Biophysical Chemistry 29 (1988) 231–243
Elsevier

BPC 01230

The determination of equilibrium constants for heterogeneous macromolecular interactions

James K. Zimmerman and Mary Lou Crowl-Powers

Department of Biological Sciences, 132 Long Hall, Clemson University, Clemson, SC 29634-1903, U.S.A.

Received 30 September 1987
Revised manuscript received 3 November 1987
Accepted 3 November 1987

Association constant; Macromolecular interaction; Light scattering

A method has been developed to determine the association constant for a heterogeneous association of the type $A + B \rightleftharpoons AB$. This method requires knowledge of the two initial concentrations and of the resulting weight-average molecular weight for each data point. Computer simulations using Gaussian-distributed error on the measured parameters show that the researcher can readily determine whether the particular concentration range chosen is appropriate for the strength of binding and therefore how reliable the calculated constant might be. It is also shown that errors in measuring molecular weight have, in general, a more profound effect than do errors in concentration.

1. Introduction

The study of reversibly interacting heterogeneous macromolecular systems has not received the same amount of attention as have homogeneous self-associations. In 1954 Steiner [1] did set forth the basic principles for light scattering and in the late 1960's and early 1970's Adams looked at the theory of heterogeneous interaction for several experimental techniques, osmotic pressure [2], ultracentrifugation [3,4] and light scattering [5]. In the same time frame, Nichol and Ogston [6] and Chun and Kim [7] also investigated the theory for the ultracentrifuge as did Aune and co-workers [8–10] in the late 1970's and early 1980's. Ackers [11], Nichol et al. [12] and, very recently, Cann and Winzor [13] have set the foundations for similar analyses using analytical gel chromatography under very specialized conditions.

Very recently, several investigators have begun to look again at heterogeneous systems using the analytical ultracentrifuge [14–19]. This renewed interest in heterogeneous association properties has arisen from realizations similar to those of Donald Brown [20], "Developmental control of genes is going to boil down to some very mundane biophysical principles. It's going to involve the concentrations of activator versus repressor molecules. It's going to involve cooperative influences between molecules and binding constants of proteins to each other and to DNA." Similar conclusions are drawn by Ptashne [21], Lenardo et al. [22], and in a summary by Robertson [23] of a recent Institute of Genetics meeting.

Not all interacting macromolecular systems are suited for study by sedimentation equilibrium which is quite time consuming. Many of these systems could be more easily studied by methods such as low-angle laser light scattering. We have developed an analytical method which would be especially useful for, but not limited to, light scattering measurements. The method examines

Correspondence address: J.K. Zimmerman, Department of Biological Sciences, 132 Long Hall, Clemson University, Clemson, SC 29634-1903, U.S.A.

the interaction of a one-to-one complex of two macromolecules.

2. Method

2.1. Fitting method

The method analyses a three-dimensional surface for the association constant. The three dimensional surface uses the total concentration of species A (A_0) and B (B_0) for two axes and the weight-average molecular weight (M_w) for the third. We have used, as our primary method of fitting K, a Gauss-Newton routine from a program provided to us by Dr. M. Johnson (University of Virginia). It requires two user-supplied subroutines. This system was restricted to physically meaningful ($K \geqslant 0$) values by defining a variable Q as ln K, rewriting the appropriate equations substituting exp(Q) for K and making use of the relationship $K \mathrm{d}Q = \mathrm{d}K$. The two user-supplied subroutines calculate the difference in the observed and theoretical values as well as values for the derivative of the function (see appendix A). We have verified the results from the Gauss-Newton analyses in selected examples using a much less sophisticated Newton-Raphson routine (see appendix A).

The derivation provided in appendix A expresses concentration in terms of mg/ml, the units most commonly encountered in the laboratory. The resulting association constant K is thus in units of ml/mg, which one can convert to molar units (K') using the expression

$$K' = K(M_A M_B / M_{AB})$$

where M_A, M_B and M_{AB} are the molecular weights of species A, species B and the complex, respectively. The result of the derivation in appendix A is a nonlinear equation with a single variable, K, to be fitted using multiple experimental points. Also, analytical expressions for the required derivative expressions are shown. Appendix B provides the derivation of $\mathrm{d}K$, which shows the sensitivity of a particular value of K to the various parameters in the system.

2.2. Specific system simulated

To test the system, computer-generated data sets were created covering a wide range of equilibrium constants and loading concentrations. Molecular weights of 90000 and 180000 were chosen for species A and B, respectively, making the molecular weight of the resulting complex 270 000. Equilibrium constants (association) ranged from quite weak (0.01 ml/mg, 6×10^2 M^{-1}) to moderately strong (100 ml/mg, 6×10^6 M^{-1}). For each experiment listed at a particular concentration, the actual points consisted of a grid of values starting at 0.1-times the listed concentration and ranging to 0.9-times the listed concentration. Since this was done for both species A and B, the result was an 81-point grid. In addition, 10 equally spaced values for each A and B were added in the absence of the other species making a final grid of 101 points. The resulting 'ideal' weight-average molecular weights were then calculated. At this stage Gaussian random error of the specified standard deviation (1, 3 or 5% of the ideal value) was imposed on each of the concentrations and on each of the weight-average molecular weights using the NORMRAND function of the SPEAKEZ language. These 'experimental' data were then used for analysis.

3. Results

3.1. Fit of data

Table 1 presents the results for fitting the simulated data under a variety of different conditions. Table 1A–E represents K values of 0.01, 0.10, 1.0, 10.0 and 100 ml/mg, respectively. Each consists of 27 data sets, nine for each of the three concentration sets, 0.1, 1.0 and 10 mg/ml. These concentration entries are further grouped by the error imposed on concentration and each of these further grouped by the error imposed on molecular weights. For each data set there are three values listed. The calculated value of K (middle entry) as well as the limits of K given by the 67% confidence surface (upper and lower entries in parentheses). It is these confidence limits that

