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Graphic rule for non-steady-state enzyme kinetics and protein folding kinetics*

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When going more deeply into the principles of enzyme action as well as protein folding, one is often confronted with transient process systems. Based on the recent progress in graphic methods of enzyme kinetics, in this article a graphic rule is described, which can be used to deal with transient processes occurring in both enzyme-catalyzed reaction systems and protein folding systems. Introducing the graphic method to non-steady-state systems can raise the efficiency of the calculations and provide an intuitive picture, helping the analysis of the mechanisms concerned. For instance, using the current graphic rule, one can immediately write out the phase concentrations of enzyme species or protein folding states. Calculation work such as setting up differential equations, making Laplace transformations, and expanding determinants, which is both tedious and liable to error, is completely avoided. The mathematical proof of the non-steady-state graphic rule is given in the appendix.

1. Introduction

Thirty-five years ago, King and Altman [1] proposed a graphic method for deriving the steady-state rate equations in enzyme kinetics. Introducing graphic methods to enzyme kinetics can make the calculation more convenient and intuitive, and hence has proved to be very useful in biochemistry. As a matter of fact, the King–Altman method has been so widely employed that it has been written into many biochemistry textbooks. However, the King–Altman method can only be used to deal with very simple enzyme-catalyzed systems. When an enzyme-catalyzed reaction system is a little more complicated, the calculation will become almost formidable without the use of a computer. Furthermore, in analyzing enzyme-catalyzed mechanisms, it is crucially important to find the analytical solutions, not only the numerical solutions (cf., e.g. refs. [2–5]). Therefore, various graphical methods [6–23] have been proposed in an attempt to improve and develop the original King–Altman method. All these methods are valid only for steady-state

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systems. However, when going more deeply into the principles of enzyme action as well as protein folding, one is often confronted with transient processes (e.g. ref. [24]) which cannot be treated in terms of the conventional graphic methods. Therefore, it is highly desirable to present a graphic method that can be used to deal with non-steady-state systems. This article is devoted to such a goal. Moreover, the algorithm described in this paper can be applied for the creation of a program for the analytical solution of linear differential equations.

In enzyme kinetics and protein folding kinetics, we often have to deal with a special non-steady-state kinetic system called a "compartment system", which can be formulated by the following equations:

$$-\frac{de_i}{dt} = e_i \sum_{j=1}^{n} k_{ij} - \sum_{j=1}^{n} k_{ji} e_j \quad (i = 1, 2, \ldots, n), \tag{1a}$$

$$\sum_{i=1}^{n} e_i = e_0 \tag{1b}$$

with the following initial conditions:

$$\left.\begin{aligned} e_1(t) &= e_0 \\ e_i(t) &= 0 \quad (i \neq 1) \end{aligned}\right\} \text{ when } t = 0, \tag{2}$$

where $e_i = [\mathrm{E}_i]$ $(i = 1, 2, \ldots, n)$ is the concentration of the ith enzyme species, with $e_1 = [\mathrm{E}_1]$ for that of the free enzyme. The total concentration of the n enzyme species is e_0, which is a constant. In this article, the concentration of a reactant X is represented by either [X] or just the corresponding lower-case letter x. Obviously, for a protein folding system in which there are n different folding states, all the above formulations remain valid too. The following equations will not, therefore, refer explicitly to the protein folding system since the current formulations will always be valid for either of these two kinetic systems.

As is well known, it is much more difficult and complicated [25–28] to find the solution for a non-steady-state system in comparison with the corresponding steady-state one. A question is naturally raised: Can we also find a graphic method to deal with the non-steady-state system as we did for the steady-state one? The answer is yes. As an approach to realize this, let us first perform the Laplace transformation for eq. (1) with the initial condition (2), which yields

$$\left(s + \sum_{j=1}^{n} k_{ij}\right) \tilde{e}_i - \sum_{j=1}^{n} k_{ji}\, \tilde{e}_j = \delta_{1i}\, e_0 \quad (i = 1, 2, \ldots, n), \tag{3a}$$

$$s \sum_{i=1}^{n} \tilde{e}_i = e_0, \tag{3b}$$

where δ_{1i} is the Kronecker delta, s is an interim parameter introduced by the Laplace transform,

