NONEQUILIBRIUM LINEAR BEHAVIOR OF BIOLOGICAL SYSTEMS

EXISTENCE OF ENZYME-MEDIATED MULTIDIMENSIONAL INFLECTION POINTS

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ABSTRACT The linear phenomenological equations of nonequilibrium thermodynamics are limited theoretically to near equilibrium although a number of biological systems have been shown to exhibit a "linear" relationship between steady-state flows and conjugate thermodynamic forces outside the range of equilibrium. We have found a multidimensional inflection point which can exist well outside the range of equilibrium around which enzyme-catalyzed reactions exhibit "linear" behavior between the logarithm of reactant concentrations and enzyme catalyzed flows. A set of sufficient conditions has been derived which can be applied to any enzyme mechanism to determine whether a multidimensional inflection point exists. The conditions do not appear overly restrictive and may be satisfied by a large variety of coupled enzyme reactions. It is thus possible that the linearity observed in some biological systems may be explained in terms of enzymes operating near this multidimensional inflection point.

INTRODUCTION

A number of biological systems have been found to exhibit a linear relationship between steady-state flows and conjugate thermodynamic forces outside the range of equilibrium (1). Examples include the systems carrying out oxidative-phosphorylation in mitochondria (2, 3), sodium transport in frog skin, toad bladder (4) and toad skin (5), and hydrogen ion transport in turtle bladder (6). Linearity has also been noted in a synthetic membrane exhibiting active transport (7). (Linearity as used in these papers and here implies the flow, J, is related to the force, $\mathcal{A}$, by an affine relation, e.g. $J = L\mathcal{A} + C$, where C and L are constants. Hence, a doubling of the force only implies a doubling of the flow when $C = 0$).

These examples suggest that the function of complex systems (outside the range of equilibrium) can under some circumstances be described by sets of linear phenomenological equations.

$$J_i = \sum_{j=1}^{N} L_{ij}\mathcal{A}_j \quad i = 1, \ldots N \tag{1a}$$

where J_i is the i^{th} flow in the system, and $\mathcal{A}_i$ its conjugate thermodynamic force, and the L_{ij}'s

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are (constant) phenomenological coefficients. However, only for a system sufficiently close to equilibrium (viz. $\mathcal{A} \ll RT$, where $\mathcal{A}$ is the chemical affinity for a reaction, R is the gas constant and T the absolute temperature) has it been demonstrated theoretically (8, 9) that Eq. 1*a* will be a valid description of the system and that the phenomenological coefficients L_{ij} obey the Onsager reciprocal relations (9)

$$L_{ij} = L_{ji} \tag{1b}$$

Outside the restricted range there is no theoretical guarantee that Eq. 1 should be valid, although these equations are widely used in nonequilibrium thermodynamics (10–12) and especially in the treatment of steady-state membrane systems (13–16).

Rottenberg (2) has pointed out that those specific enzyme-catalyzed reactions which obey approximately the Michaelis-Menten rate equation (17)

$$J = \frac{Ax}{B + x} \tag{2}$$

(where x is the activity of a substrate of the reaction and A and B are independent of x) can exhibit a high degree of linearity in the chemical affinity for certain values of substrate concentrations. He also showed that for certain conditions the rate of the reaction J can be approximated by $J = L\mathcal{A}$ (where L is a constant independent of $\mathcal{A}$), even outside the range of equilibrium.

To see whether Rottenberg's approach can be expanded to explain the experimental finding of extended regions of linearity in coupled biological systems, it is necessary to treat multiple enzyme-coupled reactions which depend on more than one reactant. In this paper we establish a set of sufficient conditions which guarantee that an enzyme mechanism will exhibit a multidimensional inflection point around which a set of linear equations will be valid over an extended range outside of equilibrium. These conditions do not appear to be overly restrictive and may be satisfied by a large variety of coupled enzyme systems. Further, we show that in a specific case which is biologically reasonable reciprocal relations (identical to those defined by Onsager near equilibrium) are obtained. Hence, the linearity observed in many biological systems may in some cases be explained in terms of enzymes operating around a multidimensional inflection point.

RESULTS AND DISCUSSION

I. *A Single Flow as a Function of One Reactant Concentration*

Each enzyme kinetic mechanism can be described by a transition diagram (18, 19), sometimes known as a Hill diagram. The simplest type of diagram corresponds to a series of enzyme catalyzed reactions without branching. In Fig. 1*a* we show one such example for a three-state enzyme

$$E_1 + \chi_1 \rightleftharpoons E_2 \rightleftharpoons E_3 \rightleftharpoons E_1 + \chi_2 \tag{3}$$

At steady state, the net flows between any two states in such a diagram must all be equal for otherwise the concentration of each enzyme state would not be constant. Thus, the system can

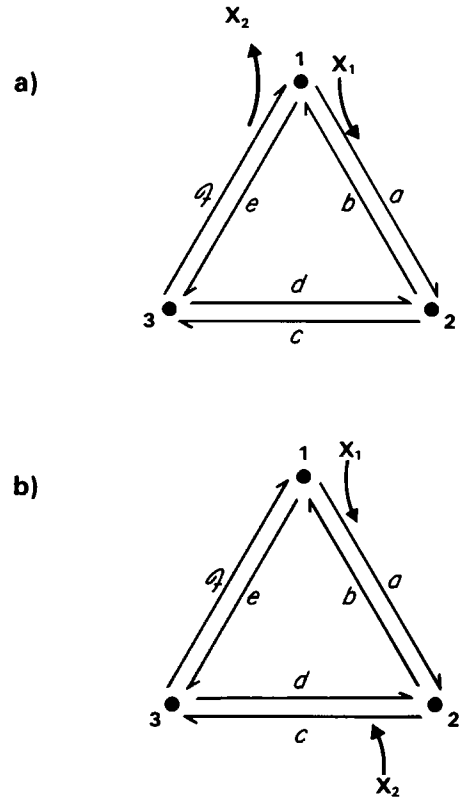

FIGURE 1

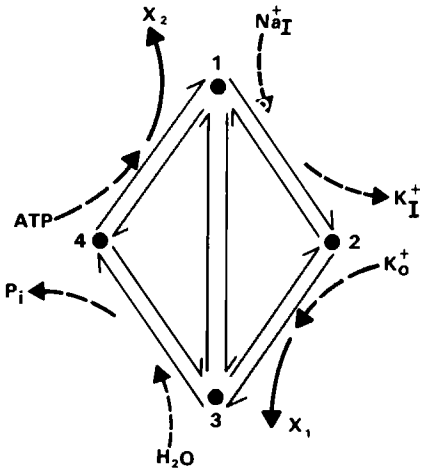

FIGURE 2

FIGURE 1 (*a*) A three-state transition diagram describing an enzyme mechanism which catalyzes a single flow reaction at steady state. Each node represents a possible enzyme state and each arrow a possible transition between states. *a*, *b*, *c*, *d*, *e*, and $\mathcal{F}$ are rate constants. The reactant variables x_1 and x_2 enter into transitions $1 \rightleftharpoons 2$ and $1 \rightleftharpoons 3$, respectively. (*b*) A three-state enzyme mechanism where the reactant variables enter the transitions $1 \rightleftharpoons 2$ and $2 \rightleftharpoons 3$.

FIGURE 2 A four-state transition diagram describing an enzyme mechanism which catalyzes two independent flow reactions at steady-state. The reactants X_1, X_2, enter into the transitions as shown. This diagram could represent a simple active transport model (dotted arrows) where $X_1 = Na_0^+$ and $X_2 =$ ADP.

be characterized by a single flow which will depend on the concentration of each of the reactants. (We shall ignore the distinction between activities and concentrations.)

In a more complex enzyme mechanism where branches occur, more than one flow is found (cf. Fig. 2). If, however, we restrict our attention to one particular flow J, then we can show (cf. Appendix A) that for an important class of reactions the dependence of the steady-state velocity J on any reactant concentration x_i is of the form

$$J(x_i) = \frac{A_i x_i + B_i}{C_i x_i + D_i} \tag{4}$$

where A_i, B_i, C_i, D_i are independent of x_j but may depend on any concentration x_i with $j \neq i$,

and where C_i, D_i, > 0. This result holds for any species χ_i that satisfies the following conditions.