Table 1

Values of K obtained by surface analysis

Concentrations (mg/ml)	Error on concentration = 1% Error on M_w			Error on concentration = 3% Error on M_w			Error on concentration = 5% Error on M_w		
	1%	3%	5%	1%	3%	5%	1%	3%	5%
(A) $K = 0.01$ ml/mg									
0.1	(0.2128)	(0.4996)	(1.1266)	(0.1599)	(0.5956)	(1.0242)	(0.4465)	(0.3741)	(1.6119)
	0.0174	0.0081	0.0023	0.0122	0.0016	0.0018	0.1950	0.0019	0.4865
	(0.0009)	(0.0003)	(0.0002)	(0.0007)	(0.0004)	(0.0002)	(0.0018)	(0.0002)	(0.0002)
1.0	(0.04121)	(0.0356)	(0.1773)	(0.0254)	(0.0748)	(0.0469)	(0.0298)	(0.0696)	(0.0859)
	0.0243	0.0003	0.0516	0.0038	0.0098	0.0004	0.0007	0.0008	0.0005
	(0.0066)	(2E-5)	(2E-5)	(8E-5)	(2E-5)	(2E-5)	(5E-5)	(3E-5)	(2E-5)
10.0	(0.0113)	(0.0154)	(0.0173)	(0.0125)	(0.0137)	(0.0203)	(0.0134)	(0.0191)	(0.0264)
	0.0092	0.0084	0.0069	0.0010	0.0081	0.0087	0.0106	0.0110	0.0134
	(0.0068)	(0.0028)	(3E-6)	(0.0078)	(0.0028)	(3E-6)	(0.0079)	(0.0036)	(0.0024)
(B) $K = 0.1$ ml/mg									
0.1	(0.233)	(0.700)	(1.432)	(0.153)	(0.454)	(0.791)	(0.524)	(0.815)	(0.895)
	0.067	0.117	0.323	0.012	0.210	0.002	0.242	0.192	0.0201
	(0.001)	(4E-4)	(1E-4)	(0.001)	(0.009)	(1E-4)	(0.006)	(3E-4)	(2E-4)
1.0	(0.124)	(0.149)	(0.195)	(0.128)	(0.159)	(0.270)	(0.130)	(0.182)	(0.287)
	0.101	0.078	0.121	0.104	0.095	0.123	0.099	0.111	0.152
	(0.076)	(0.013)	(0.051)	(0.080)	(0.039)	(0.002)	(0.069)	(0.045)	(0.055)
1.0	(0.104)	(0.107)	(0.149)	(0.105)	(0.113)	(0.130)	(0.104)	(0.114)	(0.130)
	0.099	0.092	0.120	0.100	0.096	0.102	0.097	0.097	0,102
	(0.094)	(0.078)	(0.095)	(0.094)	(0.079)	(0.078)	(0.088)	(0.081)	(0.077)
(C) $K = 1$ ml/mg									
0.1	(1.16)	(1.36)	(1.86)	(1.08)	(1.90)	(2.53)	(1.44)	(1.45)	(1.89)
	0.90	0.73	0.684	0.85	1.07	1.15	1.15	0.66	0.78
	(0.71)	(0.13)	(4E-4)	(0.59)	(0.34)	(0.04)	(0.83)	(4E-4)	(2E-4)
1.0	(1.05)	(1.15)	(1.42)	(1.06)	(1.17)	1.31	1.05	1.19	1.31
	1.00	1.03	1.09	1.00	1.02	1.00	0.98	1.02	0.98
	(0.94)	(0.90)	(0.81)	(0.92)	(0.88)	(0.73)	(0.90)	(0.85)	(0.71)
10.0	(1.08)	(1.21)	(1.36)	(1.06)	(1.23)	(1.43)	(1.12)	(1.20)	(1.41)
	1.00	1.01	1.02	0.98	0.97	0.99	1.02	0.97	1.02
	(0.92)	(0.84)	(0.78)	(0.90)	(0.77)	(0.70)	(0.91)	(0.79)	(0.74)
(D) $K = 10$ ml/mg									
0.1	(10.3)	(10.4)	(12.7)	(10.7)	(12.0)	(13.6)	(10.7)	(11.6)	(13.9)
	9.8	8.71	9.90	10.19	10.23	10.27	10.03	10.0	10.8
	(9.2)	(7.1)	(7.4)	(9.6)	(8.5)	(7.5)	(9.3)	(8.4)	(8.1)
1.0	(10.7)	(11.6)	(12.9)	(10.6)	(11.1)	(12.3)	(11.1)	(12.5)	(15.5)
	10.1	9.7	9.5	9.7	9.02	9.1	10.1	10.2	10.5
	(9.4)	(8.1)	(7.0)	(0.9)	(7.3)	(6.7)	(9.0)	(8.4)	(7.2)
10.0	(12.9)	(25.1)	(71.9)	(14.7)	(28.9)	(38.4)	(16.0)	(24.9)	(140.6)
	10.4	11.2	9.8	10.4	12.9	9.1	9.2	10.3	12.2
	(8.3)	(6.7)	(4.2)	(7.7)	(7.6)	(4.1)	(5.9)	(5.8)	(4.5)

(continued overleaf)

Table 1 (continued)

Concentrations (mg/ml)	Error on concentration = 1% Error on M_w			Error on concentration = 3% Error on M_w			Error on concentration = 5% Error on M_w		
	1%	3%	5%	1%	3%	5%	1%	3%	5%
(E) $K = 100$ ml/mg									
0.1	(106.5)	(112.8)	(121.8)	(105.2)	(131.8)	(173.4)	(116.9)	(132.4)	(151.0)
	98.6	90.3	93.1	97.6	104.3	109.8	104.7	103.8	100.6
	(91.4)	(73.4)	(71.1)	(90.4)	(83.9)	(73.7)	(94.4)	(84.6)	(69.3)
1.0	(106.3)	(188.1)	(229.1)	(130.6)	(132.9)	(634.1)	(179.9)	(202.2)	(239.6)
	84.9	77.8	73.8	99.1	74.6	96.5	103.8	79.8	66.8
	(69.3)	(44.1)	(37.2)	(77.3)	(47.9)	(39.0)	(68.4)	(43.7)	(32.4)
10.0	(394.7)	(3.4E4)	(5.4E4)	(3.1E5)	(1.2E4)	(5.0E4)	(1.5E4)	(2.2E4)	(4.5E4)
	98.5	92.1	95.7	110.4	63.0	107.8	90.3	193.8	75.3
	(43.0)	(20.5)	(10.2)	(31.4)	(17.7)	(12.5)	(24.4)	(22.8)	(10.4)

