Determination of Equilibrium Constants of Associating Protein Systems. Graphical Analysis for Discrete and Indefinite Association*

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ABSTRACT: Simplified graphical procedures for evaluation of the mode of association from weight-average molecular weight as a function of concentration in the ideal case have been derived. The procedures are applicable to several types of discrete and indefinite association. D. E. Roark and D. A. Yphantis ((1969), *Ann. N. Y. Acad. Sci.* (in press)) in graphical analysis of three species in chemical equilibrium, propose the use of the quantity: $d(M_wM_zM_n)/dM_n = A\ (d(M_nM_w)/dM_n + B$. However, the procedure requires double differentiation which introduces a high probability of error espe cially in the vicinity of zero concentration. Therefore, we propose a greatly simplified procedure which would substitute the weight fraction monomer (E. T. Adams, Jr., and J. W. Williams (1964), *J. Am. Chem. Soc. 86*, 3454) for this key quantity.

Also developed are standard plots for M_w/M_1 *vs.* M_1/M_n or M_w/M_1 *vs.* f_1 to be used for determining monomer–*n*-mer or indefinite association.

In recent years numerous theoretical treatments of associating protein systems have appeared which attempt to interpret data obtained by a variety of physical techniques. The theoretical approaches to such data are many and varied, often involving detailed and lengthy numerical manipulation to achieve the desired interpretation (Steiner, 1952; Gilbert and Jenkins, 1963; Rao and Kegeles, 1958; Bethune and Kegeles, 1961; Adams, 1965a,b, 1967b; Adams and Williams, 1964; Chun *et al.*, 1968; Chun *et al.*, 1969; Ackers and Thompson, 1965; Ackers, 1967a,b).

More recently, Roark and Yphantis (1969) described a technique for graphical analysis of two or three species involved in chemical equilibrium, estimating stoichiometry in ideal cases. For a monomer–*n*-mer association, they plot M_w *vs.* $1/M_n$ and for three species, they plot the quantity $d(M_nM_zM_w)/dM_n$ *vs.* $d(M_nM_w)/dM_n$. The evaluation of $d(M_nM_zM_w)/dM_n$ requires double differentiation, which is unwieldy and increases the probability of error in setting up the plot.

In the simplified procedure introduced in this paper, the M_z value (z-average molecular weight) is eliminated, being replaced by the more workable weight fraction monomer. Using the numerically reduced interpretation, it is possible to establish standard plots of M_w/M_1 *vs.* M_1/M_n and M_w/M_1 *vs.* f_1 for monomer–*n*-mer or indefinite association. When three species are involved, we propose a plot of $d(M_w/M_1)/df_1$ *vs.* $d(M_1/M_n)/df_1$.

The results of this graphical application to ideal cases can be checked by Adam's procedure (1967a) or by procedures for matrix transformation in evaluating BM_1 and weight fraction monomer (Chun and Kim, 1969).

The validity of the interaction parameters obtained by graphical analysis can be checked by our simplified sedimentation equilibrium boundary analysis (Chun and Kim, 1969, and in preparation, 1970).

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Enumeration of Basic Quantities and Determination of Stoichiometry of Self-Associating Solutes

Assumptions and Basic Equations. The assumptions that are used in the analysis of discrete and indefinite associations based on sedimentation equilibrium experiments are (1) that the partial specific volumes of all species are the same, (2) that the refractive index increments of all species are equal, (3) that the system is ideal in that the activity coefficient of each solute species is unity, and (4) it is assumed that the system undergoes no volume change on chemical reaction. The weight-average molecular weight (Goldberg, 1963; Adams and Williams, 1964) for the associating system is

$$\frac{M_1}{M_{w\,app}} = \frac{M_1}{M_{w(c)}} + BM_1C \tag{1}$$

The concentration of monomer for a nonideal solution as expressed by Adams and Williams (1964) is $C_1 = \alpha \exp[-BM_1C]$, where

$$\alpha = c \exp\int_0^c \left(\frac{M_1}{M_{w\,app}} - 1\right)\frac{dC}{C} \tag{2}$$

α is obtained from the integration of $(M_1/M_{w\,app} - 1)$ *vs.* C. The number-average molecular weight (Adams, 1965a), $M_{n\,app}$ is defined as

$$\frac{M_1}{M_{n\,app}} = \frac{1}{C}\int_0^c \frac{M_1}{M_{w\,app}}dC + \frac{1}{2}BM_1C \tag{3}$$

