain a correct expression, we generalize the results of Haldane [17] as established for the competition of O_2 and CO for the cooperative binding sites of hemoglobin. One has to use only one fundamental result which may be formulated as follows [7]:

When hemoglobin is exposed to a mixture of carbon monoxide and oxygen, the relative amounts of the two gases which combine are given by the relation

$$\mathrm{HbCO}/\mathrm{HbO_2} = \mathrm{apCO}/\mathrm{pO_2}. \tag{12}$$

When Y stands for CO and X stands for O_2, then in our notation expression (12) is equivalent with

$$\bar{Y}/\bar{X} = ay/x. \tag{13a}$$

The factor a is the so-called partition coefficient which is independent of the concentration of CO, O_2 and hemoglobin. When one assumes that Eqn (12) holds likewise for a mixture of three competing ligands and when one defines a second partition coefficient b by

$$\bar{Z}/\bar{X} = bz/x. \tag{13b}$$

One obtains applying Eqns (2) and (13)

$$\frac{1}{x}\frac{\partial \ln \frac{M_T}{M_0}(x,y,z)}{\partial \ln x} = \frac{1}{ay}\frac{\partial \ln \frac{M_T}{M_0}(x,y,z)}{\partial \ln y} = \frac{1}{bz}\frac{\partial \ln \frac{M_T}{M_0}(x,y,z)}{\partial \ln z} \tag{14a}$$

or

$$\frac{\partial \ln \frac{M_T}{M_0}(x,y,z)}{\partial x} = \frac{\partial \ln \frac{M_T}{M_0}(x,y,z)}{\partial ay} = \frac{\partial \ln \frac{M_T}{M_0}(x,y,z)}{\partial bz}. \tag{14b}$$

The solution of Eqn (14b) is

$$\frac{M_T}{M_0}(x,y,z) = \frac{M_T}{M_0}(x+ay+bz) \tag{15}$$

which holds for all values of x, y and z. Therefore the function M_T/M_0 is that which describes the equilibrium in the presence of only one ligand. Using the expression for M_T/M_0 which corresponds to the Hill approximation $\bar{X} = N(kx)^\alpha/(1+(kx)^\alpha)$, namely

$$\frac{M_T}{M_0} = (1+(kx)^\alpha)^{\frac{N}{\alpha}} \tag{16}$$

one obtains with the definition $l = ka$ and $m = kb$

$$\begin{aligned}\frac{M_T}{M_0} &= (1+k^\alpha(x+ay+bz)^\alpha)^{\frac{N}{\alpha}} \\ &= (1+(kx+ly+mz)^\alpha)^{\frac{N}{\alpha}}.\end{aligned} \tag{17}$$

For p classes of cooperative binding sites Eqn (17) can be generalized when the binding within each class is independent of the binding in the other classes

$$\frac{M_T}{M_0} = \prod_{i=1}^{p}(1+(k_i x+l_i y+m_i z)^{\alpha_i})^{\frac{N_i}{\alpha_i}}. \tag{18}$$

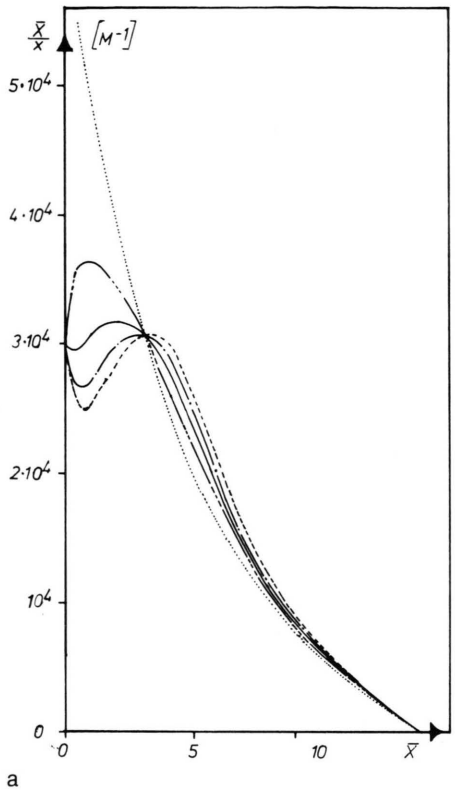
For $\alpha_i = 1$ the relation (5) for independent binding sites is obtained again.

At this point it is meaningful to restrict the further discussion to two special cases of biological relevance, particularly in the present context [3]. We will consider first the binding of a ligand X to a macromolecule having one class of cooperative and up to two classes of independent binding sites. Secondly we will treat the same binding pattern in the presence of up to two competing ligands Y and Z.