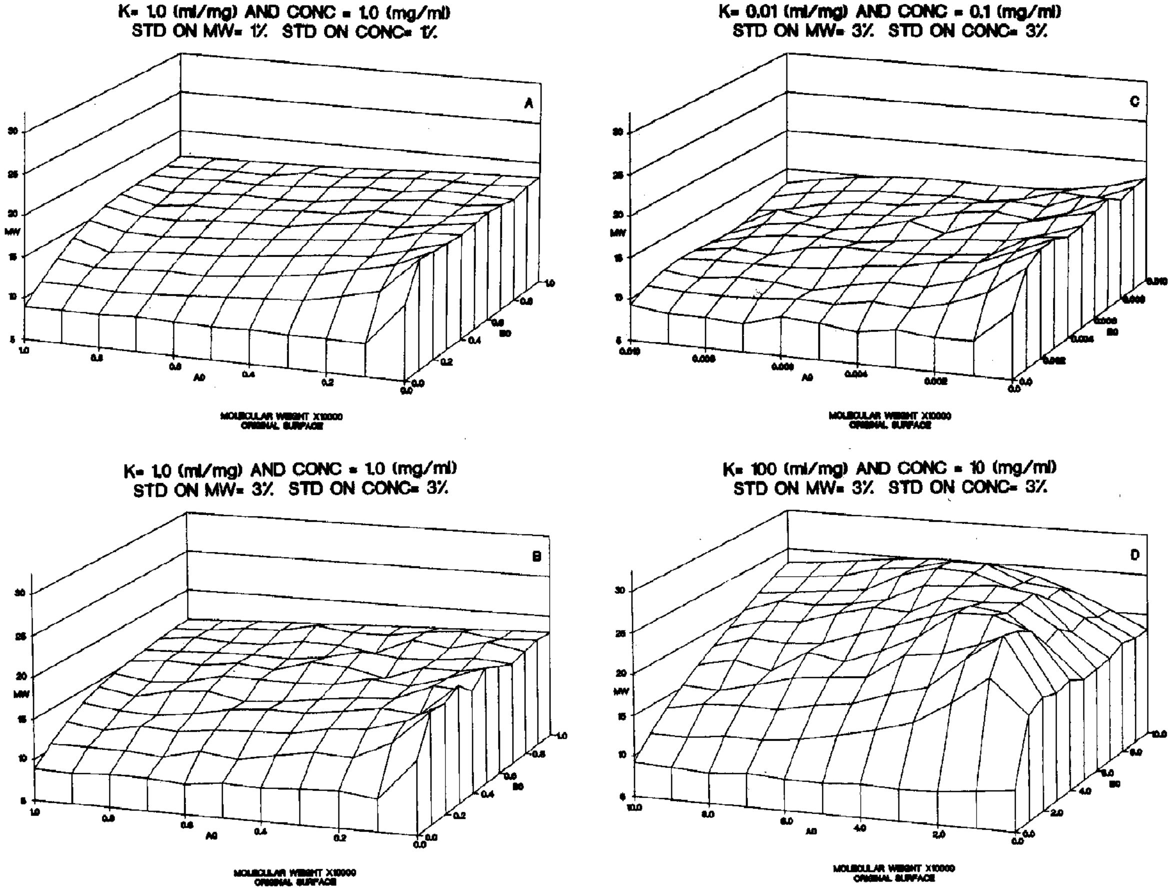

Fig. 1. Direct surface maps. Surface generated by plotting loading concentrations and the resulting weight-average molecular weights for four cases. Equilibrium (association) constants, maximum loading concentrations, and magnitude of random errors are given in each panel.

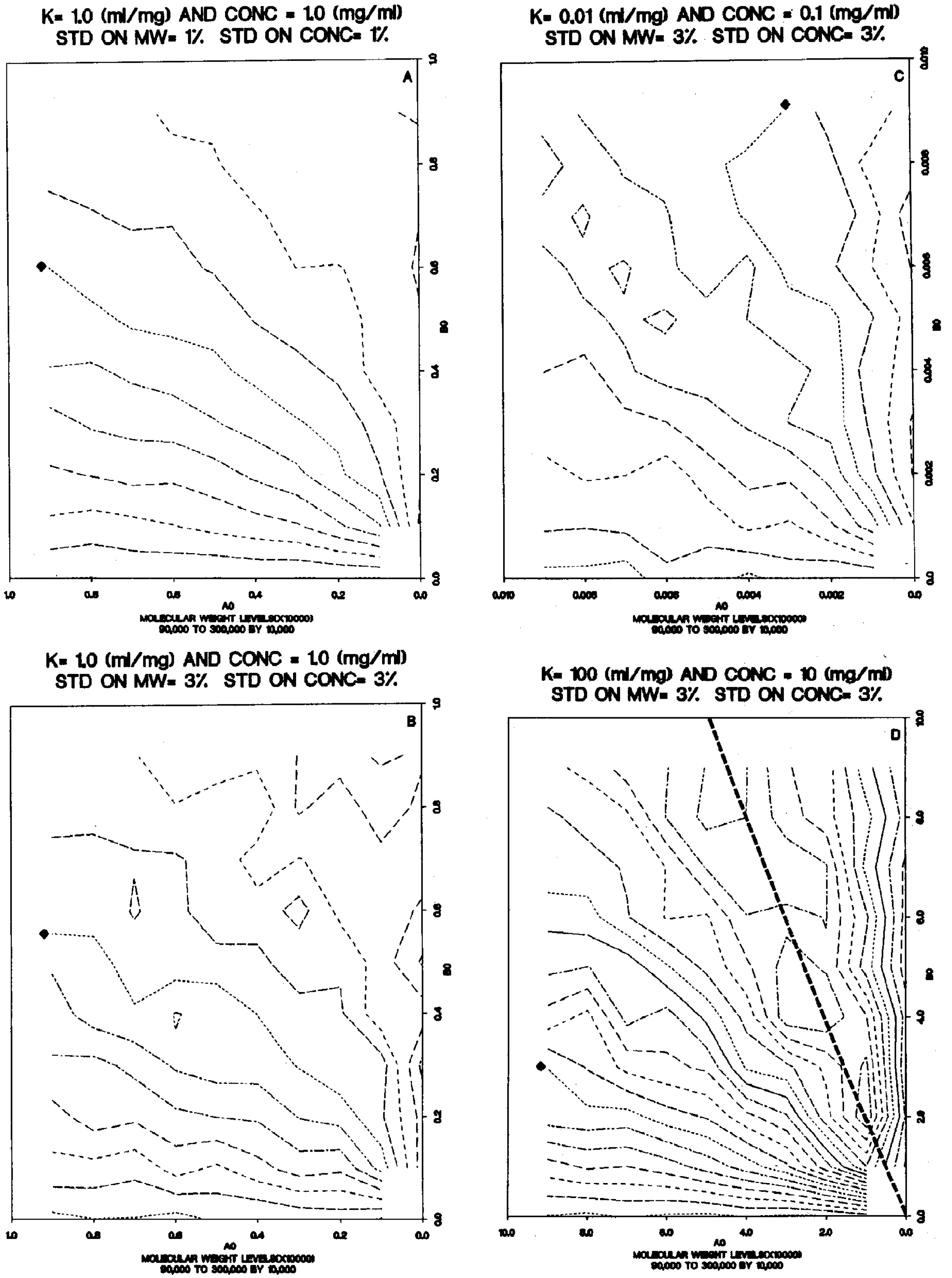

Fig. 2. Contour maps. Same data sets as in fig. 1 but represented as a contour map. Contour levels differ by steps of 10000 in molecular weight. For ease of interpretation, a molecular weight level of 150000 is indicated by a filled diamond on each map. The thick dashed line in panel D represents a 1 : 1 molar ratio transformed into a weight ratio.

