Articles

Rate-Limiting Step: A Quantitative Definition. Application to Steady-State Enzymic Reactions†

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ABSTRACT: The generality of the concept of a rate-limiting step in enzymic reactions recently has been questioned [Northrop, D. B. (1981) *Biochemistry 20*, 4056–4061] because, in simulated isotopic experiments, alterations of the step identified as rate limiting by current definitions do not *consistently* affect V_{max} in the expected manner. In this paper a definition for a rate-limiting step is posed that eliminates such inconsistencies while the thrust of the original concept is retained. Thus, for any steady-state process involving a linear reaction sequence the rate-limiting step is taken as the "most sensitive" step, or the step which, if perturbed, causes the largest change in overall velocity, v. In both V and V/K enzymic systems the most sensitive step is identified by the relative magnitude of the sensitivity function, SF_j, for the various forward steps. If forward steps are identified by k_j, SF_j is equal to $\partial(1/v)/[\partial(1/k_j)/(1/k_j)]$, when the equilibrium constant for the step involving k_j is maintained constant. The corresponding sensitivity index, SI_j, is a normalized function of SF_j (the normalizing factor is v) such that the sum of the values for SI_j is equal to 1. In addition, there is an exact relationship between the sensitivity index for the isotopic step and the fraction of the intrinsic isotopic effect that is expressed in the overall rate of the reaction (when the intrinsic effect is taken as the fractional difference in reciprocal rate constant produced by the isotope). A procedure is described for approximating the sensitivity function for the various steps in a reaction sequence on the basis of the Gibbs energy profile for that reaction and thus identifying the most sensitive step. This approach also is used to consider the general question of whether a rate-limiting step should be specified for a multistep enzymic reaction. Identifying the rate-limiting step as the most sensitive step in a reaction sequence means that *no aspect* of the concept of *minimal rate* should be *automatically considered* as a property of a rate-limiting step.

The concept of a "rate-limiting" step in a reaction that proceeds by way of a sequence of intermediates is one of the fundamentals of classical physical–organic chemistry and has been applied almost from the beginning of systematic attempts to alter reaction rates by changing the structure or the chemistry of the reactants, or both. Recently, Northrop (1981) demonstrated some of the problems that can arise when various descriptions of the rate-limiting step are applied to enzymic reactions. For example, in some (but certainly not all) cases, even when the rate constant for the isotopic step is *smaller* than that for any other step, no isotopic effect on the overall rate will be observed, contrary to what might be expected from traditional treatments of simple (nonenzymic) systems. Because of such problems, Northrop suggests that the entire concept of a rate-limiting step is "outmoded", although stating that current definitions can lead to inconsistent conclusions seems to constitute a more accurate description of his arguments. This inconsistency provides the basis for the present paper, which shows that an alternative and quantitative definition for the rate-limiting step can be formulated that consistently correlates the basic thrust of the original concept with the results obtained from isotopic studies in unbranched[1] V and V/K enzymic systems.[2] In turn, the consistency of these correlations provides a basis for evaluating the extent to which a defined enzymic reaction should be described as possessing a rate-limiting step.

The definition in question involves the concept of the "most sensitive step" in a reaction—the step which, when perturbed, causes the largest change in the rate of a reaction—and gives rise to a "sensitivity function" that can be formulated for each forward step in a reaction and used to specify the extent to which each step is rate limiting.