III.3. Binding to a macromolecule with one class of cooperative sites and two classes of independent sites

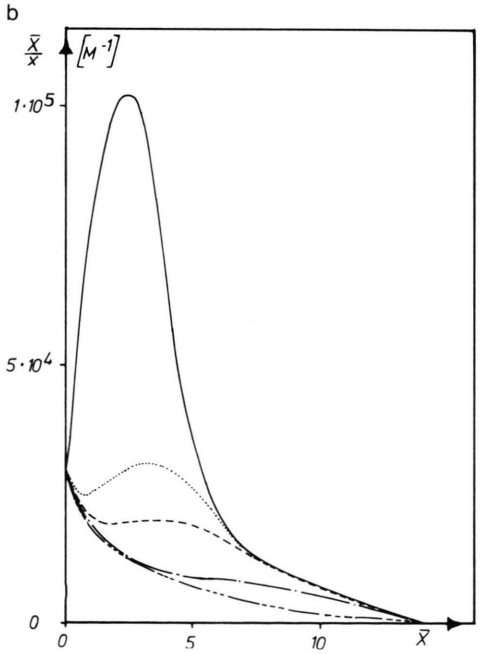
a) Absence of competing ligands

In this paragraph we will consider he binding of a ligand X to a macromolecule with one class of cooperative sites ($\alpha_1 = \alpha > 1$, k_1, N_1) and two classes of independent sites ($\alpha_2 = \alpha_3 = 1$, k_2, k_3, N_2, N_3) when



a

b



no competing ligands are present ($y, z = 0$). Application of Eqn (2) to the general relationship (18) leads to the solution

$$\bar{X} = \frac{N_1 (k_1 x)^\alpha}{1 + (k_1 x)^\alpha} + \sum_{i=2}^{3} \frac{N_i k_i x}{1 + k_i x} . \tag{19}$$

Typical Scatchard-plots derived from (19) for various sets of parameters are presented in Fig. 1.

A different analysis of the first term in Eqn (19) in terms of axis crossing and initial slope in the Scatchard-plot is given in Table I for the various ranges of Hill coefficients. Using Eqn (19) the slope in the Scatchard-plot is given by

$$\frac{\mathrm{d}\frac{\bar{X}}{x}}{\mathrm{d}\bar{X}} = \frac{\frac{N_1 k_1^\alpha x^{\alpha-2}(\alpha - 1 - k_1^\alpha x^\alpha)}{(1 + k_1^\alpha x^\alpha)^2} - \sum_{i=2}^{3} \frac{N_i k_i^2}{(1 + k_i x)^2}}{\frac{\alpha N_1 k_1^\alpha x^{\alpha-1}}{(1 + k_1^\alpha x^\alpha)^2} + \sum_{i=2}^{3} \frac{N_i k_i}{(1 + k_i x)^2}} \tag{20}$$

The slope is negative for $\alpha - 1 \leqq k_1^\alpha x^\alpha$. (Here and in the following we consider only the meaningfull case that $x \geqq 0$ and, when not stated otherwise, that all constants N_i and k_i are greater than zero.) When a concentration x exists such that the denominator in Eqn (20) becomes more than zero, then the slope becomes positive and because of the continuity of the function $\bar{X}/x = f(\bar{X})$ one finds a relative maximum in the Scatchard-plot. This is always the case for $2 > \alpha > 1$, independent of the individual N_i's and k_i's, and likewise for $\alpha = 2$ when the condition $N_1 k_1^2 > \sum_{i=2}^{p} N_i k_i^2$ is fulfilled (see Table I). For $\alpha > 2$ the initial slope is always negative (Table I) and dependent on the relative magnitude of the constants N_i, k_i and α there is no relative maximum or a relative maximum and a relative minimum in contrast to the case of $\alpha < 2$ where one finds a maximum only (Fig. 1).

Fig. 1. Scatchard-plots for the binding of ligand X to a macromolecule with one class of $N_1 = 3$ cooperative binding sites and two classes of independent sites with the binding parameters: $N_2 = 1$, $N_3 = 10$; $k_2 = 2 \times 10^4$ M^{-1}, $k_3 = 1 \times 10^3$ M^{-1}. The plotted quantities are given by the experimental observables: $\bar{X}$, the number of bound ligands X per macromolecular unit and x, the activity of ligand X.
a) Influence of the Hill-coefficient α_1 as variable parameter with fixed cooperative binding constant: $k_1 = 1 \times 10^4$ M^{-1}. – – – – – $\alpha_1 = 3.0$; —·— $\alpha_1 = 2.5$; ——— $\alpha_1 = 2.0$; —··— $\alpha_1 = 1.5$; ····· $\alpha_1 = 1.0$.
b) Influence of the binding constant k_1 as variable parameter with fixed Hill-coefficient $\alpha_1 = 3$.
——— $k_1 = 5 \times 10^4$ M^{-1}; ····· $k_1 = 1 \times 10^4$ M^{-1};
– – – – – $k_1 = 3 \times 10^3$ M^{-1}; —·— $k_1 = 1 \times 10^3$ M^{-1};
—··— $k_1 = 1 \times 10^2$ M^{-1}.