really are the measure of the correctness of the fit. Several entries show that the data return values for the association constant quite close to the input value, but upon closer examination this closeness of fit is fortuitous. As would be expected, high concentrations and high association constants give less reliable data showing up particularly in the error on the upper limits while low concentrations and low association constants give low reliability particularly noticed in the lower error limit. Runs that were unreliable were those containing data where a very large majority of the points had at least one of the three components at a concentration less than the generated experimental error for the total concentration containing that species.

3.2. Sensitivity of K to 'experimental' error

Careful examination of table 1 shows that the effect of an error on the measured molecular weight has a larger effect than do errors in concentrations. Appendix B shows the evaluation of dK as a function of error in the three independent variables M_w, A_0 and B_0. We have verified the dominating effect of errors in M_w by comparing the relative effects of the three terms in eq. B1. Propagation of error analysis using these equations also verifies that if the values for free A, free B or complex are below the percentage expected from the experimental error, the uncertainty of the final analysis becomes extreme.

3.3. Surface maps

Fig. 1 shows three-dimensional representations of experimental data for four cases. The total concentrations of species A and B are shown along the x- and y-axes and the weight-average molecular weight along the z-axis. Three-dimensional maps allow the viewer to visualize quickly and easily the overall shape of the surface and the magnitude of errors. Fig. 1A is one example of a data set for which a good fit was obtained; the association constant obtained was close to the input value and the confidence limits were close to one another (table 1C). Note the smoothness of the surface in comparison to fig. 1B–D, where the fit of the data was poorer. Fig. 1A represents an intermediate association constant and relatively large concentrations of each of the three species, as well as low errors on both molecular weight and concentrations (1%). The surfaces become more irregular as the errors on molecular weights and concentrations are increased to 3% (fig. 1B), as association constants and concentrations are decreased so that less of the complex is found (fig. 1C), and as association constants and concentrations are increased so that less free A and B are present (fig. 1D).

3.4. Contour maps

Fig. 2 shows two-dimensional contour maps of the same data sets as in fig. 1. Shown along the x- and y-axes are the total concentrations of species A and B, while the molecular weight is represented by the contour levels. Each map starts the contour levels at 90000, located along the x-axis. Contours levels increase by 10000 as indicated in the figure legend. If several of the measured M_w values are larger than the greater of M_A or M_B, the contour maps allow one to estimate very quickly the stoichiometry (in weight ratios) of the complex even under circumstances where a K value cannot be determined.

Fig. 2D is the only case illustrated that allows a determination of the stoichiometry of the complex. In fig. 2D a line has been drawn along the surface corresponding to 2 g B per g A. Since $M_A = 90000$ and $M_B = 180000$ this line corresponds to a 1:1 molar ratio. Note that this line intersects all of the local maxima. Conversely, any line that is drawn through the maxima and the origin will have a slope that closely approximates the weight stoichiometry. If the concentrations of A_0 and B_0 were expressed as molar quantities the slope of this line would directly give a very good approximation to the molar stoichiometries.

4. Conclusion

A method is presented which analyzes an A_0, B_0 and M_w surface for an association constant. The limits of the system in terms of total concentrations, association constants and the effect of experimental error on the fit have been determined. This analysis is not method-dependent and requires only knowledge of A_0, B_0 and M_w at each point. The use of two different surface representations facilitates analysis, one allowing for visual inspection of the 'goodness of fit,' while the other allows for visual inspection of the stoichiometry of the system.

Appendix A

For the system

$$A + B \rightleftharpoons (AB) = C$$

the association constant can be expressed, in units of ml/mg, as

$$K = \frac{c}{ab}$$

Let M_r of species a $= M_1$, of b $= M_2$ and of c $= M_3$, where $M_3 = M_1 + M_2$. The total concentration of species a (a_0) and species b (b_0) can be expressed as

$$a_0 = a + \frac{M_1}{M_3} c$$

$$b_0 = b + \frac{M_2}{M_3} c$$

Let $r_1 = M_1/M_3$ and $r_2 = M_2/M_3$

$$a_0 = a + r_1 abK \qquad \text{(A1)}$$

$$b_0 = b + r_2 abK \qquad \text{(A2)}$$

by definition

$$M_W(a_0 + b_0) = aM_1 + bM_2 + abKM_3 \qquad \text{(A3)}$$

Rearranging eq. A1,

$$a = \frac{a_0}{1 + r_1 bK}.$$

Substituting into eq. A2

$$b_0 = b + \frac{r_2 bKa_0}{1 + r_1 bK}$$

$$b_0 + b_0 r_1 bK = b + r_1 Kb^2 + r_2 bKa_0$$

$$b^2 r_1 K + b[1 + r_2 Ka_0 - b_0 r_1 K] - b_0 = 0$$

$$b = \frac{-(1 + r_2 Ka_0 - r_1 Kb_0) \pm \sqrt{(1 + r_2 Ka_0 - r_1 Kb_0)^2 + 4b_0 r_1 K}}{2r_1 K}$$

Note that the only variable on the right-hand side is K. Let $X = r_2 a_0 - r_1 b_0$ and $W = 1 + r_1 bK$. Therefore

$$b = \frac{-(1 + KX) \pm \sqrt{(1 + KX)^2 + 4b_0 r_1 K}}{2r_1 K}$$

Let $Q=((1+KX)^2+4b_0r_1K)^{1/2}$. Now

$$b=\frac{-(1+KX)\pm Q}{2r_1K}$$

Rewriting eq. A3

$$M_\mathrm{W}(a_0+b_0)=\frac{a_0M_1}{W}+\frac{a_0bKM_3}{W}+bM_2=\frac{a_0}{W}(M_1+bKM_3)+bM_2$$