Because this paper is primarily concerned with formulating a consistent definition of the rate-limiting step and its use to describe enzymic reactions more precisely, Results contains the following: definitions of the sensitivity function and the sensitivity index for steps in a reaction sequence together with a description of the relationship between the most sensitive step and the rate-limiting step in such a sequence; definitions of other properties that currently are used to describe steps in a reaction sequence and an illustration of the use of these in analyzing a simple, two-step, nonenzymic reaction; a rationale for the differences in analyses of V and V/K enzymic systems; an analysis of a simple V/K system to illustrate the formulation of the sensitivity function and the sensitivity index; a description of the relationship between the sensitivity index for a given step and the extent to which an isotopic effect on that step is expressed in the overall rate; revised analyses of some of the enzymic V systems which were formulated by Northrup (1981) to demonstrate the inconsistencies noted above and which now are consistently interpreted. Discussion deals with a more generalized application of the relationships

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[1] Reaction sequences are considered unbranched when, in effect, only one sequence of steps leading from free reactants to free products is functional, whether because the addition of substrates and release of products are compulsory or because of the relative concentrations of substrates and products employed in the reaction.

[2] V is used herein for $V_{max}/[E_t]$ and is equivalent to k_{cat}. A V system thus refers to an enzymic reaction at saturating substrate(s); a V/K system refers to an enzymic reaction conducted at a specified substrate concentration well below the value of its K_m.

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developed under Results to the analysis of (defined) enzymic reactions when these are represented by Gibbs energy profiles; it also addresses the question of whether the concept of a rate-limiting step should be applied to a given enzymic reaction. A number of general proofs are formalized under Appendix. In order to strike a compromise between readability and throughness of presentation, both proposed and currently used concepts that are employed in subsequent analyses are separated within the text and italicized.

Results

Formulating a definition that retains the traditional concept of a rate-limiting step for a linear reaction sequence when the process is in steady state and that always provides the expected correlations with the results of isotopic studies requires that the traditional concept be stated in a manner analogous to that given below, since, as is subsequently suggested, this statement also constitutes a description of what the isotopic effect actually expresses.

The rate-limiting step in a reaction sequence is that forward step for which a change in its rate constant produces the largest effect on the overall rate.

To quantify this statement, with the above goal in mind, requires two additional considerations: (a) it is *reciprocal* velocities and *reciprocal* rate constants that are additive; (b) it is the change in the observed quantity (reciprocal velocity) with a *fractional* change in a parameter (reciprocal rate constant) that appropriately describes sensitivity.

The sensitivity function (SF_j) for the forward step designated by k_j is the change in reciprocal velocity with a fractional change in $1/k_j$ when the equilibrium constant involving k_j (i.e., K_j) is kept constant and all reactant concentrations (C_i) remain unchanged.

$$[\partial(1/V)/[\partial(1/k_j)/(1/k_j)]]_{C_i,K_j} = \mathrm{SF}_j \qquad (1)$$

Although *generalized* expressions for various functions that describe enzymic reactions frequently are more complex when rate constants are designated in the manner suggested by Cleland (1963), this convention is used herein, in deference to common usage, except in the general treatment in the latter part of Discussion and in the Appendix:

$$1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} 3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \cdots \rightleftharpoons n \underset{k_{j+1}}{\overset{k_j}{\rightleftharpoons}} n+1 \rightleftharpoons \qquad (2)$$

In this system, $j = 2n - 1$ and $[n+1]/[n] = k_j/k_{(j+1)} = K_j$.

To identify the step in a reaction sequence for which a fractional change in its reciprocal rate constant produces the largest change in the reciprocal velocity thus requires conducting the operation specified by eq 1 for each forward step and identifying the step with the largest value of SF_j. (A shortcut procedure for evaluating the above derivative is described in Appendix I.)

That step in the forward reaction for which the sensitivity function is maximal is the most sensitive step.[3]

The first and third definitions, above, equate the properties of being "rate limiting" and "most sensitive" in a somewhat arbitrary manner that nevertheless is consistent with the goal of this treatment, as stated at the beginning of Results. In the following sections, the relationship between isotopic effects and the sensitivity function for various steps in the reaction will be defined. In the qualitative parts of these sections it is simpler to consider systems in terms of velocities and rate constants instead of the reciprocals of these. Thus, the *identity* of the step for which $(\partial V/\partial k_j)/k_j$ is maximal (under the restrictions sepecified for eq 1) is the same as that for the step where $\partial(1/V)/[\partial(1/k_j)/(1/k_j)]$ is maximal, although from a *quantitative* standpoint SF_j is a much simpler function when it is defined in terms of reciprocal velocities and reciprocal rates, as in eq 1, than when defined in terms of velocities and rate constants.