$$\tilde{e}_i(s) = \int_0^\infty e_i(t)\ \exp(-ts)\,\mathrm{d}t, \tag{4}$$

and $\tilde{e}_i$ is the *phase concentration* of E_i. The relation between the phase concentration $\tilde{e}_i$ and the usual concentration e_i can be simply expressed as

$$\left.\begin{aligned} \tilde{e}_i &= \hat{L}e_i \\ e_i &= \hat{L}^{-1}\tilde{e}_i \end{aligned}\right\}, \tag{5}$$

where $\hat{L}$ and $\hat{L}^{-1}$ are the Laplace transformation and inverse transformation operator, respectively.

Below, a graph rule is presented by which one can directly write out the phase concentrations according to the directed graph without the need of solving eqs. (3) and (4), whose operation is even more tedious and error-prone than that of the case of a steady-state system. Once the phase concentrations $\tilde{e}_i$ $(i = 1, 2, \ldots, n)$ are known, the corresponding normal concentrations $e_i(t)$ $(i = 1, 2, \ldots, n)$ can be immediately obtained according to the Laplace transform table, available in any mathematical handbook.

2. A graphic rule for calculating the phase concentrations $\tilde{e}_m$ $(m = 1, 2, \ldots, n)$

The rule can be illustrated in terms of the following four steps:

(1) According to the reaction mechanism of a system, draw a directed graph G, which consists of vertices and arcs. In such a graph, various enzyme species are represented by different vertices, and the interconversion between any two enzyme species by an arc with an arrow and weighted by a rate constant to indicate the conversion direction and rate, respectively. If $k_{ij} = 0$, then the arc from E_i to E_j is not depicted, implying no direct conversion from the ith enzyme species to the jth one.

(2) Transform the directed graph G to $\tilde{G}$ according to the following procedures: To each of the vertices E_m $(m = 1, 2, \ldots, n)$ add a loop with the weight $s + \sum_{j=1}^{n} k_{mj}$, respectively. If there are two or more arcs from one vertex to another identical vertex, then condense them into one by adding their rate constants together.

(3) The graph obtained through the above procedure is called the phase graph $\tilde{G}$. Thus, the phase concentration $\tilde{e}_m$ for the mth enzyme species E_m is given by

$$\tilde{e}_m = \frac{N_m}{\sum_{i=1}^{n} N_i}\ (e_0/s), \tag{6}$$

where N_m can be obtained as follows. From the phase graph $\tilde{G}$, find all the subgraphs each of which has one, and only one, path from E_1 to E_m, as well as all the cycles and loops that intersect with neither each other nor the path. For each such subgraph, multiply all its weights and a sign factor given by

$$(-1)^{C_y}, \tag{7}$$

where C_y is the number of the cycles (not including loops) in the respective subgraph. The sum of all these results will immediately give N_m. When $m = 1$, however, the path from E_1 to E_m will reduce to a point whose weight, in this case, should be assigned as 1 for calculations (e.g., see eq. (12)).

(4) In order to facilitate checking and to avoid missing any subgraphs, we can also predict the number of subhraphs to be counted. The method is as follows. Construct a matrix $\mathbf{C} = [c_{ij}]$, where

$$c_{ij} = \begin{cases} 1, & \text{if there is an arc from } E_i \text{ to } E_j \text{ in } \tilde{G}; \\ 0, & \text{otherwise,} \end{cases} \tag{8}$$

then the number of subgraphs involved in calculating N_m must be

$$n^{1\to m} = \text{per } \mathbf{C}_{m,1}, \tag{9}$$

where $\mathbf{C}_{m,1}$ is the submatrix obtained by removing the mth row and the first column from $\mathbf{C}$, and per $\mathbf{C}_{m,1}$ denotes the sum of all terms obtained by expanding the determinant of $\mathbf{C}_{m,1}$ but taking all the signs of the expanded terms as plus, e.g.,

$$\text{per}\begin{pmatrix} 1 & 1 \\ 1 & 1 \end{pmatrix} = 2,$$

$$\text{per}\begin{pmatrix} 1 & 1 & 0 \\ 0 & 1 & 1 \\ 1 & 1 & 1 \end{pmatrix} = \text{per}\begin{pmatrix} 1 & 1 \\ 1 & 1 \end{pmatrix} + \text{per}\begin{pmatrix} 0 & 1 \\ 1 & 1 \end{pmatrix} = 2 + 1 = 3$$

and so forth. Therefore, it is very easy to calculate the number of the subgraphs in terms of eq. (9) in which the matrix elements are either 1 or 0.