For ideal cases, the BM_1 term becomes zero and eq 1-3 become

$$\frac{M_{w\,app}}{M_1} = \frac{M_{w(c)}}{M_1} = \frac{1}{M_1}\sum_i \frac{C_iM_i}{C} = \sum_i if_i \tag{4}$$

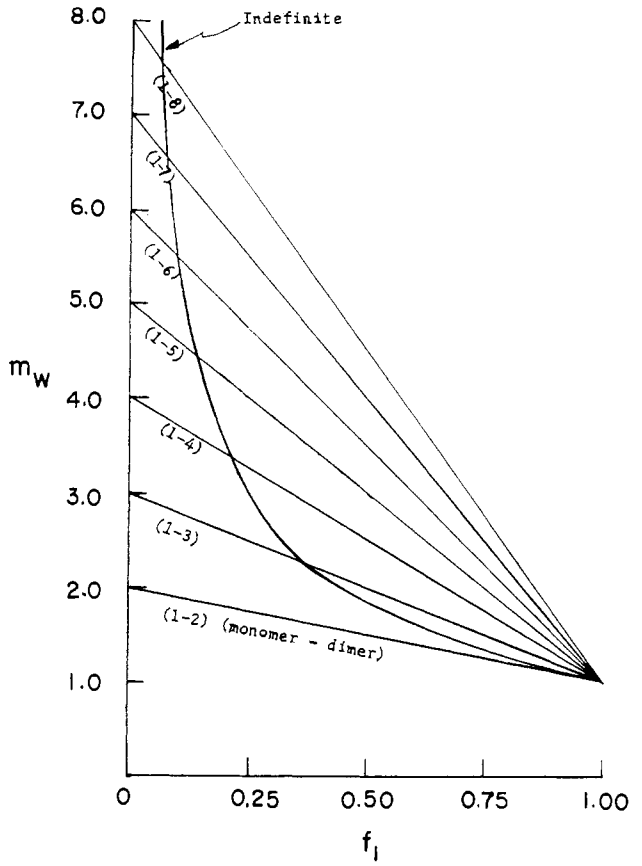

FIGURE 1: Standard plot of m_w *vs.* f_i for monomer–*n*-mer and indefinite association. Plot is based on eq 7 and $m_w = (2/(f_i)^{1/2}) - 1$.

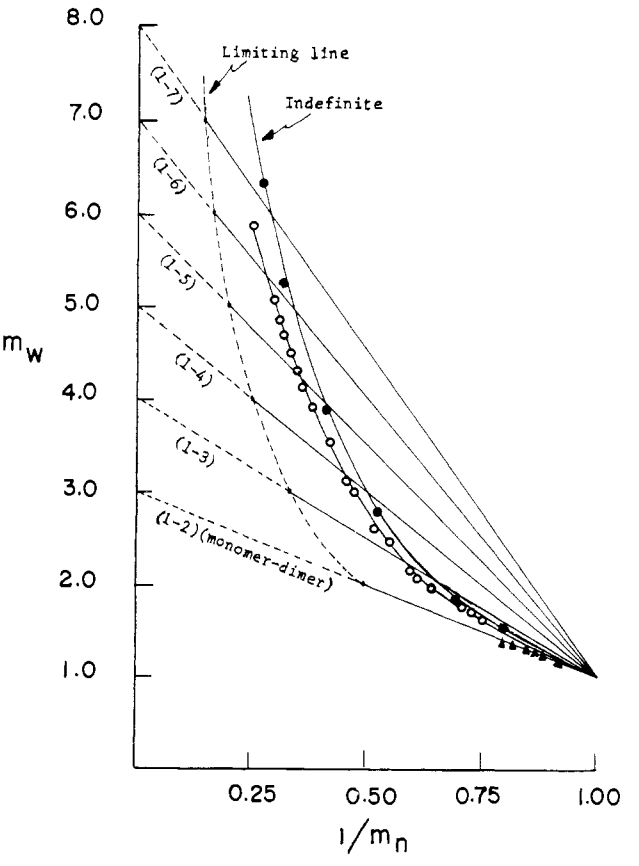

FIGURE 2: Standard plot of m_w *vs.* $1/m_n$ for monomer–*n*-mer and indefinite association. Plot is based on eq 8. Limiting values for $C \rightarrow \infty$ are shown by dotted line. Application of bovine liver L-glutamate dehydrogenase and lysozyme to the standard plot. ● represents the ideal case and ○ represents the change when nonideality is considered. Molecular weight *vs.* concentration data was taken from Eisenberg and Tompkins (1968), pH 7.0, 0.2 M phosphate buffer–0.001 M EDTA. Lysozyme data, plotted as ▲, were taken from Adams and Filmer (1966), pH 6.7, 0.005 M phosphate buffer containing 0.15 M NaCl.