[3] Actually, the "most sensitive step" is somewhat of a misnomer since, as used herein, "sensitive" actually applies to the *overall rate*; i.e., the overall rate is most sensitive to .... However, for convenience, most sensitive step will be used in the manner designated.

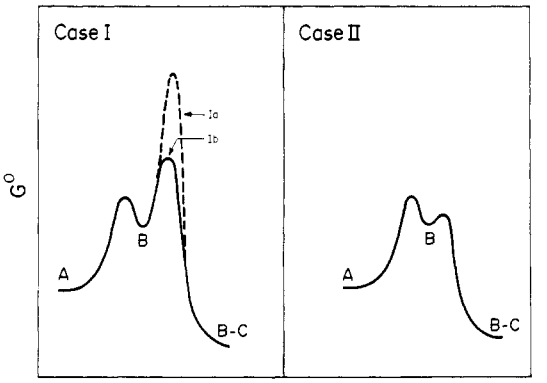


FIGURE 1: Gibbs energy profile showing the effect of two extreme conditions on the reaction in eq 3: case 1, where $k_2 \gg k_3[C]$; case 2, where $k_2 \ll k_3[C]$. In case Ia, $k_1 > k_3[C]$; in case Ib, $k_3[C] > k_1$.

Table I: Comparison of Properties of the First and Second Steps for Limiting Cases of Equation 3[a]

		step			
case	criterion	most sensitive	least conductive	most difficult	isotopically sensitive[b]
Ia	$k_3[C] \ll k_2$ $k_1 > k_3[C]$	second	first	second	second
Ib	$k_3[C] \ll k_2$ $k_1 < k_3[C]$	second	first	first	second
II	$k_3[C] \gg k_2$	first	first	first	first

[a] For definition of terms, see the text. [b] It is assumed that the isotopic effect is manifest only in one of the two steps and that it does not alter the overall equilibrium constant.

Most Sensitive, Least Conductive, and Most Difficult Steps in a Simple Reaction. The limiting cases for the following nonenzymic steady-state reaction sequence, where $k_1/k_2 \ll 1$, are represented by Gibbs energy profiles in Figure 1. The properties in the above heading, assigned either to the first or second steps on the basis of the following analysis, are summarized in Table I.

$$\mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{B} \xrightarrow{k_3[C]} \mathrm{B\text{–}C} \qquad (3)$$

When the concentration of C is kept low, case I, the rate of production of B–C will be proportional to [C], and the overall rate will be *insensitive* to a change in k_1 (when the ratio k_1/k_2 is kept constant) but *sensitive* to a change in k_3; hence, the second step is the *most sensitive*. In case II, sufficiently high concentrations of C are used so that the overall rate of the reaction becomes insensitive to further increases in [C]. Under these conditions, the overall rate will be *sensitive* to changes in k_1 but *insensitive* to changes in k_3, and the first step is the *most sensitive*. In both cases, the critical factor is the proportioning of the steady-state intermediate, B, between reactant and product (cf. Jencks, 1969): when $k_2 \gg k_3[C]$, the second step is the most sensitive; when the reverse is true, the first step is the most sensitive.

Another way of analyzing the steady-state reaction in eq 3 (as well as more complex steady-state processes) involves using *net* rate constants in the manner described by Cleland (1975).

The net rate constant, k_j', is the steady-state rate expression for the net (effective) *conversion of the intermediate reacting via the step designated by k_j into the intermediate generated by the subsequent step* (see above reference).