The mathematical proof of this rule is given in the appendix.

3. Examples and discussion

Let us illustrate the above non-steady-state graphical rule by some examples.

EXAMPLE 1

Find the non-steady-state solution for the Michaelis–Menten mechanism [29]:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E + P, \tag{10}$$

where the concentration of E is equal to e_0 and that of ES equal to 0 when $t = 0$. Let $E_1 = E$, $E_2 = ES$, $k_{12} = k_{+1}[S]$, $k_{21} = k_{-1}$, $k^*_{12} = k_{-2}[P]$, $k^*_{21} = k_{+2}$. According to (1)

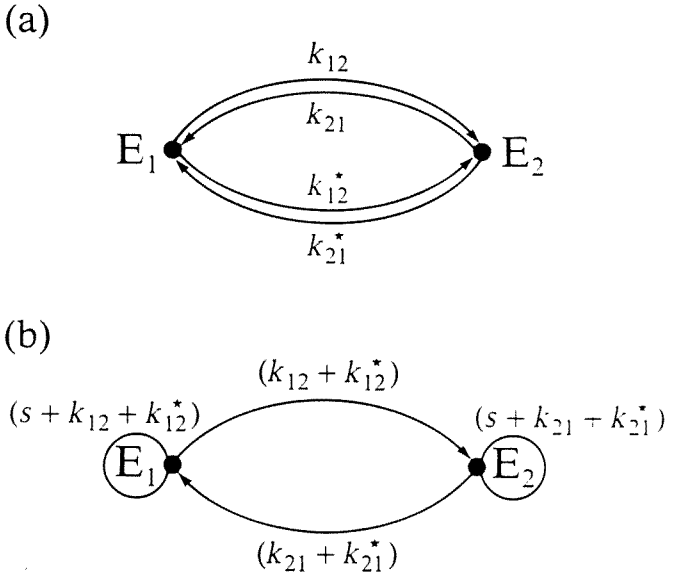


Fig. 1. (a) The directed graph G for the Michaelis–Menten mechanism as formulated in eq. (10). (b) The phase graph $\tilde{G}$ obtained from (a) according to the procedure as stated in (2) of the rule.

of the above rule, the Michaelis–Menten mechanism of eq. (10) can be expressed by the directed graph, as shown in fig. 1(a). Following the procedure as described in (2) of the rule, the directed graph G in fig. 1(a) can be transformed to the phase graph $\tilde{G}$ as given in fig. 1(b). Then, according to (3) of the rule, it follows

$$N_1 = (-1)^0 \quad \begin{matrix} 1 \\ \bullet \\ E_1 \end{matrix} \qquad (s+k_{21}+k_{21}^*) \qquad E_2 \qquad = s + k_{21} + k_{21}^*, \tag{11a}$$

$$N_2 = (-1)^0 \qquad (k_{12} + k_{12}^*) \qquad E_1 \qquad E_2 \qquad = k_{12} + k_{12}^*. \tag{11b}$$

Substituting these results into eq. (6), we obtain

$$\tilde{e}_1 = \frac{s + k_{21} + k_{21}^*}{s(s + k_{12} + k_{21} + k_{12}^* + k_{21}^*)} e_0, \tag{12a}$$

$$\tilde{e}_2 = \frac{k_{12} + k_{12}^*}{s(s + k_{12} + k_{21} + k_{12}^* + k_{21}^*)} e_0. \tag{12b}$$