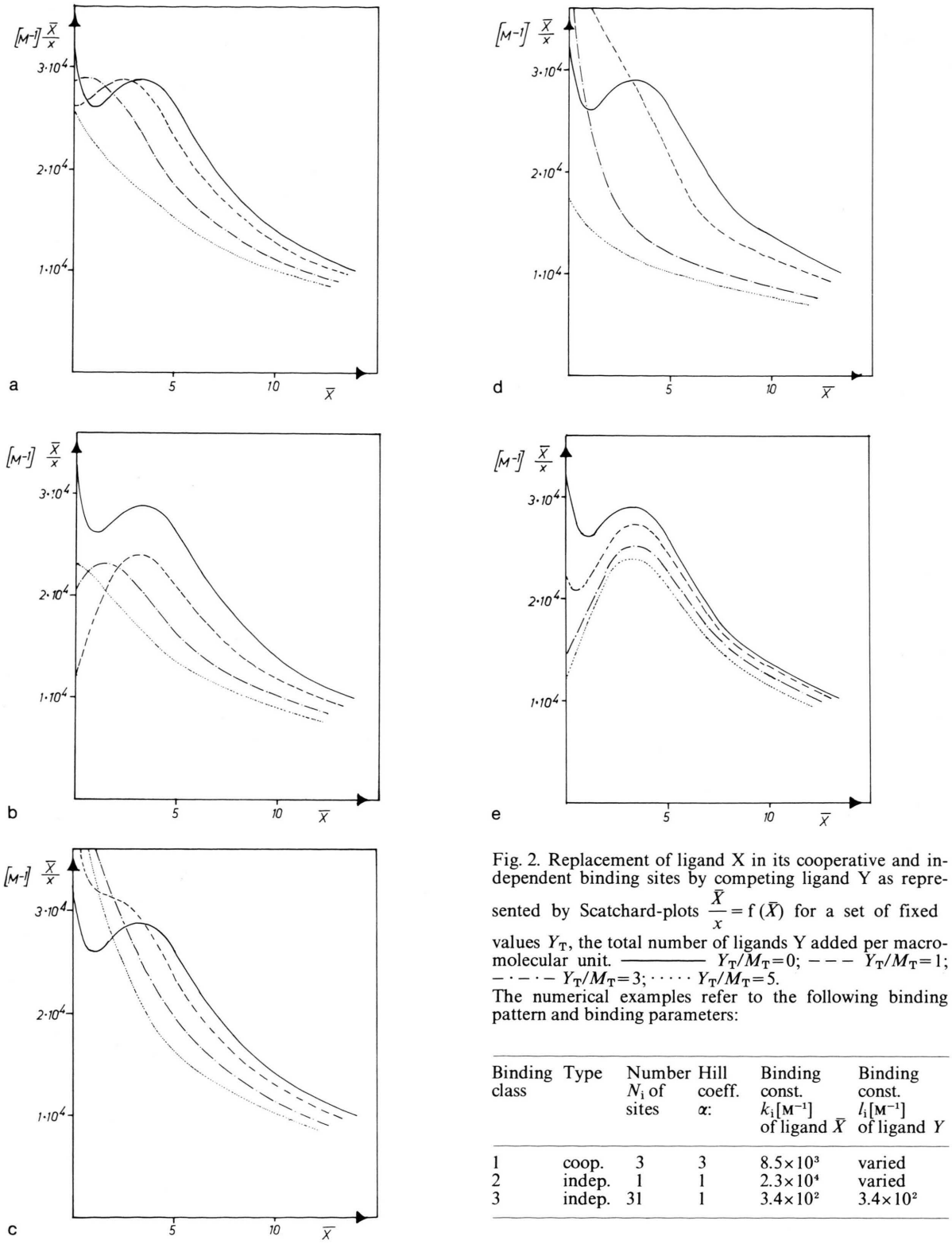


Fig. 2. Replacement of ligand X in its cooperative and independent binding sites by competing ligand Y as represented by Scatchard-plots $\frac{\bar{X}}{x} = f(\bar{X})$ for a set of fixed values Y_T, the total number of ligands Y added per macromolecular unit. ——— $Y_T/M_T=0$; – – – $Y_T/M_T=1$; – · – · – $Y_T/M_T=3$; · · · · · $Y_T/M_T=5$.
The numerical examples refer to the following binding pattern and binding parameters:

Binding class	Type	Number N_i of sites	Hill coeff. α:	Binding const. k_i[M^{-1}] of ligand $\bar{X}$	Binding const. l_i[M^{-1}] of ligand Y
1	coop.	3	3	8.5×10^3	varied
2	indep.	1	1	2.3×10^4	varied
3	indep.	31	1	3.4×10^2	3.4×10^2

Cooperative binding does not result necessarily in the occurrence of a relative maximum in the Scatchard-plot, because for any given N_1, k_1, x it is possible to choose a set of N_i's and k_i's in such a way that the denominater in Eqn (20) is less than nought. But as stated before the occurrence of regions in the Scatchard-plot with positive slope is incompatible with independent sites only.

Another characteristic feature of (19) is found with the variation of the Hill coefficient. All curves have a common point for $x=1/k_1$.

b) Presence of one competing ligand

In the following we will deal with the binding of ligand X to a macromolecule with one class of cooperative sites ($\alpha_1=\alpha>1$, N_1, k_1, l_1) and two classes of independent sites ($\alpha_2=\alpha_3=1$, N_2, k_2, l_2, N_3, k_3, l_3) when one competing ligand Y is present ($y\neq 0$, $z=0$).

Analytical expressions can no longer be found for quantities that can be plotted, rather the system of Eqn (2) obtained from the Ansatz (18) has to be solved numerically for $\bar{X}$ and $\bar{Y}$. A suitable method is the Newton-Raphson technique [18] applied to the equations for $\bar{X}$ and $\bar{Y}$.

$$\bar{X}=\frac{N_1 k_1 x\,(k_1 x+l_1 y)^{\alpha-1}}{1+(k_1 x+l_1 y)^{\alpha}}+\sum_{i=2}^{3}\frac{N_i k_i x}{1+k_i x+l_i y} \tag{21a}$$

$$\bar{Y}=\frac{N_1 l_1 y\,(k_1 x+l_1 y)^{\alpha-1}}{1+(k_1 x+l_1 y)^{\alpha}}+\sum_{i=2}^{3}\frac{N_i l_i y}{1+k_i x+l_i y}. \tag{21b}$$

Among the various alternatives for plotting the numerical and experimental results we have chosen the Scatchard-plot for the ligand X while the variation of the concentration of the competing ligand Y is restricted to a set of fixed parameters of the total ligand concentration Y_T. This choice has been discussed in somewhat more detail in ref. [3, 8]. The advantage of this representation is that it allows to be an easy qualitative analysis of the experimental data in order to derive the appropriate binding pattern and an initial set of binding parameters for the numerical analysis.

Fig. 2 presents examples of characteristic features in the Scatchard-plots associated with particular sets of binding parameters as found to apply in the experimental investigation reported earlier [3]. For instance, it is quite clear that the relative maximum due to the cooperative binding class moves towards $\bar{X}=0$ with increasing concentration Y_T of the competing ligand when the cooperative sites become occupied by competing ligands before the independent one (Fig. 2a, c, d). On the other hand, the position of the maximum remains essentially unchanged when the competing ligand occupies primarily the independent sites (Fig 2b, e).

IV. Discussion

IV.1. The mathematical framework

For fitting the experimental data a suitable mathematical formalism must be available. Whereas its derivation is straightforward for the case of independent binding, even in the presence of competing ligands, some difficulties arise in the case of competition for cooperative binding sites.

When extending the Hill-approximation for various competing ligands one must not ignore that it is only an approximation. As pointed out in chapter III.2. one has to make some assumptions about the type of competition, for example the validity of Haldane's law, which is experimentally verified for hemoglobin. With this assumption it is possible to derive the correct equations by applying relation (15) to the Hill-approximation. We would like to stress, that relation (15) can be applied to any expression which describes the binding of only one ligand to cooperative sites and that no more assumptions are necessary, for example that the Hill coefficients are the same for all competing ligands.