Define $Y=M_\mathrm{W}(a_0+b_0)$, $F=a_0(M_1+bKM_3)/W+bM_2$ and $R=\Sigma(Y-F)^2$

$$\frac{\mathrm{d}R}{\mathrm{d}K}=-2\left[\Sigma(Y-F)\frac{\mathrm{d}F}{\mathrm{d}K}\right]=0=G$$

$$G=\Sigma Y\frac{\mathrm{d}F}{\mathrm{d}K}-\Sigma F\frac{\mathrm{d}F}{\mathrm{d}K} \tag{A4}$$

$$\frac{\mathrm{d}G}{\mathrm{d}K}=\frac{\mathrm{d}}{\mathrm{d}K}\left(\Sigma Y\frac{\mathrm{d}F}{\mathrm{d}K}\right)-\frac{\mathrm{d}}{\mathrm{d}K}\left(\Sigma F\frac{\mathrm{d}F}{\mathrm{d}K}\right)=\Sigma Y\frac{\mathrm{d}^2F}{\mathrm{d}K^2}-\Sigma\left(\frac{\mathrm{d}F}{\mathrm{d}K}\right)^2-\Sigma F\frac{\mathrm{d}^2F}{\mathrm{d}K^2} \tag{A5}$$

To use eq. A5 we must evaluate $\mathrm{d}F/\mathrm{d}K$ and $\mathrm{d}^2F/\mathrm{d}K^2$

$$\frac{\mathrm{d}F}{\mathrm{d}K}=\frac{\mathrm{d}}{\mathrm{d}K}\left[\frac{a_0M_1}{W}+\frac{a_0bKM_3}{W}+bM_2\right]$$

$$=\frac{-a_0M_1}{W^2}\frac{\mathrm{d}W}{\mathrm{d}K}+\frac{a_0KM_3}{W}\frac{\mathrm{d}b}{\mathrm{d}K}+\frac{a_0bM_3}{W}-\frac{a_0bKM_3\frac{\mathrm{d}W}{\mathrm{d}K}}{W^2}+M_2\frac{\mathrm{d}b}{\mathrm{d}K} \tag{A6}$$

Since $W=1+r_1bK$, $\mathrm{d}W/\mathrm{d}K=r_1b+r_1K'\mathrm{d}b/\mathrm{d}K=r_1(b+K\cdot\mathrm{d}b/\mathrm{d}K)$ and $b=(-(1+KX)+Q)/2r_1K$ (use only the plus option for physically meaningful parameters)

$$\frac{\mathrm{d}b}{\mathrm{d}K}=\frac{\mathrm{d}}{\mathrm{d}K}\left[\frac{-1}{2r_1K}-\frac{KX}{2r_1K}+\frac{Q}{2r_1K}\right]=\frac{1}{2r_1K^2}+\frac{\frac{\mathrm{d}Q}{\mathrm{d}K}}{2r_1K}-\frac{Q}{2r_1K^2}=\frac{1-Q}{2r_1K^2}+\frac{\frac{\mathrm{d}Q}{\mathrm{d}K}}{2r_1K} \tag{A7}$$

Since $Q=[(1+KX)^2+4b_0r_1K]^{1/2}$

$$\frac{\mathrm{d}Q}{\mathrm{d}K}=\frac{\mathrm{d}}{\mathrm{d}K}\left[1+2KX+K^2X^2+4b_0r_1K\right]^{1/2}$$

$$=\frac{\frac{1}{2}(2X+2KX^2+4b_0r_1)}{[1+2KX+K^2X^2+4b_0r_1K]}=\frac{(1+KX)X+2b_0r_1}{Q} \tag{A8}$$

From eq. A7

$$\frac{\mathrm{d}b}{\mathrm{d}K}=\frac{1-Q}{2r_1K^2}+\frac{(1+KX)X+2b_0r_1}{2r_1KQ}$$

Substituting for $\mathrm{d}W/\mathrm{d}K$ in eq. A6,

$$\frac{\mathrm{d}F}{\mathrm{d}K}=\frac{-a_0M_1r_1}{W^2}\left(b+K\frac{\mathrm{d}b}{\mathrm{d}K}\right)+\frac{a_0KM_3}{W}\frac{\mathrm{d}b}{\mathrm{d}K}+\frac{a_0bM_3}{W}-\frac{a_0bKM_3r_1}{W^2}\left(b+K\frac{\mathrm{d}b}{\mathrm{d}K}\right)+M_2\frac{\mathrm{d}b}{\mathrm{d}K}$$

$$=\frac{-a_0M_1r_1}{W^2}\left(b+K\frac{\mathrm{d}b}{\mathrm{d}K}\right)+\frac{a_0KM_3(1+r_1bK)}{W^2}\frac{\mathrm{d}b}{\mathrm{d}K}+\frac{a_0bM_3(1+r_1bK)}{W^2}$$

$$-\frac{a_0bKM_3r_1}{W^2}\left(b+K\frac{\mathrm{d}b}{\mathrm{d}M}\right)+M_2\frac{\mathrm{d}b}{\mathrm{d}K}$$

$$=\frac{-a_0M_1r_1b}{W^2}-\frac{a_0M_1r_1K}{W^2}\frac{\mathrm{d}b}{\mathrm{d}K}+\frac{a_0M_3K}{W^2}\frac{\mathrm{d}b}{\mathrm{d}K}+\frac{a_0M_3r_1bK^2}{W^2}\frac{\mathrm{d}b}{\mathrm{d}K}$$

$$+\frac{a_0bM_3}{W^2}+\frac{a_0b^2M_3r_1K}{W^2}-\frac{a_0b^2KM_3r_1}{W^2}-\frac{a_0bM_3r_1K^2}{W^2}\frac{\mathrm{d}b}{\mathrm{d}K}+M_2\frac{\mathrm{d}b}{\mathrm{d}K}$$

$$=\frac{-a_0M_1r_1b}{W^2}+\frac{a_0bM_3}{W^2}-\frac{a_0M_1r_1K}{W^2}\frac{\mathrm{d}b}{\mathrm{d}K}+\frac{a_0M_3K}{W^2}\frac{\mathrm{d}b}{\mathrm{d}K}+M_2\frac{\mathrm{d}b}{\mathrm{d}K}$$

$$=\frac{a_0(M_3-M_1r_1)}{W^2}b+\frac{a_0(M_3-M_1r_1)}{W^2}K\frac{\mathrm{d}b}{\mathrm{d}K}+M_2\frac{\mathrm{d}b}{\mathrm{d}K}$$

$$=\frac{a_0(M_3-M_1r_1)}{(1+r_1bK)^2}\left[b+K\frac{\mathrm{d}b}{\mathrm{d}K}\right]+M_2\frac{\mathrm{d}b}{\mathrm{d}K} \tag{A9}$$