Condition I*a*: The reactant affects the transition rates for leaving only one of the enzyme states. Condition I*b*: The kinetics are of first order with respect to the reactant. (Condition I*b* can be generalized (cf. Appendix A))

The existence of a general expression, Eq. 4, for the dependence of the flow on the concentration of any particular reactant χ_i which obeys conditions I allows us to show that there will exist a special point x^0 around which the flow expression can be written as

$$J = J^0 + b \ln\left(\frac{x}{x^0}\right) + \mathcal{O}^3\left[\ln\left(\frac{x}{x^0}\right)\right] \tag{5}$$

(Here and henceforth in this section we drop the subscript i since there is only one reactant.) The notation $\theta^3[\ln(x/x^0)]$ denotes the correction term in a Taylor series. The corrections are of order $\ln^3(x/x^0)$ and higher. A plot of J vs. x (cf. Eq. 4) for values of the coefficient A, B, C, and D where $AD > BC$ and $C, D > 0$ is monotonically increasing with decreasing slope (e.g. cf. Fig. 3 A). If however, J is plotted vs. $Q = \ln(x)$ (cf. Fig. 3 B) there is an inflection point at $x = D/C$. In order to see this note that

$$\frac{\partial J}{\partial Q} = \frac{\partial J}{\partial x}\frac{\partial x}{\partial Q} = \frac{x(AD - BC)}{(Cx + D)^2} \geq 0 \tag{6}$$

because $AD > BC$ and

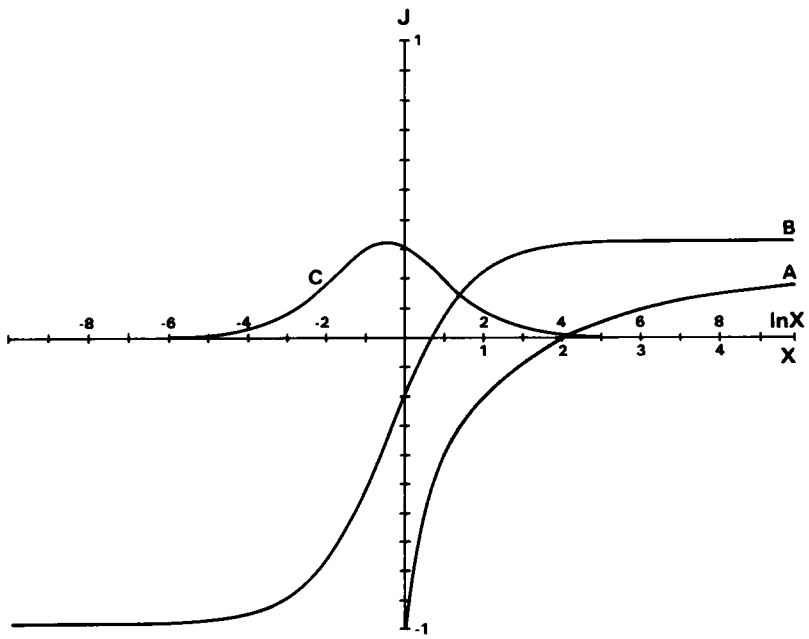

FIGURE 3 (*A*) Plot of the function $J(x) = (x - 2)/(3x + 2)$ where the scale on the x-axis $0 - 5$ is used. (*B*) J is replotted as a function of $Q = \ln x$, where $\ln x$ varies from -10 to 10. An inflection point is found at $x = \frac{2}{3}$ ($Q = -0.405$). (*C*) The slope of curve B is plotted as a function of Q. A maximum is found at $Q = \ln D/C$ where $D = 2$ and $C = 3$.

$$\frac{\partial^2 J}{\partial Q^2} = \frac{x(D - Cx)(AD - BC)}{(Cx + D)^3}\bigg|_{x=(D/C)} = 0 \tag{7}$$

(If $BC > AD$, an inflection point is again found at $x = D/C$ but now J decreases with increasing x.) If we expand $J(x)$ in a Taylor series with respect to $Q = \ln x$ about the point $Q^0 = \ln x^0$, where $x^0 = D/C$, then

$$J(Q) = J(Q^0) + \left(\frac{\partial J(Q^0)}{\partial Q}\right)(Q - Q^0) + \left(\frac{\partial^2 J(Q^0)}{\partial Q^2}\right)\frac{(Q - Q^0)^2}{2!} + \text{higher order terms} \tag{8}$$

From Eq. 7 $(\partial^2 J/\partial Q^2) = 0$ at $Q = Q^0 \equiv \ln (D/C)$. Therefore from Eqs. 4, 6, and 8, again expressing J as a function of x, we have

$$J(x) = \frac{AD + BC}{2DC} + \frac{AD - BC}{4DC}\ln\left(\frac{Cx}{D}\right) + e(\hat{x}), \tag{9}$$

where $\hat{x} \epsilon (\ell_1, \ell_2)$ and $e(\hat{x})$ is the error term obtained by truncating the Taylor series at second order. For $\ell_1 < x < \ell_2$, this error term can be shown to obey (20) the inequality

$$|e| \leq 1/3!\ \underset{x\epsilon(\ell_1,\ell_2)}{\text{Max}}\left|\frac{\partial^3 J(x)}{\partial Q^3}\right| \ln^3\left(\frac{\ell_2}{\ell_1}\right); \tag{10}$$

therefore Eq. 5 has been demonstrated.

The affinity of the general single flow reaction

$E + \chi_1 + \chi_2 \cdots \chi_n \rightleftharpoons E + \chi_{n+1} + \chi_{n+2} \cdots \chi_m$ is defined by (10)

$$\mathcal{A} = RT \ln K_{eq}\left(\frac{x_1 x_2 \cdots x_n}{x_{n+1} x_{n+2} \cdots x_m}\right) \tag{11}$$

If all the reactant concentrations are held constant except x then

$$\mathcal{A}(x) = RT \ln (x) + c \tag{12}$$

where c is independent of x. Therefore, from Eq. 9 and 12 we can write, within corrections of third order:

$J(x) = LA + constant$

$$\text{where } L \text{ is the slope } (1/RT)\frac{\partial J(Q^0)}{\partial Q} = (1/RT)\left[\frac{AD - BC}{4DC}\right]. \tag{13}$$

The two cases treated by Rottenberg are included in the above results.

II. *A Single Flow as a Function of Two Reactant Concentrations*

In this section we shall consider the case of two reactant concentrations which are allowed to vary. We begin by noting that if both χ_1 and χ_2 obey conditions I then we have from Appendix A

$$J(x_1) = \frac{A_1 x_1 + B_1}{C_1 x_1 + D_1}, \tag{14a}$$

$$J(x_2) = \frac{A_2 x_2 + B_2}{C_2 x_2 + D_2}. \tag{14b}$$

The general form of the flow equation for two reactants obeying conditions I is therefore:

$$J(x_1, x_2) = \frac{f'_{12} x_1 x_2 + f'_1 x_1 + f'_2 x_2 + f'}{f_{12} x_1 x_2 + f_1 x_1 + f_2 x_2 + f}, \tag{15}$$

where the f and f' coefficients are independent of x_1 and x_2. Comparing Eqs. 14 and 15 we have

$$\begin{aligned} C_1 &= f_{12} x_2 + f_1 \qquad & C_2 &= f_{12} x_1 + f_2 \\ D_1 &= f_2 x_2 + f & D_2 &= f_1 x_1 + f \end{aligned} \tag{16}$$

Because $J(x_1)$ and $J(x_2)$ have the form of Eq. 5 near $x_1 = D_1/C_1$ and $x_2 = D_2/C_2$ respectively, we have two simultaneous conditions to be satisfied in order for $J(x_1, x_2)$ to be approximately linear in both the ln (x_1) and ln (x_2). From Eq. 16 and the requirement that at the inflection point $x_1 = D_1/C_1$ and $x_2 = D_2/C_2$, we have

$$x_1 = \frac{D_1}{C_1} = (f_2 x_2 + f)/(f_{12} x_2 + f_1) \tag{17}$$

$$x_2 = \frac{D_2}{C_2} = (f_1 x_1 + f)/(f_{12} x_1 + f_2). \tag{18}$$

Eqs. 17 and 18 admit only one physically feasible set of values of x_1, x_2 provided f_1, f_2 and $f_{12} \neq 0$

$$x_1 = x_1^0 = \sqrt{\frac{f f_2}{f_1 f_{12}}}; \quad x_2 = x_2^0 = \sqrt{\frac{f f_1}{f_2 f_{12}}}. \tag{19}$$

The above constraints on the f's are an example of more general conditions to be considered in section III below.