Using the table of Laplace transforms (cf., e.g. ref. [30]), we immediately obtain

$$e_1(t) = e_0\left\{\frac{k_{21} + k_{21}^*}{k_{12} + k_{21} + k_{12}^* + k_{21}^*} + \frac{k_{12} + k_{12}^*}{k_{12} + k_{21} + k_{12}^* + k_{21}^*} \exp[-(k_{12} + k_{21} + k_{12}^* + k_{21}^*)t]\right\}, \tag{13a}$$

$$e_2(t) = e_0\left\{\frac{k_{12} + k_{12}^*}{k_{12} + k_{21} + k_{12}^* + k_{21}^*} - \frac{k_{12} + k_{12}^*}{k_{12} + k_{21} + k_{12}^* + k_{21}^*} \exp[-(k_{12} + k_{21} + k_{12}^* + k_{21}^*)t]\right\}. \tag{13b}$$

Now let us see how to use the check formula of eq. (9). According to the phase graph $\tilde{G}$ of fig. 1(b) we have

$$\mathbf{C} = \begin{pmatrix} 1 & 1 \\ 1 & 1 \end{pmatrix}. \tag{14}$$

Thus, it follows from eq. (10) that

$$n^{1\to 1} = \text{per } \mathbf{C}_{1,1} = [1] = 1, \quad n^{1\to 2} = \text{per } \mathbf{C}_{2,1} = [1] = 1, \tag{15}$$

which means that no subgraphs were missed in calculating the phase concentrations as illustrated in eqs. (11a) and (11b).

EXAMPLE 2

Consider the non-steady-state kinetics of the following three-state model of protein folding:

$$\mathrm{D} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{X} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{N}, \tag{16}$$

where D represents the denatured and unfold protein species, N the native and folded protein species, and X is the intermediate in the pathway between unfolded and folded states. Let $E_1 = D$, $E_2 = X$, $E_3 = N$, $k_{12} = k_1$, $k_{21} = k_{-1}$, $k_{23} = k_2$, $k_{32} = k_{-2}$. Then the three-state folding model can be expressed by the directed graph G as shown in fig. 2(a). Assume that initially only denatured protein is present, viz.,

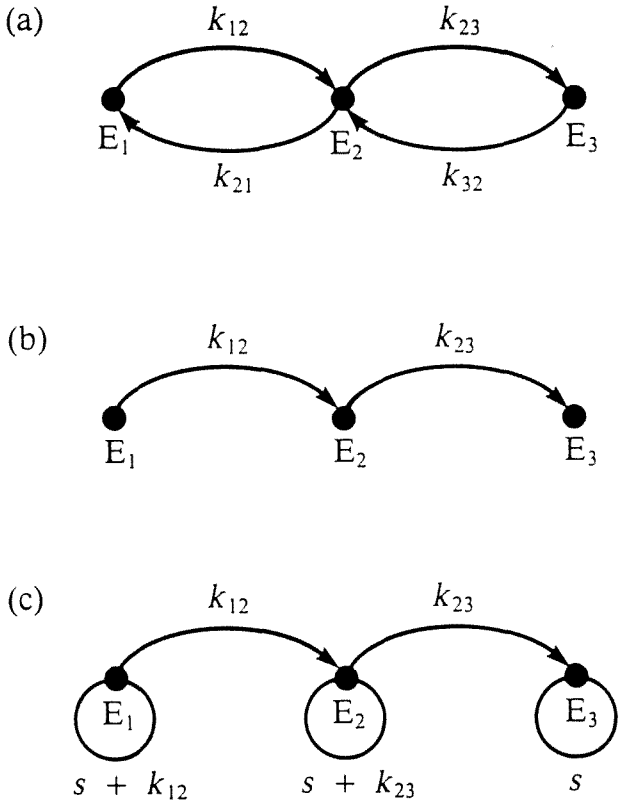
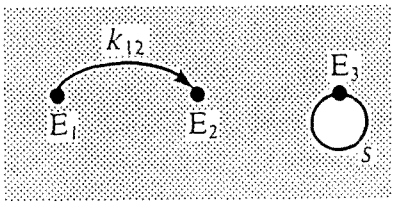


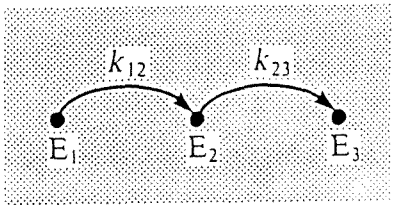
Fig. 2. (a) The directed graph for the three-state folding model as formulated in eq. (16). (b) The directed graph reduced from fig. 2(a) when $k_{12}, k_{23} \gg k_{21}, k_{32}$. (c) The phase graph $\tilde{G}$ obtained from fig. 2(b) according to (2) of the rule.