Thus, eq 3 can be rewritten as

$$A \xrightarrow{k_1'} B \xrightarrow{k_3'} B\text{–}C \qquad (4)$$

According to Cleland (1975), k_1' in eq 4 is equal to $k_1[k_3[C]/(k_3[C] + k_2)]$, where the factor in brackets describes the steady-state partitioning of B between A and B–C. In case II ($k_3[C] \gg k_2$), the first step has the smallest net rate constant, since ($k_3[C]=$) $k_3' \gg (k_2 \gg k_1 \approx) k_1'$.

The (net) *conductance of a step is proportional to the net rate constant for that step.*[4]

Thus, in case II the first step in the above reaction is the least conductive step. In fact, in case I ($k_3[C] \ll k_2$), the first step also is the least conductive, since $k_1' \approx k_1(k_3[C]/k_2) = k_3'(k_1/k_2)$, so that $k_1' \ll k_3'$.

Another property that is useful in describing steps in a reaction sequence is that of difficulty.

The difficulty of a single step in a reaction sequence is directly related to the Gibbs activation energy for that step; the step with the largest value of $\Delta G^{\ddagger}$ is the most difficult step in a sequence of steps.

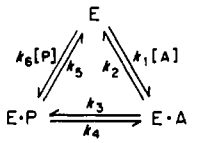
By difinition, in case II, the first step is the most difficult step (see Figure 1). However, the specification for case I ($k_3[C] \ll k_2$) does not uniquely define the most difficult step. In case Ia, Figure 1 (dashed line), the second step is the most difficult, while in case Ib (solid line) the first step is so designated.

Table I summarizes the above analysis. Clearly, the terms most sensitive, least conductive, and most difficult assess *different properties* of the various steps, although in one case, case II, each of these properties is associated with the same step (the first). In more complex reaction sequences each can be characteristic of a *different* step; i.e., the most sensitive, the least conductive, and the most difficult steps can be different (see below). Hence, only one of these properties can consistently correlate with an isotopic effect, even in the simple system described by eq 3 [if that effect is restricted to either one or the other of the two steps and does not alter the overall equilibrium constant (see below)]. In fact, if the rate-limiting step is to correlate consistently with isotopic effects, the most sensitive step must be taken as the rate-limiting step, at least in this system (see Table I). The generality of this requirement is demonstrated in a subsequent section. Intermediate cases in which no single step is clearly the most sensitive are considered under Discussion.

V and V/K Systems. Returning to the definition of the most sensitive step in eq 1, since expressions for V and V/K for an enzymic process are different, the (partial) derivatives of V and V/K with respect to the rate constant for a particular step clearly will not be the same. In many cases the difference in conditions between V and V/K systems, i.e., the difference in substrate concentration, will mean that different steps are identified as the most sensitive for reasons analogous to those considered above for the reaction in eq 3. Thus, in both V and V/K systems, altering the rate constant for a particular step will change the measured velocity only if it changes the steady-state concentration of that intermediate from which product dissociates (since rate = k_d[E·P]), by altering the relative concentrations of the various central complexes. In V systems, such a change can be produced only by changing the fraction of the central complexes present as E·P. By contrast, in a V/K system, [E·P] can be changed *either* as above (V change) *or* by keeping [E·P]/[CC] constant and changing the fraction of the total enzyme in the central complexes (K change), or both. Thus, *in general* one should not expect the rate-limiting step to be the same both at saturating substrate and at [S] $\ll K_m$. An alternative viewpoint of the basic difference between the most sensitive steps in V and V/K systems is given under Discussion. Because of the above difference, the sensitivity function for V and V/K systems is designated by ${}^{V}SF_j$ and ${}^{V/K}SK_j$, respectively, although these functions actually involve $(1/V)$ and (K/V).

Most Sensitive Step in a V/K System. The identification of the most sensitive step in an enzymic system by means of the sensitivity function is illustrated first for a V/K system with two central complexes. The operation specified by eq 1 is used, although a shortcut to this procedure is described in Appendix I.