We can now expand $J(x_1, x_2)$ around the point $x_1^0 = D_1/C_1$, $x_2^0 = D_2/C_2$ in a Taylor series taking into account the fact that $\partial^2 J(x_1)/\partial(\ln x_1)^2 = \partial^2 J(x_2)/\partial(\ln x_2)^2 = 0$ at the point (x_1^0, x_2^0). We then have

$$\begin{aligned} J(x_1, x_2) = J(x_1^0, x_2^0) &+ \left(\frac{\partial J(x_1^0, x_2^0)}{\partial \ln x_1}\right)\left(\ln \frac{x_1}{x_1^0}\right) + \left(\frac{\partial J(x_1^0, x_2^0)}{\partial \ln x_2}\right)\left(\ln \frac{x_2}{x_2^0}\right) \\ &+ \left(\frac{\partial^2 J(x_1^0, x_2^0)}{\partial(\ln x_1)\partial(\ln x_2)}\right)\left(\ln \frac{x_1}{x_1^0}\right)\left(\ln \frac{x_2}{x_2^0}\right) + \mathcal{O}^3\left(\ln \frac{x_1}{x_1^0}, \ln \frac{x_2}{x_2^0}\right). \end{aligned} \tag{20}$$

Hence, we have shown that for the case of two reactants varied an operating point can exist around which the enzyme-mediated flow will depend linearly on both ln x_1 and ln x_2. The existence of this point is guaranteed if $f_1, f_2, f_{12} \neq 0$ and conditions I are satisfied. In analogy with Eq. 5, the general form of $J(x_1, x_2)$ can be written:

$$J(x_1, x_2) = J^0 + a_1 \ln\left(\frac{x_1}{x_1^0}\right) + a_2 \ln\left(\frac{x_2}{x_2^0}\right) + a_{12} \ln\left(\frac{x_1}{x_1^0}\right) \ln\left(\frac{x_2}{x_2^0}\right) + \mathcal{O}^3\left[\ln\left(\frac{x_1}{x_1^0}\right), \ln\left(\frac{x_2}{x_2^0}\right)\right], \quad (21)$$

where the last term includes all third order and higher order corrections.

In the general case of two varied reactants it would be desirable to know when the two linear terms $f_1 x_1, f_2 x_2$ and the cross term $f_{12} x_1 x_2$ will appear in the denominator of the flow equation, thus assuring the existence of an inflection point. This could in theory be determined by deriving the flow equation for each enzyme mechanism in question. However, it is simpler to verify the existence of these terms by merely inspecting the directional diagrams associated with the Hill diagram of a given mechanism. This is true because each directional diagram corresponds to a term in the denominator of the flow equation (all terms being positive) (cf. references 18, 19 and 29). Hence by verifying that those directional diagrams which correspond to the $f_1 x_1, f_2 x_2$ and $f_{12} x_1 x_2$ terms appear in the Hill diagram, one is guaranteed that $f_1, f_2, f_{12} \neq 0$.

Directional diagrams can also be used to give a mechanistic significance to Eqs. 19, which rearranged give:

$$\begin{aligned} f_1 x_1 &= f_2 x_2 \\ f_{12} x_1 x_2 &= f. \end{aligned} \quad (22)$$

From Eqs. 22 it can be seen that the conditions for a two-dimensional inflection point are simply that (*a*) the sum of all directional diagrams involving x_1 or x_2 is equivalent to the sum of all directional diagrams not involving x_1 or x_2, (*b*) the sum of all directional diagrams involving only x_1 is equivalent to the sum of all directional diagrams involving only x_2. Because these directional diagrams are directly related to the fluxes through specific cycles in the Hill diagram (29) the rules for a two-dimensional inflection point have a mechanistic significance. An extension to higher dimensions is made in section III and Appendix B.

Application to a Three-State Model

To illustrate the theory of the preceding section we consider a three-state model of an enzyme catalyzed reaction. We consider two cases, one satisfying, the other not satisfying, the constraints on Eqs. 19.

In Fig. 1 *a* is shown the Hill diagram for a model where the two reactants to be considered, χ_1 and χ_2, act as substrates in the individual reactions:

$$\begin{aligned} E_1 + \chi_1 &\underset{b}{\overset{a}{\rightleftharpoons}} E_2 \\ E_1 + \chi_2 &\underset{\mathcal{F}}{\overset{e}{\rightleftharpoons}} E_3. \end{aligned} \quad (23)$$

At steady state, the dependence of the flow rate J on the different kinetic rate constants and reactant concentrations is given by:

$$J = \frac{E_0(ac\mathcal{F}x_1 - bedx_2)}{(ac + ad + a\mathcal{F})x_1 + (eb + ec + ed)x_2 + bd + b\mathcal{F} + c\mathcal{F}}, \tag{24}$$

where E_0 represents total enzyme concentration (for a derivation see reference 19). Comparing this expression to Eq. 15 we find that $f_{12} = 0$ and therefore the inflection point (x_1^0, x_2^0) cannot be computed using Eqs. 19.

In Fig. 1 *b* is shown a second example:

$$\begin{aligned} E_1 + \chi_1 &\underset{b}{\overset{a}{\rightleftharpoons}} E_2 \\ E_2 + \chi_2 &\underset{d}{\overset{c}{\rightleftharpoons}} E_3. \end{aligned} \tag{25}$$

It should be noted that although no products are shown in this cycle, they exist (e.g. $\chi_2 + E_2 \rightleftharpoons E_3 + Y$) but are lumped into the rate constants since their concentrations will not be varied. The steady-state flow rate for this example is given by:

$$J = \frac{E_0(ac\mathcal{F}x_1x_2 - bde)}{acx_1x_2 + (a\mathcal{F} + ad)x_1 + (ce + c\mathcal{F})x_2 + ed + db + be + \mathcal{F}b}. \tag{26}$$

By comparing Eq. 26 with Eq. 15 we find:

$$\begin{aligned} f &= ed + db + be + \mathcal{F}b \qquad f_{12} = ac \\ f_1 &= a\mathcal{F} + ad \\ f_2 &= ce + \mathcal{F}c \end{aligned} \tag{27}$$

Substituting these values into Eqs. 19 we have:

$$x_1^0 = \frac{\sqrt{f}}{a}\sqrt{\frac{(e + \mathcal{F})}{(d + \mathcal{F})}} \qquad x_2^0 = \frac{\sqrt{f}}{c}\sqrt{\frac{(d + \mathcal{F})}{(e + \mathcal{F})}}. \tag{28}$$

Around this inflection point the flow expressions will reduce to Eq. 21. To verify this, we plot in Fig. 4 A the family of curves J vs. $\ln x_1$ and in Fig. 4 B J vs. $\ln x_2$ for specific values of a, b, c, d, e, and $\mathcal{F}$. In this case a two-dimensional inflection point exists at x_1^0, x_2^0 corresponding to an affinity of $-4.37RT$. A variation in the $\ln x_1$ or $\ln x_2$ of $2.5RT$ away from this point causes an approximately linear change in J over an extended region.

It is possible for the equilibrium point to coincide with the inflection point if the rate constants are constrained. The condition for equilibrium is given by

$$\frac{x_1x_2ac\mathcal{F}}{bde} = 1. \tag{29}$$

If the inflection point conditions (Eqs. 28) are substituted into Eq. 29 we obtain the relation $f = bde/\mathcal{F}$. In Fig. 4 C, D we plot the family of curves J vs. $\ln x_1$ and $\ln x_2$, respectively, for rate constants chosen to satisfy this condition. As is seen, the inflection point now coincides with equilibrium. Under these conditions the two ln terms in the Taylor series, Eq. 21, will group into the affinity $\mathcal{A} \propto \ln (x_1x_2ac\mathcal{F})/(bde)$. This can be verified by calculating a_1 and a_2,