Now we need to evaluate $\mathrm{d}^2F/\mathrm{d}K^2$

$$\frac{\mathrm{d}^2F}{\mathrm{d}K^2}=\frac{\mathrm{d}}{\mathrm{d}K}\left[a_0(M_3-M_1r_1)\frac{\mathrm{d}}{\mathrm{d}K}\left[\frac{K\frac{\mathrm{d}b}{\mathrm{d}K}+b}{(1+r_1bK)^2}\right]+M_2\frac{\mathrm{d}}{\mathrm{d}K}\left(\frac{\mathrm{d}b}{\mathrm{d}K}\right)\right]$$

$$=a_0(M_3-M_1r_1)\left[\frac{\frac{\mathrm{d}b}{\mathrm{d}K}}{(1+r_1bK)^2}+K\frac{\frac{\mathrm{d}^2b}{\mathrm{d}K^2}}{(1+r_1bK)^2}-\frac{2K^2r_1\left(\frac{\mathrm{d}b}{\mathrm{d}K}\right)^2}{(1+r_1bK)^3}-\frac{2r_1bK\frac{\mathrm{d}b}{\mathrm{d}K}}{(1+r_1bK)^3}+\frac{\frac{\mathrm{d}b}{\mathrm{d}K}}{(1+r_1bK)^2}\right.$$

$$\left.-\frac{2br_1K\frac{\mathrm{d}b}{\mathrm{d}K}}{(1+r_1bK)^3}-\frac{2r_1b^2}{(1+r_1bK)^3}\right]+M_2\frac{\mathrm{d}^2b}{\mathrm{d}K^2}$$

$$=a_0(M_3-M_1r_1)\left[\frac{\frac{\mathrm{d}b}{\mathrm{d}K}}{W_2}+\frac{K\frac{\mathrm{d}^2b}{\mathrm{d}K^2}}{W^2}-\frac{2r_1K^2\left(\frac{\mathrm{d}b}{\mathrm{d}K}\right)^2}{W^3}\right.$$

$$\left.-\frac{2r_1bK\frac{\mathrm{d}b}{\mathrm{d}K}}{W^3}+\frac{\frac{\mathrm{d}b}{\mathrm{d}K}}{W^2}-\frac{2br_1K\frac{\mathrm{d}b}{\mathrm{d}K}}{W^3}-\frac{2b^2r_1}{W^3}\right]+M_2\frac{\mathrm{d}^2b}{\mathrm{d}K^2}$$

$$=a_0(M_3-M_1r_1)\left[\frac{2\frac{\mathrm{d}b}{\mathrm{d}K}}{W^2}-\frac{4r_1bK\frac{\mathrm{d}b}{\mathrm{d}K}}{W^3}+\frac{K\frac{\mathrm{d}^2b}{\mathrm{d}K^2}}{W^2}-\frac{2r_1K^2\left(\frac{\mathrm{d}b}{\mathrm{d}K}\right)^2}{W^3}-\frac{2b^2r_1}{W^3}\right]+M_2\frac{\mathrm{d}^2b}{\mathrm{d}K^2} \tag{A10}$$

To evaluate $\mathrm{d}^2b/\mathrm{d}K^2$

$$\frac{\mathrm{d}^2b}{\mathrm{d}K^2}=\frac{\mathrm{d}}{\mathrm{d}K}\left(\frac{\mathrm{d}b}{\mathrm{d}K}\right)=\frac{\mathrm{d}}{\mathrm{d}K}\left[\frac{1-Q}{2r_1K^2}+\frac{(1+KX)X+2b_0r_1}{2r_1KQ}\right]$$

$$=\frac{\mathrm{d}}{\mathrm{d}K}\left[\frac{1}{2r_1K^2}-\frac{Q}{2r_1K^2}+\frac{X}{2r_1KQ}+\frac{KX^2}{2r_1KQ}+\frac{2b_0r_1}{2r_1KQ}\right]$$

$$= \frac{-2}{2r_1K^3} - \frac{\frac{\mathrm{d}Q}{\mathrm{d}K}}{2r_1K^2} + \frac{2Q}{2r_1K^3} - \frac{X}{2r_1QK^2} - \frac{X\frac{\mathrm{d}Q}{\mathrm{d}K}}{2r_1KQ^2} - \frac{X^2\frac{\mathrm{d}Q}{\mathrm{d}K}}{2r_1Q^2} - \frac{b_0\frac{\mathrm{d}Q}{\mathrm{d}K}}{KQ^2} - \frac{b_0}{QK^2}$$

$$= \frac{Q-1}{r_1K^3} - \frac{X}{2r_1K^2Q} - \frac{b_0}{QK^2} - \frac{\mathrm{d}Q}{\mathrm{d}K}\left[\frac{1}{2r_1K^2} + \frac{X}{2r_1KQ^2} + \frac{X^2}{2r_1Q^2} + \frac{b_0}{KQ^2}\right]$$

$$= \frac{Q-1}{r_1K^3} - \frac{[X+2b_0r_1]}{2r_1K^2Q} - \frac{\mathrm{d}Q}{\mathrm{d}K}\left[\frac{Q^2+XK+K^2X^2+2b_0r_1K}{2r_1K^2Q^2}\right]$$

From eq. A8

$$\frac{\mathrm{d}Q}{\mathrm{d}K} = \frac{(1+KX)X+2b_0r_1}{Q}$$

Therefore

$$\frac{\mathrm{d}^2b}{\mathrm{d}K^2} = \frac{Q-1}{r_1K^3} - \frac{[X+2b_0r_1]}{2r_1K^2Q} - \frac{[(1+KX)X+2b_0r_1]}{Q}\left[\frac{Q^2+XK+K^2X^2+2b_0r_1K}{2r_1K^2Q^2}\right]$$

For a Newton-Raphson analysis

$$K_{\text{new}} = K_{\text{old}} - \frac{G}{\frac{\mathrm{d}G}{\mathrm{d}K}}$$

G and $\mathrm{d}G/\mathrm{d}K$ are evaluated in eqs. A4 and A5. All the necessary terms have now been evaluated.