$$\frac{M_1}{M_{n\,app}} = \frac{1}{C}\int_0^C \frac{M_1}{M_{w\,app}}dC = \frac{M_1\Sigma C_i/M_i}{C} = \sum_i f_i/i \qquad (5)$$

$$f_i = \frac{C_i}{C}, \sum_i f_i = 1 \qquad (6)$$

The weight fraction monomer

$$f_1 = C_1/C = \alpha/C = \exp\int_0^C \left(\frac{M_1}{M_{w\,app}} - 1\right)\frac{dC}{C}$$

Basic equations given to this point will suffice for determination of equilibrium constants and the weight fraction monomer when as many as three species are involved in chemical equilibrium.

In order to illustrate the use of the basic quantities outlined here, their graphical application to several cases of discrete and indefinite association will be considered.

CASE I: $q[P_1] \rightleftarrows r[P_n]$, when $n > 1$.

If two species are involved in chemical equilibrium, the mode of association is evaluated from three basic plots, the first being a plot of $(M_{w\,app}/M_1)$ *vs.* f_1, as shown in Figure 1. Letting $M_{w\,app}/M_1 = m_w$, $M_1/M_{n\,app} = 1/m_n$

$$m_w = -(n-1)f_1 + n \qquad (7)$$

Equation 7 is derived by substitution of eq 6 into eq 4. A second plot of m_w *vs.* $1/m_n$ results from proper substitution of eq 4–6 to yield

$$m_w = -n\left(\frac{1}{m_n}\right) + n + 1 \qquad (8)$$

This equation is similar to a quantity first derived by Roark and Yphantis (1969). The resulting standard graph is shown in Figure 2.

The third standard graph is obtained by plotting $1/m_n$ *vs.* f_1 as shown in Figure 3, obtained from eq 5 and 7 which may be combined to yield

$$\frac{1}{m_n} = \left(1 - \frac{1}{n}\right)f_1 + \frac{1}{n} \qquad (9)$$

Equations 7–9 provide the basis for the three permanent plots for monomer–*n*-mer association. Once they have been evaluated, the data for any ideal model system may be applied to these permanent plots.

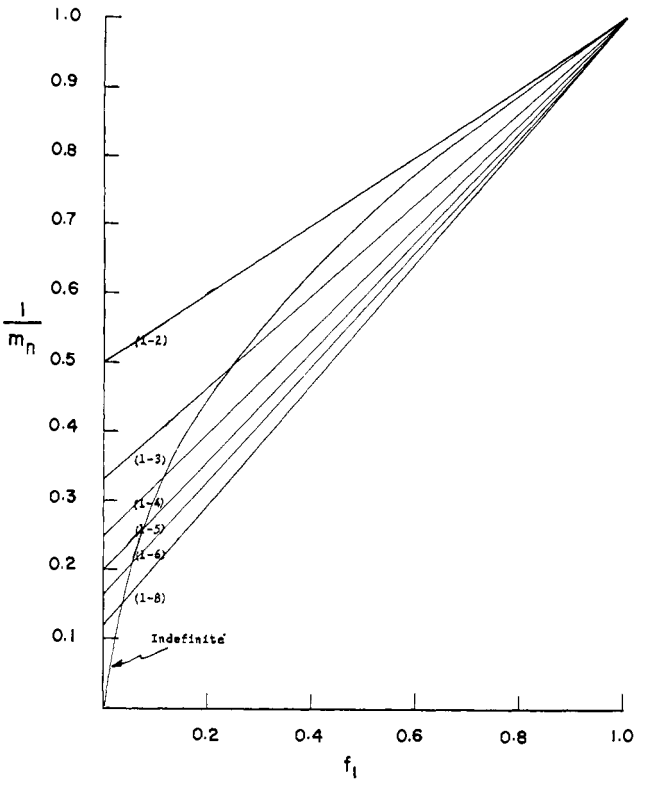

FIGURE 3: Standard plot of $1/m_n$ *vs.* f_1 for monomer–*n*-mer and indefinite association based on eq 9.