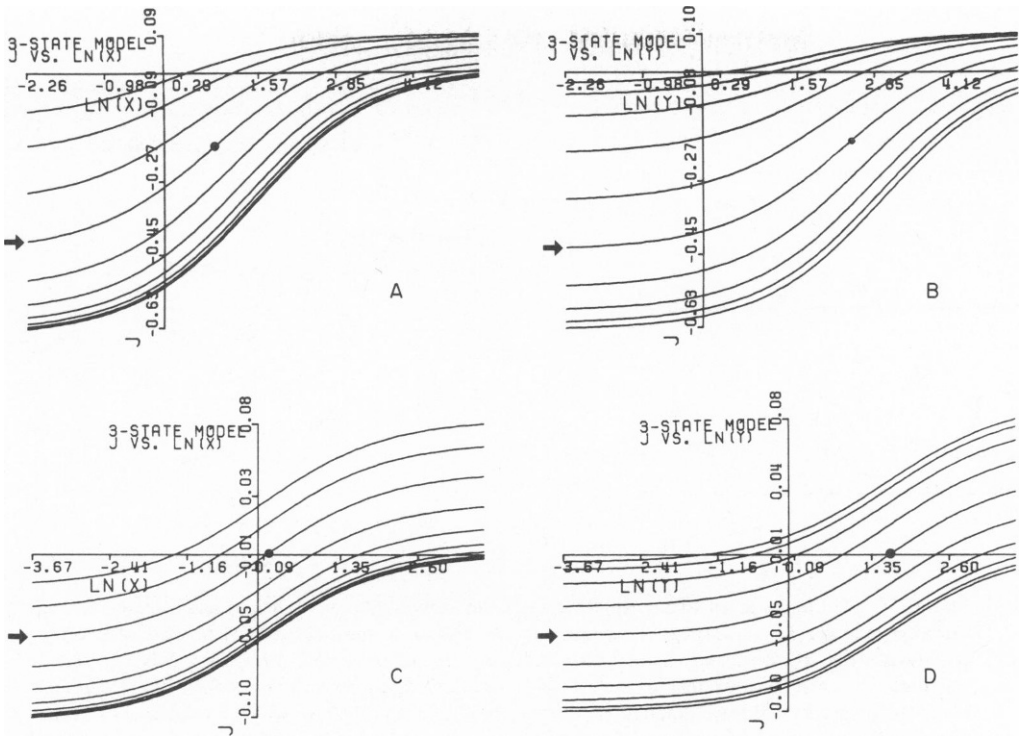

FIGURE 4 (A, B) Plots of Eq. 26 for the rate constants $a = 1, b = 3, c = 1, d = 5, e = 1, \mathcal{F} = 0.1$. (A) J plotted as a function of $\ln x_1 = \ln X$ for constant values of $\ln x_2 = \ln Y$. Starting from the upper curve $\ln Y = 4.63, 3.87, 3.11, 2.34, 1.57, 0.81, 0.04, -0.73, -1.49, -2.26$. The dot denotes the two-dimensional inflection point at $\ln X = 0.81$, $\ln Y = 2.34$. (B) J plotted as a function of $\ln x_2 = \ln Y$ for constant values of $\ln X$ (same as above) (C, D) Plots of Eq. (26) for the rate constants $a = 1, b = 0.114, c = 1, d = 5, e = 1, \mathcal{F} = 0.1$. In this case $\ln Y$ (*C*) or $\ln X$ (*D*) has the constant values starting from the upper curve of 3.11, 2.35, 1.63, 0.85, 0.10, −0.66, −1.41, −2.16, −2.91, −3.66. The dot marks the two-dimensional inflection point at $\ln X = 0.10$ and $\ln Y = 1.63$. In this case the rate constants were chosen so that X^0, Y^0 coincides with equilibrium.

which are equal in this case. The constant term J^0 is also zero, as expected. Finally, for this particular model $a_{12} = 0$ when the inflection point coincides with equilibrium. Hence, the enzyme will obey this simple relation $J = L\mathcal{A}$ over an extended range both near equilibrium and outside the range of equilibrium. That is, in this case all second order terms in the Taylor series expansion around equilibrium are zero.

III. *M Flows as a Function of N* Reactants

The extension of the analysis in Section II to M flows as a function of N reactants is straightforward and developed in Appendix B. The major result is that an N-dimensional inflection point will exist around which all M flows are linearly related to the logarithm of the N reactants provided this set of reactants satisfies both conditions I and a second set of conditions II:

For each possible combination of reactants considered there must be at least one directional graph of the Hill diagram containing only that combination of reactants and no others.

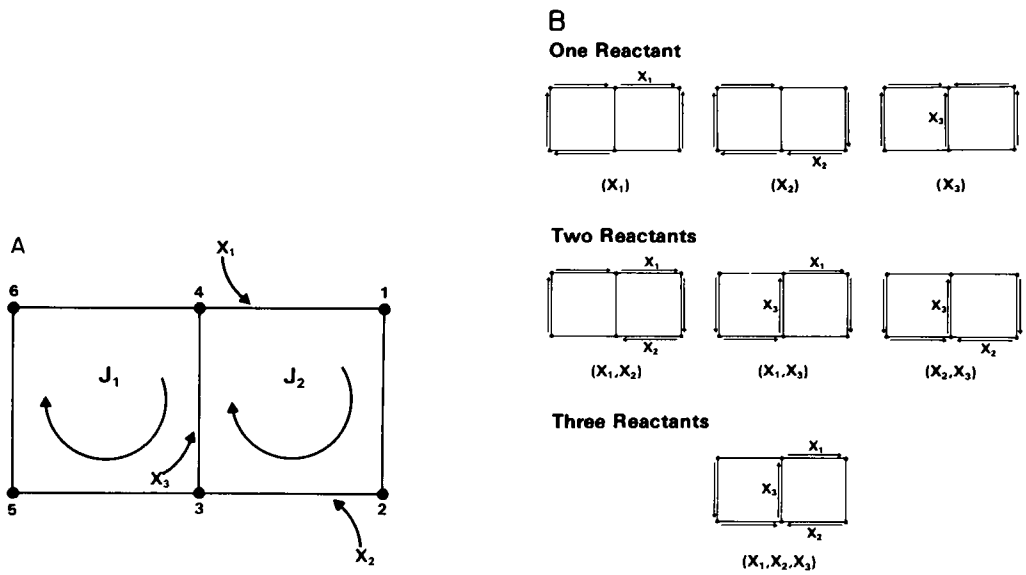

FIGURE 5 (*A*) Illustrates a six-state Hill diagram for an enzyme. Out of all the possible reactants which can affect the enzyme transitions, three reactant variables are chosen (x_1, x_2, x_3) to see if they obey conditions I and II. Because each variable affects the rate of transitions for leaving only one state, conditions I are obeyed separately for each variable. (*B*) Each figure represents a possible directed graph in the Hill diagram. A directed graph is formed by a set of transitional arrows which connect every state in the Hill diagram but do not form any closed cycles. Each arrow labeled by a particular variable denotes the unidirectional transition for which that reactant variable is involved. All transitional arrows must flow towards a single state in the Hill diagram. Thus, two transitional arrows cannot leave from a single state inside a directed graph. Each directed graph represents a term in the denominator of the enzyme flow expression which is formed by the product of all the kinetic rate coefficients for each transition in the directed graph. Examples of directed graphs are shown which involve one reactant, two reactants and three reactants. Hence, condition II is obeyed for this set of reactant variables.

Fig. 5 illustrates the Hill diagram for an enzyme mechanism with three reactants which meet conditions I and II. In particular, Fig. 5 B lists graphically all the classes of directional diagrams necessary to satisfy condition II. It should be noted that although χ_1, χ_2, and χ_3 obey conditions I and II, there could be other reactants entering into the enzyme mechanism which do not fulfill these conditions. This would not affect the overall existence of an inflection point with respect to x_1, x_2, x_3.

Because condition I and condition II are sufficient to guarantee the existence of a multidimensional inflection point, $\mathbf{x}^0$, we can in analogy with the two variable cases express the dependence of J_k (the k^{th} flow) on the N reactants near the inflection point $\mathbf{x}^0$ by

$$J_k = J_k^0 + \sum_{i=1}^{N} a_{ki} \ln\left(\frac{x_i}{x_i^0}\right) + \sum_{i \neq j} a_{kij} \ln\left(\frac{x_i}{x_i^0}\right) \ln\left(\frac{x_j}{x_j^0}\right) + \sum \mathcal{O}_k^3\left[\ln\left(\frac{x_i}{x_i^0}\right), \ln\left(\frac{x_j}{x_j^0}\right)\right] \quad k = 1, 2 \ldots M \quad (30)$$

where J_k^0, a_{ki}, and a_{kij} are independent of all x_i and the last sum includes all third and higher order corrections.

Eq. 30 illustrates that, in general, there can exist states of a multiflow enzyme system outside the range of equilibrium where a new set of linear equations is valid.

The key differences between Eqs. 1*a* and 30 are the following: (*a*) In Eqs. 30, only reactant concentrations of species which simultaneously obey conditions I and II are varied, whereas in Eqs. 1*a* very near equilibrium all concentrations may vary. (*b*) Because Eqs. 30 are obtained by expanding the flows about a multidimensional inflection point, they are valid up to third order in $\ln(x_i)$, whereas Eqs. 1 are based on an expansion about equilibrium and are only justified on a theoretical basis (as far as is presently known) up to the second order. (*c*) The flows J_k do not necessarily become zero when the system is at the inflection point $\mathbf{x}^0$ as is the case when the system is at equilibrium. (*d*) Cross terms of the form $a_{kij} \ln(x_i/x_i^0) \ln(x_j/x_j^0)$ appear in Eqs. 30. This means that although the flows J_k will depend linearly on any given $\ln(x_i)$, the slope will depend on the other x_j variables. This can be seen by simply factoring out $\ln(x_i/x_i^0)$, which gives:

$$J_k = J_k^0 + \sum_{i-1}^{N} \left[a_{ki} + \sum_{j \neq i} a_{kij} \ln\left(\frac{x_j}{x_j^0}\right) \right] \ln\left(\frac{x_i}{x_i^0}\right) + \sum \mathcal{O}_k^3 \left[\ln\left(\frac{x_i}{x_i^0}\right), \ln\left(\frac{x_j}{x_j^0}\right) \right]. \qquad (31)$$

(*e*) If $\mathbf{x}^0$ does not coincide with the condition for equilibrium, we are not guaranteed that the terms in Eqs. 31 can be grouped into the proper conjugate affinities as in Eqs. 1*a*. However, in cases where the inflection point $\mathbf{x}^0$ also satisfies the condition for equilibrium, as will be discussed, Eqs. 31 and Eq. 1*a* are equivalent, and in this case linearity and Onsager reciprocity obtain up to third order.