In the more recent literature one finds an extension of the Hill-approximation which differs remarkably from ours [16]. Unfortunately the author gives only a formula without showing the derivation. Assuming that the Hill coefficient α equals the

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The sequences of figures demonstrates the change in the Scatchard-plots when ligand X is replaced by ligand Y in various typical situations:

a) equal binding constants: $k_i=l_i$; b) ligand Y has *higher* affinity than X for strong *independent* binding sites (class 2): $l_2=10^2 k_2$, otherwise: $l_1=k_1$, $l_3=k_3$; c) ligand Y has *lower* affinity than X for class 2: $l_2=10^{-2} k_2$, otherwise like b); d) ligand Y has *higher* affinity than X for *cooperative* binding sites (class 1): $l_1=10^2 k_1$, otherwise: $l_2=k_2$, $l_3=k_3$; e) ligand Y has *lower* affinity than X for class 1: $l_1=10^{-2} k_1$, otherwise like d).

number of cooperative sites N and that it is unchanged for both competing ligands, he obtains an expression which is in our notation,

$$\left(\frac{\bar{X}}{x}\right)^{N}\left(1+l^{N}\left(Y_{T}-M_{T}(N-\bar{X})+\frac{M_{T}\left(\frac{\bar{X}}{x}\right)^{N}}{k^{N}\bar{X}^{N-1}}\right)^{N}\right)=k^{N}(N-\bar{X})\,\bar{X}^{N-1}. \tag{22}$$

When neglecting the fact that the Hill-approximation is only an approximation, one can derive Eqn (22) in a manner which seems convincing. From Eqn (19) one obtains for the case of only cooperative binding with $\alpha_1=N_1$ and $Y_T=0$ ($N_2=N_3=0$).

$$\bar{X}=\frac{N(kx)^{N}}{1+(kx)^{N}} \tag{23a}$$

or

$$k^{N}=\frac{\bar{X}\frac{M_T}{N}}{\left(M_T-\bar{X}\frac{M_T}{Nx}\right)x^{N}}. \tag{23b}$$

Analogously one obtains for the ligand Y in absence of competing ligands

$$l^{N}=\frac{\bar{Y}\frac{M_T}{N}}{\left(M_T-\bar{Y}\frac{M_T}{N}\right)y^{N}}. \tag{23c}$$

These equations may be interpreted as a description of an equilibrium between macromolecules with all binding sites occupied, ligandfree macromolecules and free ligands in the absence of competing ligands. When both ligands X and Y are present simultaneously, one has to substitute in Eqns (23b) and (23c) as concentration of ligandfree macromolecules the expression $(M_T-\bar{X}M_T/N-\bar{Y}M_T/N)$ By combining the new equations one obtains as result

$$\bar{X}=\frac{N k^{N}x^{N}}{1+k^{N}x^{N}+l^{N}y^{N}} \tag{24a}$$

and

$$\bar{Y}=\frac{N l^{N}y^{N}}{1+k^{N}x^{N}+l^{N}y^{N}}. \tag{24b}$$

After some algebra, Eqn (22) given by Danchin [16] can be obtained from Eqn (24 a, b). The consistency of relations (22) and (24), respectively, can be checked by the following simple procedure:

Suppose X and Y be two types of ligands which cannot be distinguished with respect to their binding to a macromolecule. All relevant binding parameters should be equal for each of the ligands. Due to symmetry requirements the relation holds:

$$\bar{X}(x_1,0)=\bar{X}(x_0,y_0)+\bar{Y}(x_0,y_0) \tag{25a}$$

with $x_1=x_0+y_0$, x_0, y_0 arbitrary.

For $x_0=y_0$ one finds

$$\bar{X}(x_0,y_0)=\frac{1}{2}\bar{X}(x_1,0) \tag{25b}$$

and using the Hill-approximation:

$$\bar{X}(x_0,y_0)=\frac{1}{2}\bar{X}(x_1,0)=\frac{1}{2}\frac{N k^{N}x_1^{N}}{1+k^{N}x_1^{N}}. \tag{25c}$$

With condition (25c), Eqn (22) results in

$$1-\left(\frac{1}{2}\right)^{N-1}-\left(\frac{1}{2}\right)^{N}k^{N}x_1^{N}+ +k^{N}\left(M_T N\frac{2^{N-1}-1}{1+k^{N}x_1^{N}}+\frac{1}{2}x_1\right)^{N}=0 \tag{26a}$$

and Eqn (24) results in

$$\frac{1}{2}\frac{N k^{N}x_1^{N}}{1+k^{N}x_1^{N}}=\frac{N k^{N}\cdot\left(\frac{1}{2}x_1\right)^{N}}{1+k^{N}\left(\frac{1}{2}x_1\right)^{N}+k^{N}\left(\frac{1}{2}x_1\right)^{N}}. \tag{26b}$$

Eqns (26a and b) should be true for alle sets of values k, x_1, M_T. This cannot be fulfilled in general and therefore the solutions (22) and (24) cannot be valid.