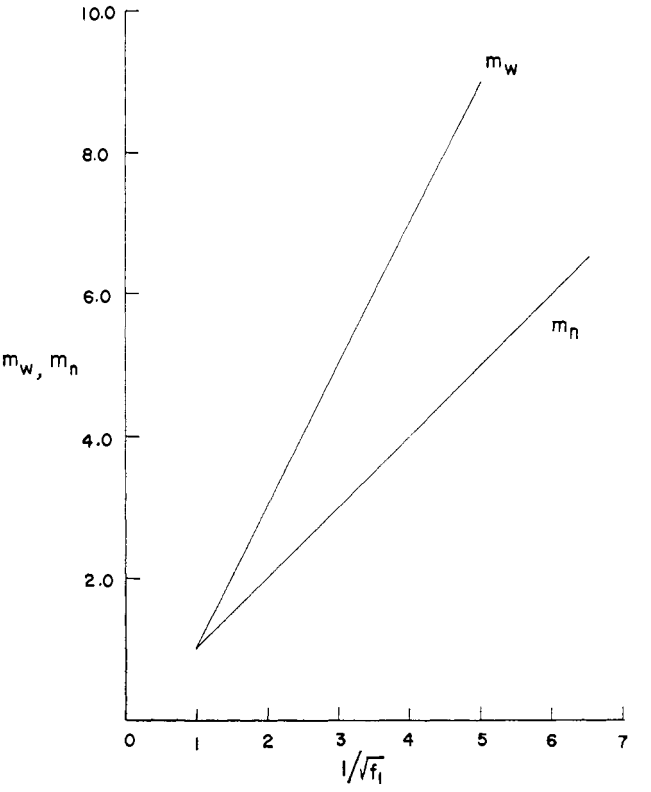

FIGURE 4: Standard plot of m_w or m_n *vs.* $1/(f_1)^{1/2}$ for indefinite association based on eq 11 and 12.

CASE II. INDEFINITE ASSOCIATION. In cases of indefinite association, the basic assumption and notation is consistent with that proposed by Adams (1967a) in which a single equilibrium constant pertains to monomer addition for all species.

$$\frac{1}{m_w} = \frac{1 - kC_1}{1 + kC_1} \tag{10a}$$

$$\frac{1}{m_n} = 1 - kC_1 \tag{10b}$$

where

$$C = C_1/(1 - kC_1)^2 \tag{10c}$$

The following expression may be obtained from eq 10a–c or from eq 4–6. The resulting values may be incorporated

$$m_w = 2m_n - 1 = \frac{2}{\sqrt{f_1}} - 1 \tag{11}$$

$$f_1 = \left(\frac{1}{m_n}\right)^2 \tag{12}$$

into the three standard plots to give a characteristic curve which differs perceptibly from the monomer–*n*-mer curve. A plot of m_n, m_w, *vs.* $1/(f_1)^{1/2}$ is seen in Figure 4. The values of m_w *vs.* m_n may be plotted, giving a straight line with a slope of 2.

Application of the Standard Plots for 1 — m Association, m > 1. From an experimental curve of weight-average molecular weight as a function of concentration for a particular model system, evaluate m_w and f_1 for a number of given concentrations. Plot the resulting coordinates on the standard graph shown in Figure 1. It is essential that a wide range of concentrations be considered, preferably 0.1 to 10 g per l. Points at highly similar concentrations will give a deceptive line which may appear to fit the standard plot, but on a wider concentration scale would be obviously deviant.

Provided only two species are involved in chemical equilibrium, the resulting values will lie along one of the lines of the standard graph. For example, should the coordinates lie on the 1–4 line, monomer–tetramer association is operating in the system. Should the values lie along the standard curve, indefinite association is involved. However, values which do not correspond to any of the standard lines would indicate more than two species are involved. As seen from eq 8, the conditions $1/n < 1/m_n < 1$ and $1 < m_w < n$ must be met. Therefore, as C approaches to zero, $m_w \rightarrow 1$, and when $c \rightarrow \infty$, m_w and $m_n \rightarrow n$. The resulting ordinates give a limiting point n on the line of each of the various modes of association. Joined, these limiting values of n yield a hyperbolic curve, shown as a dotted line in Figure 2. Therefore, all calculations for the mode of association must lie between 1 and n.