Application to an Active Transport Model with Two Flows and Two Reactants

A simple model of active transport treated by several authors (15, 22, 23) both near and outside the range of equilibrium is analyzed below to illustrate some of the theory above. In this model, two flows are present; as will be shown, they can be treated using the same formalism as in the preceding section.

In Fig. 6, we show the Hill diagram for this model. If the enzyme makes the transitions

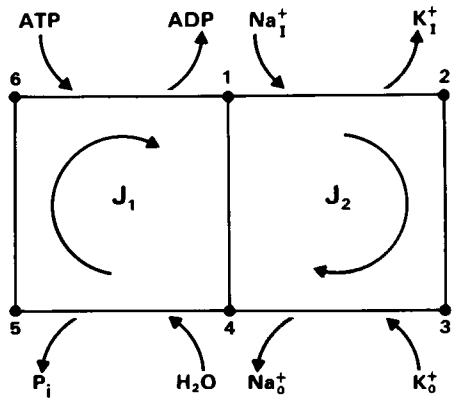

FIGURE 6 Hill diagram for enzyme model of $Na^+ - K^+$ active transport (cf. references 15 and 22). Each reactant variable is shown either as a substrate (arrow entering diagram) or as a product (arrow leaving diagram). The subscript *I* denotes inside concentrations and the subscript 0 outside concentrations.

$1 \rightarrow 2 \rightarrow 3 \rightarrow 4 \rightarrow 1$, this results in the net movement of one Na^+ from the inside to the outside of the membrane and one K^+ from the outside to the inside of the membrane, whereas the transitions $6 \rightarrow 1 \rightarrow 4 \rightarrow 5 \rightarrow 6$ result in the hydrolysis reaction: $ATP + H_2O \rightarrow ADP + P_i$. In this example, we shall focus on two variables, $x \equiv [P_i]$ = concentration of inorganic phosphate $y \equiv [K_I^+]$ = concentration of K^+ ions on the inside of the membrane. We assume all other reactant concentrations are held fixed. We shall study the flows, $J_1 \equiv$ the net rate of ATP hydrolysis, $J_2 \equiv$ the net rate of exchange of Na^+ and K^+ across the membrane.

Because P_i and K_I^+ satisfy condition I and three directional diagrams (satisfying condition II) can be found which correspond to the factors P_i, K_I^+, and $P_i K_I^+$, we know that there must exist a two-dimensional inflection point $([P_i]^0, [K_I^+]^0)$ around which a set of linear equations can be written of the form

$$J_1 = L_{11} \ln [P_i] + L_{12} \ln [K_I^+] + \alpha_1 \ln [P_i] \ln [K_I^+] + \beta_1$$
$$J_2 = L_{21} \ln [P_i] + L_{22} \ln [K_I^+] + \alpha_2 \ln [P_i] \ln [K_I^+] + \beta_2. \tag{32}$$

However, to know the explicit equations relating J_1 and J_2 to the variables $[P_i]$, $[K_I^+]$, it is necessary to solve the steady-state equations for the different enzyme states, which yields an unwieldy expression with over 100 terms in the denominator. Following Blumenthal et al. (22), we can simplify the problem by considering the case where the net transitions between states 5–6, 1–4, and 2–3 are rate limiting, so that the reactions between states 6–1, 1–2, 3–4, and 4–5 are near equilibrium. It is further assumed that the equilibrium constants for the different reactions are given by K_D for $4 \rightleftharpoons 5$, K_e for $1 \rightleftharpoons 2$ and $3 \rightleftharpoons 4$, and K_T for $6 \rightleftharpoons 1$. The forward and backward rate constant for the reactions $5 \rightleftharpoons 6$, $1 \rightleftharpoons 4$, and $2 \rightleftharpoons 3$ is p. Under these conditions the flow expressions are given by:

$$J_1 = P\left[\frac{abY - (1 + b)XY + ab}{(1 + a + b + ab)XY + (a + ab)X + (b + ab)Y + ab}\right] \tag{33a}$$

$$J_2 = P\left[\frac{abX - (1 + a)XY + ab}{(1 + a + b + ab)XY + (a + ab)X + (b + ab)Y + ab}\right], \tag{33b}$$

where

$$X \equiv [P_i]/K_D = x/K_D \quad Y \equiv K_e([K_I^+]/[Na_I^+]) = (K_e/Na_I^+)y$$
$$a \equiv K_T([ATP]/[ADP]), b \equiv K_e([K_0^+]/[Na_0^+])$$
$$P \equiv pc, \tag{34}$$

and c denotes the total concentration of all enzyme forms.

Comparing the denominator of Eqs. 33 with that of Eq. 15, and noting that $x_1 = X$, and $x_2 = Y$, we find

$$f_1 = a + ab,\ f_2 = b + ab,\ f = ab \text{ and } f_{12} = 1 + a + b + ab. \tag{35}$$

Inserting these coefficients into Eqs. 19 we can find the inflection point (X^0, Y^0), which as noted above must be the same for J_1 and J_2, because the denominators of Eqs. 33*a* and 33*b* are identical.

$$X^0 = \left\{\frac{ab(b + ab)}{(1 + a + b + ab)(a + ab)}\right\}^{1/2} = b/(1 + b)$$

$$Y^0 = \left\{\frac{ab(a + ab)}{(1 + a + b + ab)(b + ab)}\right\}^{1/2} = a/(1 + a). \tag{36}$$

In Figs. 7 A and B, we plot the family of curves, J_1 (ln X) and J_1 (ln Y), respectively for the case of $a = 0.1$, $b = 3.0$. In Figs. 7 C and D we plot J_2 (ln$X$) and J_2 (ln$Y$), respectively. We find a two dimensional inflection point at $X = 3/4$, $Y = 1/11$, as predicted by Eqs. 36. Because the affinities reduce to

$$\mathcal{A}_x = RT \ln \frac{K_0 K_T[\mathrm{ATP}]}{[\mathrm{P_i}]\,[\mathrm{ADP}]} = -RT \ln X + a$$

$$\mathcal{A}_y = RT \ln \frac{K_e[\mathrm{K}_I^+][\mathrm{Na}_0^+]}{[\mathrm{K}_0^+][\mathrm{Na}_I^+]K_e} = RT \ln Y - b, \tag{37}$$

one can see that the flow rates are linear in the affinities over a large range near the inflection