It is verified immediatly that the symmetry requirement (25) is fulfilled for our solutions given in chapter III. for cooperative binding.

We tried to evaluate our experimental data we obtained for the binding of Ca^{2+}, Mg^{2+} and Mn^{2+} to sarcoplasmic vesicles [3] by using a generalisation of Danchin's formalism which admits also Hill coefficients less than N as well as the formalism (21). As expected, we did not obtain a satisfactory fit of our

data, when describing the competition for cooperative sites by Danchin's formalism. Using relationship (18) we were able to fit all data within experimental error, as shown in ref. [3].

IV.2. Practical aspects

As mentioned before the quantitative evaluation of complex binding patterns can only be achieved in general by using a suitable computer program. The efficiency and reliability of the optimalisation procedure can be increased considerably, when before starting as much information as possible is available about the type of binding, the number of binding sites and the magnitude of the corresponding association constants. This information can come from a first analysis of experimental data by use of the Scatchard-plot.

a) Binding in absence of competing ligands

When no competing ligands are in the macromolecular solution a positive slope in the Scatchard-plot tells us that we have to consider cooperative binding sites (chapter III.1.) When the slope is negative throughout, it does not imply that only independent binding sites exist, but several important cases are to distinguish:

1. When the plotting of data results in a straight line one has to assume one class of independent binding sites.
2. When the plot of data does not result in a straight line, the macromolecule may have different classes of independent sites and/or of negatively cooperative sites. In this paper we did not deal with negative cooperativity, but nevertheless by using a Hill-coefficient $\alpha < 1$ all equations we derived for positive cooperativity can be applied for describing negative cooperativity. It is readily seen from Eqn (20) that for negative cooperativity one obtains a curve with negative slope throughout in the Scatchard-plot.
3. In the presence of tight independing sites cooperative sites with medium affinity do not yield a curve with positive slope.

When no other indications suggest the existence of cooperative sites and when the slope in the Scatchard-plot is negative throughout the most reasonable procedure seems to assume first that only independent sites are present in the macromolecule. The situation changes when additional information indicates the possible existence of cooperative sites. A method for obtaining such informations is *e.g.* the use of analogs, which bind to the same sites, but with different relative affinities.

When the number and the type of binding classes are determined, the slope and the intercepts of axis can be used for a first approximation of the number of binding sites and of the magnitude of the binding constants (see *e.g.* Table 1).

b) Binding in the presence of competing ligands

As long as the concentrations of competing ligands and of macromolecules are kept constant, the above statement holds, *i.e.* that a positive slope cannot be explained by independent binding only.

However, when the relative concentrations of competing ligands and/or macromolecules are changed during the titration, it is possible to obtain a curve with a positive slope in the Scatchard-plot even when only independent sites exist. This results trivially from the fact that when varying the total concentrations of competing ligands and/or of macromolecules one obtains a family of curves in the Scatchard-plot.

Although the presence of competing ligands complicates the binding pattern considerably, competition studies can be helpful in decomposing a complex binding curve.

This may be demonstrated by the interpretation of experimental results reported in ref. [3]. Here manganese ions bound to sarcoplasmic vesicles were replaced by magnesium in a way typified by Fig. 2e while competitive replacement by calcium ions follows the pattern of Fig. 2c.

These binding studies were particularly relevant because fortunately the highest experimental accuracy could be achieved in the region where the cooperative sites become occupied by the manganese ions. In addition, the relative maximum in the Scatchard-plot is well pronounced because it is not disturbed by any binding to strong binding sites. Therefore the manganese binding is here especially suited to a study of the binding of the biologically relevant ions magnesium and calcium by means of manganese replacement. Indeed, it was clear from the results that a direct detection of the cooperative calcium binding sites would have been impossible as a consequence of the high affinity of the strong independent binding sites (Fig. 3).

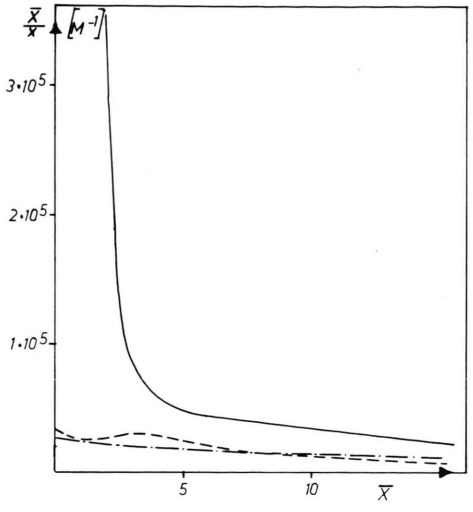

Fig. 3. Binding of calcium (——), magnesium (– · –) and manganese (– – –) to sarcoplasmic vesicles as represented by the Scatchard-plot. For the computation of the curves the binding constants of ref. 3 are used.