$e_1 = [D] = e_0$, $e_2 = [X] = 0$ and $e_3 = [N] = 0$ when $t = 0$. Here, e_0 is the total concentration of protein. The protein is then subjected to a rapid temperature jump, a sudden change in solvent, or some other quick change that causes the protein to fold. To simplify illustration, suppose $k_{12}, k_{23} \gg k_{21}, k_{32}$, which means the tendency of the protein towards folding is dominant over its tendency towards unfolding after such a sudden change. In this case, the directed graph G in fig. 2(a) can be reduced to that of fig. 2(b). According to (1) of the rule, the directed graph G in fig. 2(b) can be transformed to the phase graph $\tilde{G}$ of fig. 2(c). It follows thus by (2) of the rule that

$$N_1 = (-1)^0 \quad \begin{matrix} E_1 \\ \bullet \\ 1 \end{matrix} \qquad \begin{matrix} E_2 \\ \circ \\ s + k_{23} \end{matrix} \qquad \begin{matrix} E_3 \\ \circ \\ s \end{matrix} \qquad = (s + k_{23})s, \tag{17a}$$

$$N_2 = (-1)^0$$ 

$$= k_{12}s, \tag{17b}$$

$$N_3 = (-1)^0$$ 

$$= k_{12}k_{23}. \tag{17c}$$

Substituting the above results into eq. (6) yields

$$\tilde{e}_1 = \frac{(s + k_{23})s}{s[s^2 + (k_{23} + k_{12})s + k_{12}k_{23}]} e_0 = \frac{(s + k_{23})s}{s(s + k_{12})(s + k_{23})} e_0 = \frac{1}{s + k_{12}} e_0, \tag{18a}$$

$$\tilde{e}_2 = \frac{k_{12}}{(s + k_{12})(s + k_{23})} e_0, \tag{18b}$$

$$\tilde{e}_3 = \frac{k_{12}k_{23}}{s(s + k_{12})(s + k_{23})} e_0. \tag{18c}$$

Using the table of Laplace transforms (cf., e.g. ref. [30]), we immediately obtain the transient concentrations of the protein at three different folding states. The solutions may be classified into the following two cases:

(a) When $k_{12} \neq k_{23}$, we have

$$e_1(t) = \mathrm{e}^{-k_{12}t} e_0, \tag{19a}$$

$$e_2(t) = \frac{k_{12}}{k_{23} - k_{12}} (\mathrm{e}^{-k_{12}t} - \mathrm{e}^{-k_{23}t}) e_0, \tag{19b}$$

$$e_3(t) = \left[\frac{1}{k_{23} - k_{12}} (k_{12}\mathrm{e}^{-k_{23}t} - k_{23}\mathrm{e}^{-k_{12}t}) + 1 \right] e_0. \tag{19c}$$

(b) When $k_{12} = k_{23} = k$, we have

$$e_1(t) = \mathrm{e}^{-kt} e_0, \tag{20a}$$

$$e_2(t) = kt\,e^{-kt}e_0, \tag{20b}$$

$$e_3(t) = (1 - e^{-kt} - kt\,e^{-kt})\,e_0. \tag{20c}$$

From examples 1 and 2, we see that neither the operation of expanding determinants nor the operation of solving differential equations is needed; we can directly write out the transient concentration of enzyme species or protein folding stated just by looking up the Laplace transform table as well as its directed graph. A great deal of complicated and difficult mathematical derivations can be avoided.

An application of the non-steady-state graphic rule in analyzing the mechanism of glucokinase slow transition upon of glucose was reported recently by Lin and Neet [24].