The standard graph of Figure 1 resembles that of Figure 2 except that no limiting values are present. This graph can be used as a check on the plot in Figure 2.

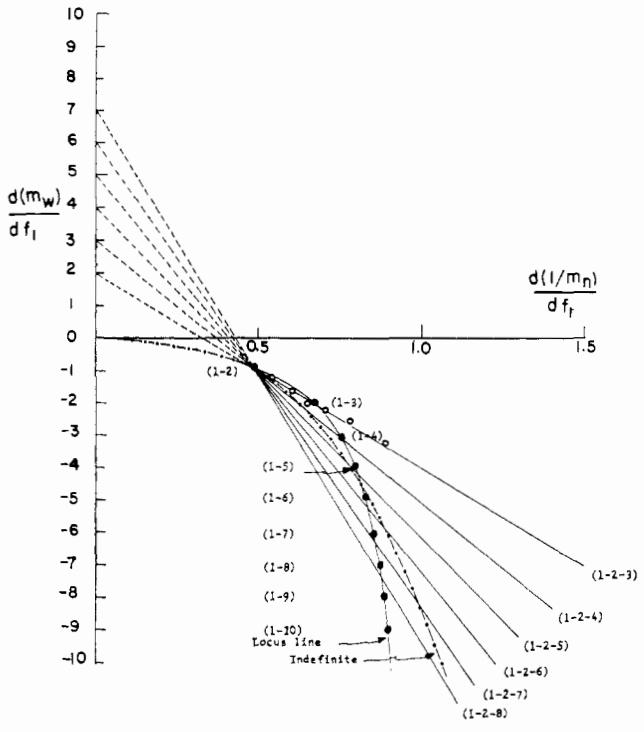

FIGURE 5: Standard plot of dm_w/df_1 *vs.* $d(1/m_n)/df_1$ for $1 - m - n$ association based on eq 17. The locus for $1 - m - n$ association is indicated by ●. Application of α chymotrypsin to standard plot is indicated by ○. Molecular weight versus concentration data from Rao and Kegeles (1958). The broken line with ● indicates indefinite association. Data for bovine liver glutamate dehydrogenase fall along this line.

With the exception that different initial values are used to obtain coordinates, the plots shown in Figures 2 and 3 may be similarly applied, giving three possible checks of the mode of association operating in any model system.

The application of bovine liver L-glutamate dehydrogenase as a model system (Chun *et al.*, 1969; Chun and Kim, 1969) to the standard plot is incorporated in Figure 2. In the ideal case, experimental values from molecular weight data fall on the standard curve. When the nonideality term BM_1 is considered, the values fall short of the standard curve but show a strong correlation, as may be seen in Figure 2.

Plotting experimental values of m_w *vs.* $1/m_n$ for a lysozyme model system indicates the operation of monomer–dimer association which is consistent with reported experimental results (Sophianopoulos and Van Holde, 1964; Adams and Filmer, 1966).

CASE III: $q[P_i] \rightleftarrows s[P_m] + r[P_n]$, $n > m > 1$.

When three species are involved in chemical equilibrium, Roark and Yphantis (1969) proposed the plotting of $d(M_nM_z - M_w)/dM_n$ *vs.* $d(M_nM_w)/dM_n$. We propose, however, a simplified procedure which would eliminate double differentiation in setting up the plot. The basic quantities which are to be plotted are dm_w/df_1 *vs.* $d(1/m_n)/df_1$.

From eq 4–6 the following simultaneous equations are set up (see Appendix):

$$\frac{1}{m_n} = f_1 + \frac{f_m}{m} + \frac{f_n}{n}$$

$$1 = f_1 + f_m + f_n \tag{13}$$

$$m_w = f_1 + mf_m + nf_n$$

Eliminating f_n and f_m from eq 13 gives

$$m\left(\frac{n}{m_n} - 1\right) - (n - m_w) = (m - 1)(n - 1)f_1 \tag{14}$$

Rearrangement yields

$$f_1 = \frac{1}{(m - 1)(n - 1)}\left[m_w + \frac{mn}{m_n} - m - n\right] \tag{15}$$

Differentiation of eq 16 with respect to f_1 gives

$$\frac{dm_w}{df_1} = -mn\left[\frac{d\left(\frac{1}{m_n}\right)}{df_1}\right] + (m - 1)(n - 1) \tag{16}$$