Appendix B

$$K = \frac{M_w(a_0+b_0) - aM_1 - bM_2}{abM_3}$$

$$= \frac{M_w(a_0+b_0)}{abM_3} - \frac{r_1}{b} - \frac{r_2}{a}$$

Therefore, the independent variables are a_0, b_0 and M_w

$$\mathrm{d}K = \frac{\partial K}{\partial M_w}\,\mathrm{d}M_w + \frac{\partial K}{\partial a_0}\,\mathrm{d}a_0 + \frac{\partial K}{\partial b_0}\,\mathrm{d}b_0 \qquad \text{(B1)}$$

The individual terms are

$$\frac{\partial K}{\partial M_w} = \frac{a_0+b_0}{abM_3} - \frac{M_w(a_0+b_0)bM_3}{(abM_3)^2}\frac{\mathrm{d}a}{\mathrm{d}M_w} - \frac{M_w(a_0+b_0)aM_3}{(abM_3)^2}\frac{\mathrm{d}b}{\mathrm{d}M_w} + \frac{r_1}{b^2}\frac{\mathrm{d}b}{\mathrm{d}M_w} + \frac{r_2}{a^2}\frac{\mathrm{d}a}{\mathrm{d}M_w}$$

$$\frac{\partial K}{\partial a_0} = \frac{M_w}{abM_3} - \frac{M_w(a_0+b_0)bM_3}{(abM_3)^2}\frac{\mathrm{d}a}{\mathrm{d}a_0} - \frac{M_w(a_0+b_0)aM_3}{(abM_3)^2}\frac{\mathrm{d}b}{\mathrm{d}a_0} + \frac{r_1}{b^2}\frac{\mathrm{d}b}{\mathrm{d}a_0} + \frac{r_2}{a^2}\frac{\mathrm{d}a}{\mathrm{d}a_0}$$

$$\frac{\partial K}{\partial b_0} = \frac{M_w}{abM_3} - \frac{M_w(a_0+b_0)bM_3}{(abM_3)^2}\frac{\mathrm{d}a}{\mathrm{d}b_0} - \frac{M_w(a_0+b_0)aM_3}{(abM_3)^2}\frac{\mathrm{d}b}{\mathrm{d}b_0} + \frac{r_1}{b^2}\frac{\mathrm{d}b}{\mathrm{d}b_0} + \frac{r_2}{a^2}\frac{\mathrm{d}a}{\mathrm{d}b_0}$$

Collected together

$$\mathrm{d}K=\frac{(a_0+b_0)\,\mathrm{d}M_\mathrm{w}}{abM_3}+\frac{M_\mathrm{w}\,\mathrm{d}a_0}{abM_3}+\frac{M_\mathrm{w}\mathrm{d}b_0}{abM_3}-\frac{M_\mathrm{w}(a_0+b_0)bM_3}{(abM_3)^2}\left[\frac{\mathrm{d}a}{\mathrm{d}M_\mathrm{w}}\,\mathrm{d}M_\mathrm{w}+\frac{\mathrm{d}a}{\mathrm{d}a_0}\,\mathrm{d}a_0+\frac{\mathrm{d}a}{\mathrm{d}b_0}\,\mathrm{d}b_0\right]$$

$$-\frac{M_\mathrm{w}(a_0+b_0)aM_3}{(abM_3)^2}\left[\frac{\mathrm{d}b}{\mathrm{d}M_\mathrm{w}}\mathrm{d}M_\mathrm{w}+\frac{\mathrm{d}b}{\mathrm{d}a_0}\,\mathrm{d}a_0+\frac{\mathrm{d}b}{\mathrm{d}b_0}\,\mathrm{d}b_0\right]$$

$$+\frac{r_1}{b^2}\left[\frac{\mathrm{d}b}{\mathrm{d}M_\mathrm{w}}\,\mathrm{d}M_\mathrm{w}+\frac{\mathrm{d}b}{\mathrm{d}a_0}\,\mathrm{d}a_0+\frac{\mathrm{d}b}{\mathrm{d}b_0}\,\mathrm{d}b_0\right]$$

$$+\frac{r_2}{a^2}\left[\frac{\mathrm{d}a}{\mathrm{d}M_\mathrm{w}}\,\mathrm{d}M_\mathrm{w}+\frac{\mathrm{d}a}{\mathrm{d}a_0}\,\mathrm{d}a_0+\frac{\mathrm{d}a}{\mathrm{d}b_0}\,\mathrm{d}b_0\right]$$

Define $X=r_2a_0-r_1b_0$; $Q=\sqrt{(1+KX)^2+4b_0r_1K}$

$$\frac{\mathrm{d}X}{\mathrm{d}a_0}=r_2$$

$$\frac{\mathrm{d}X}{\mathrm{d}b_0}=-r_1$$

From appendix A

$$b=\frac{-(1+KX)+Q}{2r_1K}$$

$$\frac{\mathrm{d}b}{\mathrm{d}a_0}=\frac{-K\dfrac{\mathrm{d}X}{\mathrm{d}a_0}}{2r_1K}+\frac{\dfrac{\mathrm{d}Q}{\mathrm{d}a_0}}{2r_1K}\qquad\frac{\mathrm{d}Q}{\mathrm{d}a_0}=\left(\tfrac{1}{2}\left[2K(1+KX)\frac{\mathrm{d}X}{\mathrm{d}a_0}\right]\right)/Q=(K(1+KX)r_2)/Q$$

$$=\frac{-Kr_2}{2r_1K}+\frac{K(1+KX)r_2}{2r_1KQ}$$

$$=-\frac{1}{2}\frac{r_2}{r_1}+\frac{1}{2}\frac{r_2}{r_1}\frac{(1+KX)}{Q}$$

so that

$$\frac{\mathrm{d}b}{\mathrm{d}a_0}=\frac{r_2}{2r_1}\left(\frac{1+KX}{Q}-1\right)$$

and

$$\frac{\mathrm{d}b}{\mathrm{d}b_0}=\frac{-K\dfrac{\mathrm{d}X}{\mathrm{d}b_0}}{2r_1K}+\frac{\dfrac{\mathrm{d}Q}{\mathrm{d}b_0}}{2r_1K}\qquad\frac{\mathrm{d}Q}{\mathrm{d}b_0}=\left(\tfrac{1}{2}\left[2K(1+KX)\frac{\mathrm{d}X}{\mathrm{d}b_0}+4r_1K\right]\right)/Q$$