Appendix: The mathematical principle of the non-steady-state graphic rule

First, let us point out that an $n \times n$ matrix $\mathbf{X} = [x_{ij}]$ can be expressed by a weighted *digraph* (i.e. directed graph) $G(\mathbf{X})$ with n vertices $(V_1, V_2, \ldots, V_n)$. The method is as follows. If $x_{ij} \neq 0$ $(i \neq j)$, draw an arc from vertex V_i to vertex V_j, and weight it with x_{ij}; if $x_{ij} = 0$ $(i \neq j)$, do not depict an arc from V_i to V_j. If $x_{ii} \neq 0$, draw a loop at vertex V_i, i.e. an arc from vertex V_i to itself, and weight it with x_{ii}; if $x_{ii} = 0$, however, no loop should be drawn at vertex V_i. Through such a procedure, a one-to-one correspondence between the matrix and the digraph is established [31]. This is the essence of why some calculations relevant to a matrix can be approached through the graphic method as well.

Second, let us prove that for any $n \times n$ matrix $\mathbf{X} = [x_{ij}]$, we have

$$\det \mathbf{X} = \sum_u (-1)^{q_u} f(G_u), \tag{A.1}$$

where G_u is the uth of those subgraphs each of which contains all disjoint cycles and loops of $G(\mathbf{X})$, q_u is the number of cycles with an even number of arcs in G_u, and $f(G_u)$ denotes the product of the weights of all the arcs in G_u. The proof of eq. (A.1) is as follows. According to the definition of a determinant,

$$\det \mathbf{X} = \sum_\sigma \operatorname{sgn}\sigma\; x_{1j_1} x_{2j_2} \ldots x_{nj_n}, \tag{A.2}$$

where $\sigma = \{j_1, j_2, \ldots, j_n\}$ is a permutation of $\{1, 2, \ldots, n\}$, and

$$\operatorname{sgn}\sigma = \begin{cases} 1, & \text{for even permutation;} \\ -1, & \text{for odd permutation.} \end{cases} \tag{A.3}$$

If $x_{1j_1}x_{2j_2}\ldots x_{nj_n} \neq 0$, it must also be equal to the product of weights of the arcs

$$\widehat{V_1V_{j_1}},\ \widehat{V_2V_{j_2}},\ \ldots,\ \widehat{V_nV_{j_n}}$$

in $G(\mathbf{X})$, where each given subscript appears only twice: one is in the initial point of an arc, and the other is in the terminal point of an arc. Consequently, $\widehat{V_1V_{j_1}}$, $\widehat{V_2V_{j_2}}, \ldots, \widehat{V_nV_{j_n}}$ must correspond to a G_u of eq. (A.1). In other words, there is a one-to-one correspondence between the non-zero terms of $\det \mathbf{X}$ and G_u ($u = 1, 2, \ldots$) of $G(\mathbf{X})$; i.e. we have

$$\det \mathbf{X} = \sum_u \operatorname{sgn} \sigma_u \ f(G_u), \tag{A.4}$$

where $\operatorname{sgn} \sigma_u$ is the sign which can be determined as follows. As mentioned above, each G_u ($u = 1, 2, \ldots$) consists of disjoint cycles and loops. Suppose V_{i_1}, $\widehat{V_{i_1}V_{i_2}}$, V_{i_2}, $\widehat{V_{i_2}V_{i_3}}, \ldots, V_{i_l}, \widehat{V_{i_l}V_{i_1}}, V_{i_1}$ is a cycle formed by l vertices and l arcs in G_u. According to eq. (A.3), the term $x_{i_1i_2}x_{i_2i_3}\ldots x_{i_li_1}$, which is actually the product of the weights of all the arcs of such a cycle, will contribute a factor of $(-1)^{l-1}$ to $\operatorname{sgn} \sigma_u$. Therefore, if we take all disjoint cycles of G_u into account, we have

$$\operatorname{sgn} \sigma_u = \prod_{\{l\}} (-1)^{l-1}, \tag{A.5}$$

where l is the number of arcs in each of the disjoint cycles in G_u. Since

$$(-1)^{l-1} = \begin{cases} -1, & \text{when } l \text{ is an even number;} \\ 1, & \text{when } l \text{ is an odd number,} \end{cases} \tag{A.6}$$

it follows that

$$\operatorname{sgn} \sigma_u = (-1)^{\{\text{the number of cycles in } G_u \text{ that have even number of arcs}\}}. \tag{A.7}$$

Substitution of eq. (A.7) into eq. (A.4) completes the proof of eq. (A.1).