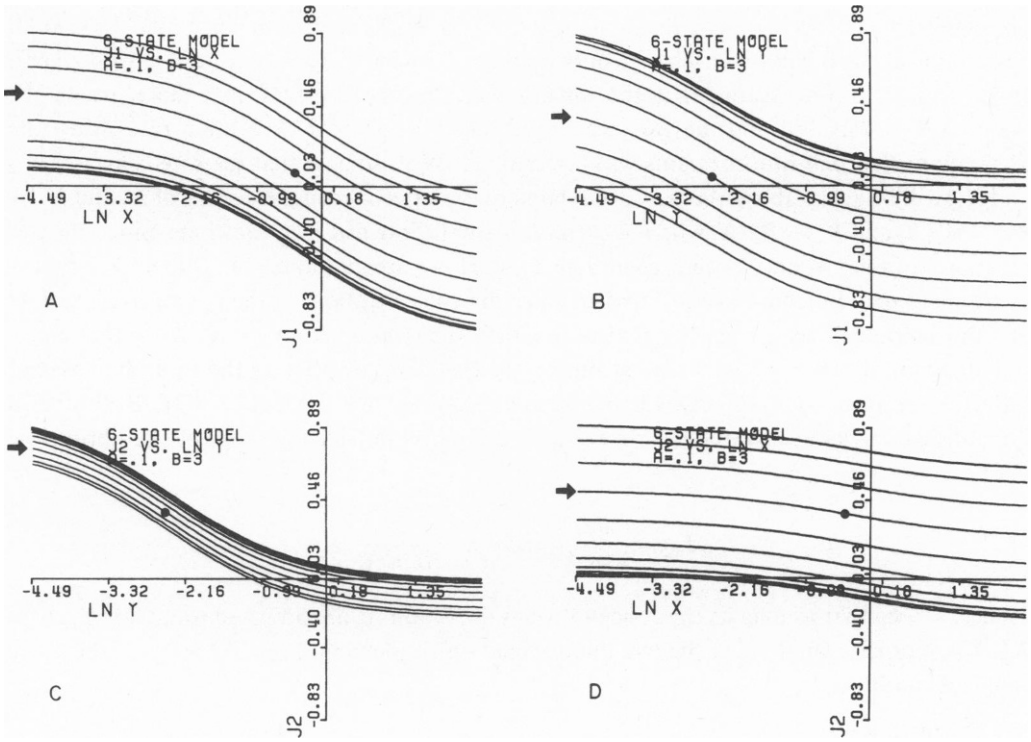

FIGURE 7 (A, B) Plots of Eq. 33*a* for $a = 0.1$, $b = 3.0$. In Fig. 7 A, J_1 is plotted as a function of ln X for constant values of ln $Y = -4.49, -3.79, -3.09, -2.39, -1.68, -0.99, -0.29, 0.41, 1.14, 1.82$ (starting from the upper curve). In Fig. 7 B J_1 is plotted as a function of ln Y for the same constant values of ln X listed above. (C, D) Plots of Eq. 33*b* for $a = 0.1$, $b = 3.0$. In Fig. 7 C J_2 is plotted as a function of ln X for constant values of ln Y (same as listed above.) In Fig. 7 D J_2 is plotted as a function of ln Y for constant values of ln X listed above. The dots represent inflection points.

point ($\mathcal{A}_x$ over $2RT$, $\mathcal{A}_y$ over $-1.3RT$). In addition, it is found for this particular case that $L_{12} \neq L_{21}$ (reciprocity is not obtained) although $\alpha_1 = \alpha_2 = 0$ (cf. Eqs. 32).

Existence of Reciprocity at a Two-Dimensional Inflection Point

In the previous model we found that reciprocity is in general not obtained near the multidimensional inflection point. This is in agreement with several theoretical studies of coupled enzyme and transport systems including those of Mikulecky (24), Bunow (25), and Oster and Perelson (26). It is possible to demonstrate however that in specific cases which are biologically plausible reciprocity can exist at a multidimensional inflection point. We consider below two cases involving a four state, two flow-two force model which could be representative of a number of facilitated transport or coupled reaction processes (cf Fig. 2). In case one, reciprocity is the result of the equivalence of certain kinetic constants in the mechanism. In the second, more general case, we show that it is possible for the multidimensional inflection point to coincide with equilibrium by adjusting a small number of kinetic parameters.

Case I

Consider the four state mechanism in Fig. 2 where some of the rate constants in flow J_1 and J_2 are equivalent (i.e. $K_{31} = K_{13}$, $K_{41} = K_{23}$, and $K_{12} = K_{34}$). Such a model might be a valid description of the facilitated exchange of two similar ions across a membrane by an ionophore. In particular, if we assume that the diffusion of the uncomplexed ionophore across the membrane is unbiased and the two exchanged ions have similar desorption rates, then the above equivalence of rate constants will be obtained. We also note that the rate constants K_{14} and K_{32} will be adjustable since they are related to the external concentrations of X_1 and X_2 by $K_{14} = K'_{14}X_2$ and $K_{32} = K'_{32}X_1$ where K'_{32} and K'_{14} are the intrinsic rate constants. Since our two reaction variables X_1 and X_2 obey conditions I and II, we are guaranteed that there will exist a two-dimensional inflection point. Further, since the concentrations of the reactants X_1 and X_2 are unconstrained, we can specify that they satisfy the relation $K'_{14}X_2 = K'_{32}X_1$ so that $K_{14} = K_{32}$. It then follows because of the symmetry of this diagram that at the multidimensional inflection point $J_1 = J_2$. It can further be demonstrated for this model (Dr. Rothschild's unpublished observations) that if $J_1 = J_2$, $L_{12} = L_{21}$, where outside the range of equilibrium we define

$$L_{12} = \frac{\partial J_1}{\partial \ln X_2} \text{ and } L_{21} = \frac{\partial J_2}{\partial \ln X_1}.$$

Hence, we see that as long as the concentrations of X_1 and X_2 are adjusted to satisfy $K'_{14}X_2 = K'_{32}X_1$ reciprocity will hold at the two-dimensional inflection point even if it occurs outside the range of equilibrium.

Case II

In the second example, Onsager reciprocity is obtained by forcing the two-dimensional inflection point to coincide with equilibrium. As discussed previously, in this case Eqs. 30 will become identical to the phenomenological Eqs. 1. The requirement that the system is at equilibrium and at a two-dimensional inflection point introduces additional constraints to those of Eqs. 19. In the case of the four state, two flow-two force model of Fig. 2, the system

will be at equilibrium provided: (note we have dropped primes on K_{14} and K_{32} for simplicity)

$$X_2 = \frac{K_{13}K_{34}K_{41}}{K_{14}K_{43}K_{31}}$$

$$X_1 = \frac{K_{12}K_{23}K_{31}}{K_{13}K_{32}K_{21}} \tag{38}$$

In general it is not possible to satisfy both Eqs. 19 and 38 simultaneously by simply finding a suitable point (X_1, X_2). It is thus necessary to impose special values or relations on some of the rate constants, K_{ij}. However, because there are 10 rate constants, the system can be made to satisfy the four constraining equations in a number of ways. For example, it can be proven (Rothschild and Ellias, unpublished observations) that by suitably adjusting the rate constants K_{41} and K_{23}, an inflection point can always be found which will coincide with equilibrium providing the remaining constants are related to each other by:

$$K_{31}^2 K_{43} K_{12}(K_{21} + K_{13} + K_{12}) = K_{13}^2 K_{21} K_{34}(K_{43} + K_{34} + K_{31})$$

$$K_{34} K_{12}(K_{21} + K_{43}) > K_{43} K_{21}(K_{34} + K_{31} + K_{12} + K_{13}). \tag{39}$$

The relation in Eq. 39 can be satisfied in a number of ways, for example by adjusting only two constants (e.g., K_{21} and K_{43}). Hence, a maximum of 4 out of the 10 rate constants must have special values to guarantee that the multidimensional inflection point will coincide with equilibrium and therefore Onsager reciprocity will be obtained. This method is particularly plausible if some of the rate constants in the diagram, e.g. K_{41} and K_{23} can be externally adjusted by varying the specific reactant concentrations. For example, if the active transport model of Fig. 6 is simplified into a four-state model (cf. Fig. 2 dotted arrows), it is then observed that:

$$X_2 = [\mathrm{ADP}] \qquad K_{41} = K'_{41}[\mathrm{ATP}] \qquad K_{21} = [K_I^+]K'_{21}$$

$$X_1 = [\mathrm{Na}_0] \qquad K_{23} = K'_{23}[\mathrm{K}_0^+] \qquad K_{43} = [\mathrm{P}_i]K'_{43} \tag{40}$$

Hence, it follows that for specific values of [ATP], $[\mathrm{K}_0^+]$, $[\mathrm{K}_I^+]$ and $[\mathrm{P}_i]$ the two-dimensional inflection point will coincide with equilibrium. This demonstration shows that even for a simple active transport model, the system possesses many degrees of freedom which can allow a coincidence of equilibrium and the two-dimensional inflection point either through proper evolution of the enzyme's rate constants or by adjustment of the external substrate concentrations. In this case we would expect an extended region of linearity to exist around equilibrium.

CONCLUSIONS

We have presented in this paper a general mathematical proof that there exists a special steady-state operating point, $\mathbf{x}^0$, around which an extended region of linearity exists between the logarithm of the concentrations x_i and the enzyme-mediated flows, provided the reactants obey two general conditions.

Condition I*a*: The reactant affects the transitions rates for leaving only one of the enzyme

states. Condition I*b*: The kinetics are of first order with respect to the reactant. (For a generalization of Condition I*b* cf. Appendix A) Condition II: For each possible combination of reactants considered there must be at least one directional graph of the Hill diagram containing only that combination of reactants and no others.

This represents a generalization of the demonstration by Rottenberg (2) that single-flow enzyme-mediated reactions characterized by Michaelis-Menten kinetics will always exhibit an inflection point for flow vs. ln x. The existence of a multidimensional inflection point may have important implications for the analysis of enzyme reactions as well as other steady-state processes operating outside the range of equilibrium. We have also demonstrated that in some cases where specific values of the rate constants are adjusted the inflection point can coincide with equilibrium. In these cases, the use of linear phenomenological equations beyond the range of equilibrium appears to be justified up to third order.