Certainly other characteristic features in the Scatchard-plots can be described for specific sets of binding parameters using the formalism outlined here. However, this would be of interest only in connection with corresponding experimental results.

It should be emphasized how important a qualitative analysis of suitable plotted data can be as the basis of a reliable quantitative analysis with a numerical fitting procedure and that in this way biochemically relevant parameters can be derived, even for fairly complex binding patterns.

Appendix A

Eqn (11) may be derived as follows:
For $p=1$ Eqn (6) renders

$$\bar{X}=\frac{N_1 k_1 x}{1+k_1 x+l_1 y+m_1 z}. \tag{27a}$$

Applying operation (2) on Eqn (5) one obtains the corresponding expression for $\bar{Y}$ and $\bar{Z}$.

$$\bar{Y}=\frac{Y_T-y}{M_T}=\frac{N_1 l_1 y}{1+k_1 x+l_1 y+m_1 z} \tag{27b}$$

$$\bar{Z}=\frac{Z_T-z}{M_T}=\frac{N_1 m_1 z}{1+k_1 x+l_1 y+m_1 z}. \tag{27c}$$

Using $1+k_1 x+l_1 y+m_1 z=N_1 k_1/(\bar{X}/x)$ (Eqn 27a) one obtains for $l_1 y$ and $m_1 z$ with Eqn (27b) and (27c)

$$l_1 y=\frac{l_1\frac{Y_T}{M_T}}{\frac{1}{M_T}+\frac{N_1 l_1}{N_1 k_1}\frac{\bar{X}}{x}} \tag{27d}$$

$$m_1 z=\frac{m_1\frac{Z_T}{M_T}}{\frac{1}{M_T}+\frac{N_1 m_1}{N_1 k_1}\frac{\bar{X}}{x}}. \tag{27e}$$

Substituting expressions (27d) and (27e) in Eqn (27a) and dividing with x results in

$$\frac{\bar{X}}{x}=\frac{N_1 k_1}{1+k_1\frac{\bar{X}}{x}+\frac{l_1\frac{Y_T}{M_T}}{\frac{1}{M_T}+\frac{l_1}{k_1}\frac{\bar{X}}{x}}+\frac{m_1\frac{Z_T}{M_T}}{\frac{1}{M_T}+\frac{m_1}{k_1}\frac{\bar{X}}{x}}}. \tag{27f}$$

Finally the transformation of Eqn (27f) renders Eqn (11).

Appendix B

We want to show that for a macromolecule M with p classes of independent sites which combines with three competing ligands X, Y, Z the slope $\mathrm{d}(\bar{X}/x)/\mathrm{d}\bar{X}$ remains negative in the Scatchard-plot when the total concentrations Y_T, Z_T and M_T are kept constant. The slope is given by

$$\frac{\mathrm{d}\frac{\bar{X}}{x}}{\mathrm{d}\bar{X}}=\frac{\partial\frac{\bar{X}}{x}}{\partial x}\frac{\mathrm{d}x}{\mathrm{d}\bar{X}}+\frac{\partial\frac{\bar{X}}{x}}{\partial y}\frac{\mathrm{d}y}{\mathrm{d}\bar{X}}+\frac{\partial\frac{\bar{X}}{x}}{\partial z}\frac{\mathrm{d}z}{\mathrm{d}\bar{X}}. \tag{28a}$$

From Eqn (6) one obtains

$$\frac{\partial\frac{\bar{X}}{x}}{\partial x}=-\sum_{i=1}^{p}\frac{N_i k_i^2}{(1+k_i x+l_i y+m_i z)^2}; \tag{28b}$$

$$\frac{\partial\frac{\bar{X}}{x}}{\partial y}=-\sum_{i=1}^{p}\frac{N_i k_i l_i}{(1+k_i x+l_i y+m_i z)^2}; \tag{28c}$$

$$\frac{\partial\frac{\bar{X}}{x}}{\partial z}=-\sum_{i=1}^{p}\frac{N_i k_i m_i}{(1+k_i x+l_i y+m_i z)^2}. \tag{28a}$$