For eq 5, $K/V = 1/k_1 + k_2/(k_1k_3) + k_2k_4/(k_1k_3k_5)$ (cf.



(5)

$$K_1 = k_1/k_2 \qquad K_3 = k_3/k_4$$

Cleland, 1963). When the right-hand side of this equation is differentiated with respect to the forward step, k_j (i.e., to k_1, or to k_3, or to k_5), and is divided by $1/k_j$, the expressions for ${}^{V/K}SF_j$ in eq 6–8 are obtained. (In order that K_j remain unchanged by this operation, as is required by eq 1, each term in which k_{j+1}/k_j occurs is treated as a constant: i.e., its differential with respect to k_j is zero.)

$${}^{V/K}SF_1 = 1/k_1 \qquad (6)$$

$${}^{V/K}SF_2 = 1/(k_3K_1) \qquad (7)$$

$${}^{V/K}SF_3 = 1/(k_5K_1K_3) \qquad (8)$$

For such a system, the step with the largest value of ${}^{V/K}SF$ is the most sensitive step. For unbranched reaction sequences, this step can be readily identified from a Gibbs energy profile for the reaction.

In V/K systems the most sensitive step crosses the highest point on a Gibbs energy profile for the reaction.

A generalized proof of this statement is given in Appendix IV.

In this example it can be seen that the sum of the expressions on the right-hand side of eq 6–8 is equal to K/V (where j is odd):

$$\sum_j {}^{V/K}SF_j = K/V \qquad (9)$$

Hence

$$\sum_j {}^{V/K}SF_j/(K/V) = 1 \qquad (10)$$

and V/K is a normalizing factor. This factor, when used as

[4] By analogy with electrical circuitry, it seems reasonable to designate k_j as the conductance of a specified step and k_j' as its *net* conductance. Thus, conductance represents the property of an isolated step; net conductance involves this property in conjunction with the properties of subsequent steps (cf. Cleland, 1975).