$$=\frac{-K(-r_1)}{2r_1K}+\frac{K[(1+KX)(-r_1)+2r_1]}{2r_1KQ}$$

$$=\frac{1}{2}+\frac{[-1-KX+2]}{2Q}$$

$$=\frac{1}{2}+\frac{1-KX}{2Q}$$

so that

$$\frac{\mathrm{d}b}{\mathrm{d}b_0}=\frac{1}{2}\left[1+\frac{1-KX}{Q}\right]$$

$$a=\frac{-(1+r_1Kb_0-r_2Ka_0)+\sqrt{(1+r_1Kb_0-r_2Ka_0)^2+4a_0r_2K}}{2r_2K}$$

Let $X'=r_1b_0-r_2a_0=-X$; $\mathrm{d}X'/\mathrm{d}a_0=-r_2$; $\mathrm{d}X'/\mathrm{d}b_0=r_1$ and $Q'=[(1+KX')^2+4a_0r_2K]^{1/2}$. Therefore

$$a=\frac{-(1+KX')+Q'}{2r_2K}$$

$$\frac{\mathrm{d}a}{\mathrm{d}b_0}=\frac{-K\frac{\mathrm{d}X'}{\mathrm{d}b_0}}{2r_2K}+\frac{\frac{\mathrm{d}Q'}{\mathrm{d}b_0}}{2r_2K}$$

$$\frac{\mathrm{d}Q'}{\mathrm{d}b_0}=\frac{\frac{1}{2}\left[2K(1+KX')\frac{\mathrm{d}X'}{\mathrm{d}b_0}\right]}{Q'}$$

$$=\frac{-Kr_1}{2r_2K}+\frac{K(1+KX')r_1}{2r_2KQ'}$$

$$=\frac{-r_1}{2r_2}+\frac{(1+KX')r_1}{2Q'r_2}$$

$$\frac{\mathrm{d}a}{\mathrm{d}b_0}=\frac{r_1}{2r_2}\left[-1+\frac{1+KX'}{Q'}\right]$$

$$\frac{\mathrm{d}a}{\mathrm{d}a_0}=\frac{-K(-r_2)}{2r_2K}+\frac{\frac{\mathrm{d}Q'}{\mathrm{d}a_0}}{2r_2K}\qquad\frac{\mathrm{d}Q'}{\mathrm{d}a_0}=\left(\frac{1}{2}\left[2K(1+KX')+\frac{\mathrm{d}X'}{\mathrm{d}a_0}+4r_2K\right]\right)/Q'$$

$$=\frac{-K(-r_2)}{2r_2K}+\frac{K(1+KX')(-r_2)+2r_2K}{2r_2KQ'}$$

$$=\frac{1}{2}+\frac{-(1+KX')+2}{2Q'}$$

$$=\frac{1}{2}+\frac{-1-KX'+2}{2Q'}$$

$$=\frac{1}{2}+\frac{1-KX'}{2Q'}$$

$$\frac{\mathrm{d}a}{\mathrm{d}a_0}=\frac{1}{2}\left[1+\frac{1-KX'}{Q'}\right]$$

Starting with $M_\mathrm{w}(a_0+b_0)=aM_1+bM_2+abKM_3$ and using $a=a_0/(1+r_1bK)$

$$bM_2+\frac{ba_0KM_3}{1+r_1bK}=M_\mathrm{w}(a_0+b_0)-\frac{a_0M_1}{1+r_1bK}$$

$$(1+r_1bK)bM_2+ba_0KM_3=M_\mathrm{w}(a_0+b_0)(1+r_1bK)-a_0M_1$$

$$bM_2+r_1KM_2b^2+ba_0KM_3=M_\mathrm{w}(a_0+b_0)+M_\mathrm{w}(a_0+b_0)r_1bK-a_0M_1$$

$$r_1KM_2b^2 + b\left(M_2 + a_0KM_3 - M_w(a_0+b_0)r_1K\right) - M_w(a_0+b_0) + a_0M_1 = 0$$

$$R = M_2 + a_0KM_3 - M_w(a_0+b_0)r_1K$$

$$Z = \left[R^2 - 4r_1KM_2\left(a_0M_1 - M_w(a_0+b_0)\right)\right]^{1/2}$$

$$b = \frac{-R+Z}{2r_1KM_2}$$

$$\frac{\mathrm{d}b}{\mathrm{d}M_w} = \frac{\mathrm{d}}{\mathrm{d}M_w}\left[\frac{-R+Z}{2r_1KM_2}\right] = \frac{1}{2r_1KM_2}\left[\frac{-\mathrm{d}R}{\mathrm{d}M_w} + \frac{\mathrm{d}Z}{\mathrm{d}M_w}\right]$$

$$\frac{\mathrm{d}R}{\mathrm{d}M_w} = \frac{\mathrm{d}}{\mathrm{d}M_w}\left[M_2 + a_0KM_3 - M_w(a_0+b_0)r_1K\right] = -r_1K(a_0+b_0)$$

$$\frac{\mathrm{d}Z}{\mathrm{d}M_w} = \frac{\mathrm{d}}{\mathrm{d}M_w}\left[\left(R^2 - 4r_1KM_2\left(a_0M_1 - M_w(a_0+b_0)\right)\right)^{1/2}\right]$$

$$= \frac{\frac{1}{2}\left[2R\frac{\mathrm{d}R}{\mathrm{d}M_w} + 4r_1KM_2(a_0+b_0)\right]}{Z}$$

$$= \frac{R\left(-r_1K(a_0+b_0)\right) + 2r_1KM_2(a_0+b_0)}{Z} = \frac{2r_1KM_2(a_0+b_0) - r_1RK(a_0+b_0)}{Z}$$

$$\frac{\mathrm{d}b}{\mathrm{d}M_w} = \frac{1}{2r_1KM_2}\left[\frac{-\mathrm{d}R}{\mathrm{d}M_w} + \frac{\mathrm{d}Z}{\mathrm{d}M_w}\right] = \frac{1}{2r_1KM_2}\left[r_1K(a_0+b_0) + \frac{2r_1KM_2(a_0+b_0) - r_1KR(a_0+b_0)}{Z}\right]$$

$$\frac{\mathrm{d}b}{\mathrm{d}M_w} = \frac{(a_0+b_0)}{2M_2}\left[1 + \frac{2M_2 - R}{Z}\right] = \frac{(a_0+b_0)}{2M_2}\left[1 + \frac{M_2 - a_0KM_3 + M_w(a_0+b_0)r_1K}{Z}\right]$$

$\mathrm{d}a/\mathrm{d}M_w$ could be handled in a symmetric fashion with M_2 being replaced by M_1; R by R', and Z by Z' where the parameters denoted by primes are symmetrical with the unprimed parameters.

Acknowledgements

Part of this work was supported by a grant from NIAID (AI23099). We also wish to thank the staff at the Clemson University Computing Center for their help.

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