An important remaining question is whether the linear behavior observed in complex biological systems may indeed in some cases be attributable to mechanisms of the type considered here. The present analysis deals only with single enzyme systems. For example, a Na^+ – ATPase enzyme which actively transports Na^+ across the plasma membrane could exhibit a linear dependence of both J_{Na}, the net rate of Na^+-active transport, and J_R, the net rate of metabolic reaction, viz. ATP hydrolysis, on both ln (Na_0^+) and ln (ATP), if it is operating near the multidimensional inflection point (cf., the example in the preceding section). It is also possible that the linear behavior observed for large enzyme complexes such as those in the inner mitochondrial membrane are explicable on this basis (27, 28). For example, Hill diagrams have been recently proposed for oxidative and phosphorylative (29) complexes of the mitochondria. The conditions governing the existence of a multidimensional inflection point $\mathbf{x}_0$ for a complex are identical to those governing a single enzyme.

Additional complexity arises in analysis of the metabolism of mitochondria, which exhibit a wide range of linear dependence of the rates of both oxidative and phosphorylation and oxygen consumption on the phosphorylation and oxidation affinities (2, 30). Since the present analysis deals only with single enzymes or enzyme complexes, it might appear that oxidative-phosphorylation would not be amenable to the above treatment. In particular, in the chemiosmotic hypothesis there are two discrete enzyme complexes whose functions are linked by circulation of proton flow (31). However, it can be shown (32) that as long as the two separate components of oxidative-phosphorylation obey linear phenomenological equations, in the steady state, when net proton flow $J_H = 0$, the overall system will also obey linear equations (32). In a similar manner it should be possible to extend the present analysis to long chains of series enzymatic reactions by considering states in which the reactants linking each enzyme have reached their steady-state concentrations. The more general treatment of multienzyme complexes sharing common reactants remains to be developed.

The present model of linearity outside the range of equilibrium as based on the existence of a multidimensional inflection point offers an alternative to the suggestion of Prigogine (33) which envisions a series of coupled reactions each close to equilibrium. There is at present only limited experimental evidence which might help test the two hypotheses. Recent studies by Rottenberg and Gutman (34) on reverse electron transport in submitochondrial particles do reveal large regions of linear dependence of the oxidation-reduction rate on phosphorylation potential and redox potential which appear centered around inflection points. More data

would be necessary, however, to determine if this system is operating around a true two-dimensional inflection point.

In a future paper we shall further explore some of the implications of steady-state systems operating around a multidimensional inflection point.

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APPENDIX A

In this appendix we will prove the following result: if condition I is satisfied by any particular reactant χ_i of an N-state enzyme system, which is defined below, all steady-state flows in the system will depend on the concentration of that reactant as shown in Eq. A1

$$J = \frac{A_i x_i + B_i}{C_i x_i + D_i}, \tag{A1}$$

where A_i, B_i, C_i, D_i are independent of x_i.

To demonstrate this result, we will first state several assumptions and definitions that will help characterize the enzyme kinetic system. Consider an ensemble of identical enzymes where each individual enzyme can be in any one of N states E_i; $i = 1, 2, \ldots N$. Let $[E_i]$ be defined as the concentration of states E_i and p_i defined as the ratio $[E_i]/\Sigma_{k=1}^{N}[E_k]$. Consequently, p_i is the probability that the enzyme is in state E_i and

$$\sum_{k=1}^{N} p_k = 1. \tag{A2}$$

Furthermore, it is assumed that the enzyme obeys the following time-invariant mass action laws and admissible initial conditions.

$$dp_i/dt = -\sum_{j=1}^{N} p_i K_{ij} + \sum_{m=1}^{N} p_m K_{mi} \quad i = 1, 2, \ldots N \tag{A3}$$

for all i and j, $p_i(0) \geq 0$, $K_{ij} \geq 0$, $K_{jj} = 0$.

In Eq. A3, K_{ij} denotes the total directional transition rate constant from E_i to E_j. Furthermore, each K_{ij} may depend on some reactant concentrations. The reaction rate J_{ij} is defined as the net rate of advancement of the reaction from E_i to E_j, and is given by $J_{ij} \equiv (p_i K_{ij} - p_j K_{ji})E$, where E is the total enzyme concentration.

It can be shown that Eq. A3 implies that each $p_k(t)$ tends to a unique steady state, p_k^*, as $t \to \infty$ if the K_{ij}'s are time invariant regardless of the particular initial conditions. Thus for the "quasi-static" case where reactant concentrations and K_{ij} are almost time-invariant, it is assumed that the system is at steady state $dp_k/d_t = 0$, $(k = 1, 2, \ldots N)$. In this steady state, let

$$J_{ij}^* \equiv (p_i^* K_{ij} - p_j^* K_{ji})E. \tag{A4}$$

We now express Eqs. A3 for the steady state case in matrix form:

$$0 = \bar{\bar{B}}\bar{P} \tag{A5}$$

$$1 = \sum_{i=1}^{N} P_i \tag{A6}$$

where $\bar{\bar{B}}$ is the $N \times N$ matrix with elements

$$B_{ij} = \begin{cases} -\sum_{m=1}^{N} K_{im} & \text{if} \quad i = j \\ K_{ji} & \text{if} \quad i \neq j \end{cases}$$

and the elements of the column vector $\bar{P}$ are given by $P_i \equiv p_i^*$, $i = 1, 2, \ldots N$.

To solve the system of Eqs. A5 and A6 we can replace the m^{th} row of Eq. A5 with Eq. A6 yielding

$$\bar{I}^{(m)} = \bar{\bar{B}}^{(m)}\bar{P} \tag{A7}$$

where $\bar{I}^{(m)}$ is a column vector of elements

$$I_k^{(m)} = \begin{cases} 0 & m \neq k \\ 1 & m = k \end{cases}$$

and

$$B_{ij}^{(m)} = \begin{cases} B_{ij} & i \neq m \\ 1 & i = m \end{cases}.$$

Solving Eq. A7 by Cramer's rule, (20), gives, after simplification,

$$P_m = \frac{\text{Det}(\bar{\bar{A}}^{(m)})}{\text{Det}(\bar{\bar{B}}^{(m)})} \tag{A8}$$

where $A^{(m)}$ is the $(n-1) \times (n-1)$ matrix obtained by deleting the m^{th} column and m^{th} row from $\bar{\bar{B}}^{(m)}$.

The explicit solution of Eq. A8 depends upon evaluating the two determinants. However, a diagramatic method of writing down these terms upon inspection of the enzyme graph is well known (18, 19). For the purposes of this section it is sufficient to note the following properties:

(*a*) $P_i = N_i\Delta^{-1}$, where $N_i = |\text{Det}(\bar{\bar{A}}^{(i)})|$, and $\Delta \equiv |\text{Det}(\bar{\bar{B}}^{(m)})| = \Sigma_{k=1}^{N} N_k$.

(*b*) Each N_i is a sum of positive terms. Each term is a product of $N-1$ different $K_{\ell m}$ (cf. references 12, 18, 19).

(*c*) N_i does not contain any K's of the form $K_{\lambda i_\chi}$ $\chi = 1, 2, \ldots N$. This is true because the $\bar{\bar{A}}^{(i)}$ matrix does not contain any of these factors.

(*d*) For any square matrix $\bar{\bar{M}}$, $\text{Det}(\bar{\bar{M}})$ can be expanded about any column k according to $\text{Det}(\bar{\bar{M}}) = \Sigma_i M_{ik} C_{ik}$, where C_{ik} is the cofactor of M_{ik} and the C_{ik} are independent of the M_{jk} for all j (20).

The form of J in Eq. A1 can be obtained by using these properties ($a - d$) in Eq. A4: The numerator of P_i does not contain any $K_{i\lambda}$, $\lambda = 1,2, \ldots N$ (see *a* and *c* above). Furthermore, from (*d*), the numerator and denominator of P_i can be expanded as a linear combination of the r^{th} column of $\bar{\bar{A}}^{(i)}$ and $\bar{\bar{B}}^{(i)}$, respectively. Thus, the numerator of P_iK_{ij} in Eq. A4 can be written as a linear combination of $K_{r\lambda}$, $\lambda = 1, 2, \ldots N$ for any fixed r: i.e. no transition rate will ever appear to a power other than one. This is also true of the denominator, Δ, of P_iK_{ij} and for the numerator and denominator of P_jK_{ji} in Eq. A4. Therefore,

observing that $P_i K_{ij}$ and $P_j K_{ji}$ have the same denominator, Δ, (from [a]), we can rewrite J_{ij}^* as

$$J_{ij}^* = \frac{c_r + \sum_{\lambda=1}^{N} K_{r\lambda} d_{r\lambda}}{e_r + \sum_{\lambda=1}^{N} K_{r\lambda} g_{r\lambda}}, \tag{A9}$$

where c_r, $d_{r\lambda}$, e_r, $g_{r\lambda}$ are independent of K_{rj} for all j and from (a) and (b) e_r, $g_{r\lambda} \geq 0$.