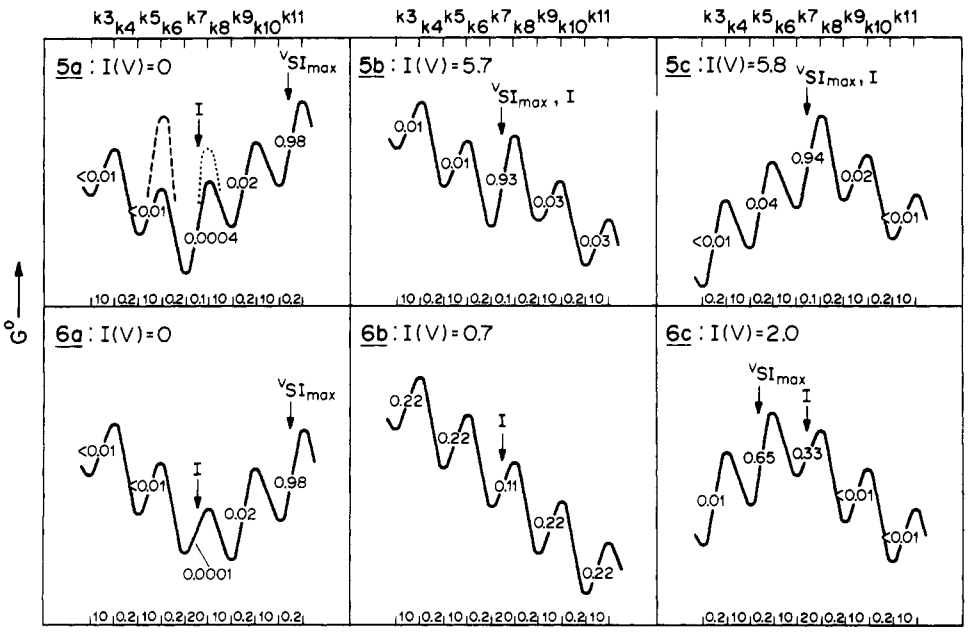


FIGURE 2: Gibbs energy profiles for six different combinations of the rate constants in eq 17. Relative Gibbs energies are designated at values of the abscissa arbitrarily chosen to represent the designated intermediates and transition states for the reaction in the above equation. Values of the "dimensionless rate constants" (top of figure) that are used are given at the bottom of each panel (see also footnote 8); these, together with the case numbers, were taken from Table I of Northrop (1981). Along each profile are given the sensitivity indexes for each step calculated according to eq 22. In each case the most sensitive step, designated by SI_{max}, is indicated by an arrow, together with the isotopic step, I. The actual value of the isotopic effect on V, I(V), also is given, where $I(V) = ({}^{D}V - 1)$ so that the maximal value of I(V) is 6.0 [since $({}^{D}k_i - 1)$ is taken as 6.0]. The dotted and dashed lines in case 5a represent variations that are described in the text.

a multiplier of ${}^{V/K}SF_j$, produces a dimensionless fraction, the sensitivity index, SI_j, or ${}^{V/K}SI_j$ in a V/K system:

$$\left[\frac{\partial(K/V)}{K/V} \Big/ \frac{\partial(1/k_j)}{1/k_j}\right]_{C_i,K_j} = (V/K)^{V/K}SF_j \quad (11)$$

$$(V/K)^{V/K}SF_j = {}^{V/K}SI_j \quad (12)$$

$$\sum_j {}^{V/K}SI_j = 1 \quad (13)$$

The fractional change in reciprocal velocity with a fractional change in $1/k_j$ is the sensitivity index (SI_j) for the step designated by k_j; this index is a dimensionless ratio whose sum for all steps is 1 (when K_j and C_i remain unchanged).[5]

The above definition is considered in a more general manner for both V and V/K systems in Appendix I.

Relationship between an Isotopic Effect and the Sensitivity Index of the Isotopic Step: V/K Systems. Although the size of the deuterium isotope effect can be specified as $(V/K)_H/(V/K)_D$ or ${}^{D}V/K$ (cf. Northrop, 1981), in the following discussion the isotopic effect on V/K will be expressed as ${}^{D}V/K - 1$, and its intrinsic effect on the rate constant for the isotopic step as $k_H/k_D - 1$, or ${}^{D}k_i - 1$,[6] where subscript, i, refers to a specific step (the isotopic step), as opposed to j, a general step. [The expressions ${}^{D}V/K - 1$ and ${}^{D}k_i - 1$ are the fractional changes in $1/(V/K)$ and $1/k_i$ produced by deuterium substitution.]

The sensitivity index for the isotopic step is equal to the fraction of the isotopic effect that is expressed in V/K when there is no equilibrium isotope effect:

$${}^{V/K}SI_i = \frac{{}^{D}V/K - 1}{{}^{D}k_i - 1} \quad (14)$$

A general proof of the above statement and its quantitative formulation (eq 14) is given in Appendix II.

The isotopic effect on the equilibrium constant $({}^{D}K_e - 1)$ may either increase or decrease the *apparent* sensitivity index, as can be seen from the treatment in Appendix II [cf. eq 30; see also Northrop (1977)]. The equilibrium isotope effect is considered further under Discussion.

Sensitivity Function for V Systems. Expressions for the sensitivity function for steps in V systems, ${}^{V}SF_j$, can be formulated by differentiating the expression for $1/V$ in the manner specified by eq 1 and illustrated, above, for K/V in a V/K system. However, the shortcut procedure in Appendix I can be used to formulate both ${}^{V}SF_j$ and ${}^{V/K}SF_j$ for uni-uni reactions.[7] By analogy with the sensitivity index in V/K systems

$${}^{V}SI_j = {}^{V}SF_jV \quad (15)$$

Relationship between the Sensitivity Index of the Isotopic Step and the Isotopic Effect in V Systems. The relationship between the isotopic effect on V and the sensitivity index for the isotopic step in a V system, ${}^{V}SI_i$, is analogous to that described above for a V/K system.