On the other hand, according to the definition of the *permanent* of a matrix, we have

$$\operatorname{per} \mathbf{X} = \sum_\sigma x_{1j_1}x_{2j_2}\ldots x_{nj_n}. \tag{A.8}$$

In comparison with $\det \mathbf{X}$ of eq. (A.2), the only difference is in the sign factor $\operatorname{sgn} \sigma$ which appears in $\det \mathbf{X}$ but not in $\operatorname{per} \mathbf{X}$. Therefore, if $\mathbf{C}$ is built from $G(\mathbf{X})$ according to rule 2(3), it is obvious that

$$\begin{aligned} \operatorname{per} \mathbf{C} &= \{\text{the number of non-zero terms in } \det \mathbf{X}\} \\ &= \{\text{the number of } G_u \text{ in } G(\mathbf{X})\}. \end{aligned} \tag{A.9}$$

Now we can use eqs. (A.1) and (A.9) to prove the rule of calculating the phase concentrations. The phase concentrations are the solutions of eqs. (3a), (3b). However, the $n+1$ equations in eq. (3) are not independent. The first equation in eq. (3a) is equal to eq. (3b) minus all the other equations, i.e. $i = 2, 3, \ldots, n$ of eq. (3a). Therefore, instead of solving eqs. (3a), (3b), we can consider the following equations:

$$\sum_{i=1}^{n} \tilde{e}_i = e_0/s, \tag{A.10a}$$

$$\left(s + \sum_{j=1}^{n} k_{ij}\right)\tilde{e}_i - \sum_{j=1}^{n} k_{ji}\,\tilde{e}_j = 0 \quad (i = 2, 3, \ldots, n). \tag{A.10b}$$

Thus, according to Cramer's rule, the solutions of eq. (A.10) can be expressed as

$$\tilde{e}_m = \frac{N_m}{\sum_{i=1}^{n} N_i}(\tilde{e}_0/s), \tag{A.11}$$

where

$$N_m = \begin{pmatrix} 1 & 1 & \cdots & 1 & \cdots & 1 \\ -k_{12} & s + \sum_{j=1}^{n} k_{2j} & \cdots & 0 & \cdots & -k_{n2} \\ \vdots & \vdots & & \vdots & & \vdots \\ -k_{1m} & -k_{2m} & \cdots & 0 & \cdots & -k_{nm} \\ \vdots & \vdots & & \vdots & & \vdots \\ -k_{1n} & -k_{2n} & \cdots & 0 & \cdots & s + \sum_{j=1}^{n} k_{nj} \end{pmatrix}. \tag{A.12}$$

Observing eq. (A.12), we discover the following:

(1) According to the relation between terms and graphs as given in eq. (A.1), in all the graphs corresponding to N_m there is no arc starting from E_m. Therefore, any cycle containing point E_m must be reduced to a path. On the other hand, in all the corresponding graphs, there is no arc ending at the vertex E_1. Therefore, the reduced cycle must be a path from point E_1 to E_m. This is actually reflected by selecting E_1 as a starting point to calculate N_m as stated in (3) of the graphic rule. In a particular case, if $m = 1$, i.e. the vertex E_m is the starting point itself, the path is further reduced to a point with weight of 1, as implied in eq. (A.12). (Note that according to eq. (A.1), if the path is reduced from a cycle with an even number of arcs, then a factor of (-1) will be added; if the path is reduced from a cycle with an odd number of arcs, no such factor should be added.)

(2) For brevity, all the minus signs before the elements of N_m in eq. (A.12) can be removed through an appropriate adjustment in sign. Such an adjustment, combined with $(-1)^{q_u}$ in eq. (A.1) as well as the sign contributed from the path as described in (1) of this section, will eventually lead to eq. (7).

(3) Again, from eqs. (A.9) and (A.12) it is obvious that if E_1 is selected as a starting point, the number of subgraphs to counted for calculating N_m is given by eq. (9).

This completes the proof of the graphic rule for calculating the phase concentrations.

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