Applying operation (2) to Eqn (5) we obtain a system of equations for $\bar{X}$, $Y_T/M_T = \bar{Y}+y/M_T$ and for $Z_T/M_T=\bar{Z}+z/M_T$. Their derivation $d/d\bar{X}$ results in

$$1=\sum_{i=1}^{p}\frac{N_i k_i(1+l_i y+m_i z)}{(1+k_i x+l_i y+m_i z)^2}\frac{dx}{d\bar{X}}+\sum_{i=1}^{p}\frac{-N_i k_i l_i x}{(1+k_i x+l_i y+m_i z)^2}\frac{dy}{d\bar{X}}+\sum_{i=1}^{p}\frac{-N_i k_i m_i x}{(1+k_i x+l_i y+m_i z)^2}\frac{dz}{d\bar{X}} \tag{28c}$$

$$0=\sum_{i=1}^{p}\frac{-N_i l_i k_i y}{(1+k_i x+l_i y+m_i z)^2}\frac{dx}{d\bar{X}}+\left(\left(\sum_{i=1}^{p}\frac{N_i l_i(1+k_i x+m_i z)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)\frac{dy}{d\bar{X}}$$

$$+\sum_{i=1}^{p}\frac{-N_i l_i m_i y}{(1+k_i x+l_i y+m_i z)^2}\frac{dz}{d\bar{X}} \tag{28f}$$

$$0=\sum_{i=1}^{p}\frac{-N_i m_i k_i z}{(1+k_i x+l_i y+m_i z)^2}\frac{dx}{d\bar{X}}+\sum_{i=1}^{p}\frac{-N_i m_i l_i z}{(1+k_i x+l_i y+m_i z)^2}\frac{dy}{d\bar{X}}$$

$$+\left(\left(\sum_{i=1}^{p}\frac{N_i m_i(1+k_i x+l_i y)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)\frac{dz}{d\bar{X}}. \tag{28g}$$

According to Kramer's rule the solution of this system of Eqns (28e, f, g) renders for $dx/d\bar{X}$, $dy/d\bar{X}$ and $dz/d\bar{X}$

$$\frac{dx}{d\bar{X}}=\frac{A_1}{A}=\frac{1}{A}\left(\left(\sum_{i=1}^{p}\frac{N_i l_i m_i z}{(1+k_i x+l_i y+m_i z)^2}\right)\left(\left(\sum_{i=1}^{p}\frac{N_i m_i(1+k_i x)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)\right.$$

$$+\left(\sum_{i=1}^{p}\frac{N_i m_i l_i y}{(1+k_i x+l_i y+m_i z)^2}\right)\left(\left(\sum_{i=1}^{p}\frac{N_i l_i(1+k_i x)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)$$

$$\left.+\left(\left(\sum_{i=1}^{p}\frac{N_i l_i(1+k_i x)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M}\right)\left(\left(\sum_{i=1}^{p}\frac{N_i m_i(1+k_i x)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)\right). \tag{28h}$$

$$\frac{dy}{d\bar{X}}=\frac{A_2}{A}=\frac{1}{A}\left(\left(\sum_{i=1}^{p}\frac{N_i l_i k_i y}{(1+k_i x+l_i y+m_i z)^2}\right)\left(\left(\sum_{i=1}^{p}\frac{N_i m_i(1+k_i x+l_i y)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)\right.$$

$$\left.+\left(\sum_{i=1}^{p}\frac{N_i l_i m_i y}{(1+k_i x+l_i y+m_i z)^2}\right)\left(\sum_{i=1}^{p}\frac{N_i m_i k_i z}{(1+k_i x+l_i y+m_i z)^2}\right)\right); \tag{28i}$$

$$\frac{dz}{d\bar{X}}=\frac{A_3}{A}=\frac{1}{A}\left(\left(\sum_{i=1}^{p}\frac{N_i l_i k_i y}{(1+k_i x+l_i y+m_i z)^2}\right)\left(\sum_{i=1}^{p}\frac{N_i m_i l_i z}{(1+k_i x+l_i y+m_i z)^2}\right)\right.$$

$$\left.+\left(\sum_{i=1}^{p}\frac{N_i m_i k_i z}{(1+k_i x+l_i y+m_i z)^2}\right)\left(\left(\sum_{i=1}^{p}\frac{N_i l_i(1+k_i x+m_i z)}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)\right). \tag{28j}$$

A is given by

$$A=A_1\left(\sum_{i=1}^{p}\frac{N_i k_i}{(1+k_i x+l_i y+m_i z)^2}\right)+A_2\left(\left(\sum_{i=1}^{p}\frac{N_i l_i}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right)$$

$$+A_3\left(\left(\sum_{i=1}^{p}\frac{N_i m_i}{(1+k_i x+l_i y+m_i z)^2}\right)+\frac{1}{M_T}\right). \tag{28k}$$

From Eqns (28a), (28b–d) and (28h–k) follows the above assertion that the slope $d(\bar{X}/x)/d\bar{X}$ is always negative.

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