In order to establish a standard plot for three species in chemical equilibrium, it is necessary to evaluate the quantities dm_w/df_1 and $d(1/m_n)/df_1$. Differentiating eq 4 and 5 with respect to f_i will yield

$$\frac{dm_w}{df_1} = \sum_i i\frac{df_i}{df_1} = -(m - 1) \tag{17}$$

$$\frac{d\left(\frac{1}{m_n}\right)}{df_1} = \sum_i \frac{1}{i}\frac{df_i}{df_1} = \left(1 - \frac{1}{m}\right) \tag{18}$$

Thus it is possible to determine the locus points for three species association, where $dm_w/df_1 = -(m - 1)$ and $d(1/m_n)/df_1 = (1 - (1/m))$. These points are indicated in Figure 5.

Graphical Analysis of 1 − m − n Association. It is obvious from eq 16 that $d(m_w)/df_1$ is a linear function of $d(1/m_n)/df_1$. The resulting straight line for any $1 - m - n$ mode of association thus must initiate at the locus point, $[-(m - 1), (1 - (1/m))]$ with a slope of $-mn$ and a hypothetical intercept of $(m - 1)(n - 1)$ on the dm_w/df_1 axis.

Note from Figure 5 that all lines plotted for three species, monomer–dimer–*n*-mer association, would initiate at the locus point indicated as 1–2, and similarly a monomer–trimer–*n*-mer association would initiate at the 1–3 locus. Differentiation of eq 7 and 9 with respect to f_1 yields $-(m - 1)$ and $(1 - 1/m)$, the quantities which specify the locus points. Monomer–dimer association, then, is graphically represented by the 1–2 locus, monomer–trimer by the 1–3 locus, etc. Thus the placement of these loci is a necessary first step to plotting the mode of association on the standard graph.

The curve characteristic of indefinite association can be added to the standard plot of $1 - m - n$ association for comparison purposes if desired. Differentiation of eq 11 with respect to f_1, that is $m_w = (2/(f_1)^{1/2}) - 1$ or $1/m_n = (f_1)^{1/2}$, results in the quantities $dm_w/df_1 = -(1/(f_1)^{1/2})^3$ and $d(1/m_n) \cdot df_1 = 0.5(1/(f_1)^{1/2})$. Combining these two yields $dm_w/df_1 = -8[d(1/m_n)/df_1]^3$ which may be plotted on the standard graph of Figure 5.

Application of data for α-chymotrypsin (Rao and Kegeles, 1958) indicates the operation of monomer–dimer–trimer association. The high correlation of the experimental data to the standard plot may be seen in Figure 5. Our results reconfirm the conclusion of both earlier researchers as to the mode of association of this model system (Rao and Kegeles, 1958; Adams and Filmer, 1966).

Discussion

The graphical analysis described herein simplifies the determination of mode of association and equilibrium constants (see Appendix) of any ideal associating protein model system. To verify the theoretical calculations which we have put forth, we applied data for bovine liver L-glutamate dehydrogenase, lysozyme, and α-chymotrypsin association. The results reconfirmed those modes of association previously indicated by the experimental data.

Appendix

Evaluation of equilibrium constants of $1 - m - n$ association:

From eq 13

$$1 = f_1 + f_m + f_n$$

$$m_w = f_1 + mf_m + nf_n$$

$$\frac{1}{m_n} = f_1 + f_m/m + f_n/n$$

By determinants, the solution to the equation set above is given by

$$\begin{bmatrix} 1 - f_1 \\ m_w - f_1 \end{bmatrix} = \begin{bmatrix} 1 & 1 \\ m & n \end{bmatrix} \begin{bmatrix} f_m \\ f_n \end{bmatrix}$$

Note that $f_m + f_n = 1 - f_1$ and $mf_m + nf_n = m_w - f_1$. Thus the weight fraction, f_m, becomes

$$f_m = \frac{n(m_w - f_1) - m(1 - f_1)}{n - m} = \frac{nm_w - m - (n - m)f_1}{n - m}$$

$$= \frac{nm_w - m}{n - m} - f_1$$

and $f_n = 1 - f_1 - f_m$. Hence $C_m = K_m C_1^m$ and $C_n = C - C_1 - C_m = K_n C_1^n$.

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