Now, from conditions Ia single reactant is only allowed to enter the transition rates for leaving one enzyme state, and it can only enter these rates linearly or not enter at all, hence, if x_r is the reactant associated with some of the transition rates $K_{r\lambda}$, $\lambda = 1, 2, \ldots N$; then, factoring out this dependence in Eq. A9 we obtain

$$J = J_{ij}^*(x_r) = \frac{A_r x_r + B_r}{C_r x_r + D_r}, \tag{A10}$$

where A_r, B_r, C_r, D_r are independent of x_r and C_r, $D_r \geq 0$.

This entire argument is independent of the value of r. Therefore, Eq. A10 is the general form of the flow dependence for any reactants obeying conditions I. It is noted that if the reactant x_r enters into a transition bimolecularly we substitute x_r^2 for x_r in Eq. A10. In this case the requirement for unimolecular reactions can be dropped. In the most general case, condition Ib can be generalized to "the kinetics are of fixed order, ℓ_i, with respect to the i^{th} reactant, x_i."

APPENDIX B

In section II we treated the case of a single flow as a function of two reactant concentrations. In order to find a point (x_1^0, x_2^0) around which $J(x_1, x_2)$ varied linearly with ln x_1 and ln x_2 up to third order, it was necessary to simultaneously solve Eqs. 17 and 18. These equations are an expression of the condition that $[\partial^2 J(x_1^0, x_2^0)]/[\partial(\ln x_1)^2] = [\partial^2 J(x_1^0, x_2^0)]/[\partial(\ln x_2)^2] = 0$. In the more general case of N reactants we desire that $[\partial^2 J(\mathbf{x}^0)/\partial(\ln x_k)^2] = 0$ for $k = 1, 2, \ldots, N$ where $\mathbf{x}^0 = (x_1^0, x_2^0 \ldots x_N^0)$ is the multidimensional inflection point. This condition leads to a set of N equations (Eqs. B1) which unlike Eqs. 17 and 18 may not be solvable explicitly. However, it is still possible to demonstrate by using the Brouwer's fixed point theorem (21) that a solution to Eqs. B1 will exist under certain conditions. These conditions are analogous to the condition for the case of two reactants which requires that a directional graph corresponding to the terms $f_1 x_1, f_2 x_2$ and $f_{12} x_1 x_2$ appear in the Hill diagram of the enzyme.

In the more general case where M-independent enzyme-catalyzed flows depend on N-reactant concentrations, the generalized flow expressions can be expressed simply as: $J_k = J_k(x_i)$ $i = 1, N$; $k = 1, M$. The analysis in Appendix A shows that any of the M flows, J_k, of a generalized enzyme-mediated reaction which is dependent on a substrate χ_i which obeys condition I will have the characteristic form of Eq. 4:

e.g.
$$J_k = (A_{ki} x_i + B_{ki})/(C_i x_i + D_i) \qquad \begin{matrix} k = 1, \mathrm{M} \\ i \in \{i\}_I \end{matrix}$$

where $\{i\}_I$ denotes the set of all reactants χ_i which satisfy condition I. Furthermore, because we show in Appendix A that the denominator of each flow expression J_k must be identical, the inflection point $\mathbf{x}_i^0 = D_i/C_i$ is independent of k, i.e., $\mathbf{x}^0$ will be the same for all J_k, $k = 1, M$. Therefore the entire analysis can be generalized to the case of M flows and we can write Eq. 30 provided a multidimensional inflection point can be found which satisfies Eq. B1.

We will now prove the following result: If conditions I and II are satisfied by a set of reactants χ_k, then

there will exist an inflection point $\mathbf{x}^0$, which is the solution to the N simultaneous algebraic equations

$$x_i = \frac{D_i(\mathbf{x})}{C_i(\mathbf{x})} \quad i = 1, 2 \ldots N \tag{B1}$$

where C_i and D_i are uniquely defined by representing the denominator of the flow expression, Δ, (see Eq. A10) as

$$\Delta \equiv C_i x_i + D_i \tag{B2}$$

Our proof is based on the Brouwer's Fixed Point Theorem which applies to equations of the form

$$\mathbf{x} = \mathbf{F}(\mathbf{x}). \tag{B3}$$

This theorem guarantees that there exists at least one solution, $\mathbf{x}^*$, to Eq. B3 providing $\mathbf{F}$ is a continuous function which maps a set Ω into Ω, where Ω is some closed, bounded convex set.

We will show that Brouwer's Fixed Point Theorem applies to Eq. B1. Assume that the reactants χ_k are consecutively labeled (i.e. $x_1, x_2, \ldots x_N$) and that their concentrations are all non-negative. Moreover define $\mathbf{F}(\mathbf{x})$ as $F_i(\mathbf{x}) \equiv D_i(\mathbf{x})/C_i(\mathbf{x})$.

In order to demonstrate that the Brouwer's Fixed Point Theorem applies it is sufficient to show that (*a*) for each i, $F_i(\mathbf{x})$ is continuous for $\mathbf{x}$ in some set Ω_N(*b*) Ω_N can be chosen so that it is closed, bounded, convex and if $\mathbf{x} \in \Omega_N$ then $\mathbf{F}(\mathbf{x}) \in \Omega_N$.

(*a*) Since all the reactants χ_i obey conditions I we can infer from Eq. A10 that the general form of the denominator of the flow expression, Δ, is

$$\Delta = \sum_j \Delta_j \prod_{k \in J} x_k, \quad \Delta_j \geq 0 \tag{B4}$$

where Σ_j represents the sum over all possible combinations (2^N) of sets of indices, J (were set J is indexed by j), and includes the term Δ_0 which contains no x_k. Comparing the form of Eq. B4 to Eq. B2, D_i and C_i have the general forms

$$D_i(\mathbf{x}) = \sum_j d_j^i \prod_{k \in J} x_k, \quad d_j^i \geq 0$$

$$C_i(\mathbf{x}) = \sum_j c_j^i \prod_{k \in J} x_k, \quad c_j^i \geq 0 \tag{B5}$$

$F_i(\mathbf{x})$ is therefore a ratio of two polynomials and is continuous in any bounded region provided that $C_i(\mathbf{x}) \neq 0$ for $i = 1, 2, \ldots N$. Because each x_i is non-negative then $C_i(\mathbf{x}) = 0$ only if $c_0^i = 0$. However $c_0^i > 0$ because condition II requires that there exists a directional diagram, and hence a denominator term, involving x_i and no other x_k. Thus $\mathbf{F}(\mathbf{x})$ must be continuous over any set of $\mathbf{x}$ for which each x_i is non-negative.

(*b*) To show that there exists a closed bounded convex set, Ω_N, such that $F(\Omega_N) \epsilon \Omega_N$, it is sufficient to show there exists some rectangular set R_N with each x_j coordinate limited to region $[0, x_j^+]$, $x_j^+ < \infty, j = 1,2, \ldots N$ such that $F_j(\mathbf{x}) \leq x_j^+$ for all $\mathbf{x} \in R_N$. This follows because R_N is a closed, bounded, convex set.

We first define

$$N_j \equiv \prod_{k \in J} x_k, \quad x_k \geq 0 \quad k = 1, 2, \ldots N \tag{B6}$$

Substituting Eq. B6 into Eq. B5 $F_i(\mathbf{x})$ can be rewritten as

$$F_i(\mathbf{x}) = \frac{\sum_j d_j^i N_j^i}{\sum_j c_j^i N_j} \tag{B7}$$

where, by definition, N_j, d_j^i, and $c_j^i \geq 0$.

Condition II requires that there exists a directional diagram (and hence a term in Δ) for each possible combination of variables x_k, consequently, all the d_j^i and c_j^i are non-zero ($i = 1, 2, \ldots N$).

Let us choose x_i^+

$$x_i^+ = \operatorname*{Max}_j (d_j^i)\epsilon_2^{-1}, \tag{B8}$$

where ϵ_2 is the minimum value of c_j^i for all i and j. If $F_i(\mathbf{x}) \leq x_i^+$, then, from Eqs. B7 and B8 we must have

$$\frac{\sum_j d_j^i N_j}{\sum_j c_j^i N_j} \leq \operatorname*{Max}_j (d_j^i)\epsilon_2^{-1},$$

Rearranging terms we have the inequality

$$0 \leq \sum_j N_j \left[\frac{c_j^i}{\epsilon_2} \operatorname*{Max}_j (d_j^i) - d_j^i\right]. \tag{B9}$$

Eq. B9 is true because $c_j^i \geq \epsilon_2$ by definition. Therefore, we are guaranteed that there always exists an $x_i^+ < \infty$ such that $F_i(\mathbf{x}) \leq x_i^+$ for $\mathbf{x}$ non-negative.

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