[5] ${}^{V}SI_j$ is the same whether defined in terms of reciprocal velocity and reciprocal rate constant or in terms of velocity and rate constant. The former definition is used because of the relationship between ${}^{V}SI_j$ and ${}^{V}SF_j$.

[6] It is assumed that only one step in a sequence of steps will involve an isotope effect.

[7] In an ordered bi-bi reaction, sensitivity indexes formulated for the various steps in a V system (saturating A and B) will differ from those in a V/K_B system (saturating A) in a manner analogous to differences in V and V/K systems for a uni-uni reaction. Sensitivity indexes for steps in a $V/(K_{iA}K_B)$ system ($[A] \ll K_{iA}$) will be the same as those for a V/K_B systems while the sensitivity index for k_1 in a V/K_{iA} system ($[B] \gg K_B$) is 1. In noncompulsory bi-bi reactions, sensitivity indexes for a V/K_A system will be the same as for a V/K_B system whether the reaction is rapid equilibrium or not. In ping-pong reactions, sensitivity indexes for V, V/K_A, and V/K_B systems all will be different.

The sensitivity index for the isotopic step is equal to the fraction of the intrinsic isotopic effect that is expressed in V when there is no equilibrium isotope effect.

$$^{V}SI_i = \frac{^{D}V - 1}{^{D}k_i - 1} \quad (16)$$

This relationship in verified in Appendix II. When there is an equilibrium isotope effect on the reaction, a modification of eq 16 analogous to that described for V/K systems must be made (see Appendix II and Discussion).

To visually illustrate the correspondence between $^{V}SI_i$ and $^{D}V - 1$ in enzymic systems, examples 5a–c and 6a–c from Northrop (1981) are used, although in view of the relationship in eq 16 such a comparison is somewhat redundant. These examples represent special cases of the following hypothetical reaction scheme.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}S \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\cdot}I \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E{\cdot}P \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E{\cdot}P \xrightarrow{k_{11}} E + P \quad (17)$$

Gibbs energy profiles for these cases are given in Figure 2, where Northrop's case number is retained. Also given (below each profile) are the relative rate constants selected by Northrop for the various steps. (Since only ratios of rate constant are important, no units are required for these constants.)[8] In each case, $^{D}k_i - 1$ is taken as equal to 6.0, $^{D}K_e - 1 = 0$, and the step designated by k_7 (vertical arrow, I) is the isotopic step.[6] The sensitivity index for each (forward) step, calculated according to eq 22, is shown along the profiles. Values for $^{D}V - 1$ [abbreviated as I(V) in Figure 2] given by Northrop also are shown.

In view of the lack of a *consistent* correspondence in the simple reaction in eq 3, between the least conductive and most difficult steps, on one hand, and the isotopically sensitive step, on the other, it is not surprising that a *consistent* relationship between these is not observed for the enzymic reactions whose energy profiles are shown in Figure 2. Thus, although in case 5b a large deuterium isotope effect on V, 5.7, is observed when the isotopic step also is the least conductive step, in case 5a essentially no isotopic effect on V is produced by the same correspondence. (Net rate constants for identifying the least conductive step are given by Northrop; however, a shortcut procedure for identifying such steps is given under Discussion.) In addition, although in case 5c a large isotopic effect on V is observed when the isotopic step is the most difficult, in 5a′ (case 5a of Figure 2, with steps 7 and 8 represented by the dotted line), essentially no isotopic effect is observed, even though the isotopic step also is the most difficult. Thus, an isotopic effect on V is not consistently produced when the isotopic step *either* is the least conductive or the most difficult. The reader is referred to Northrop (1981) for a further discussion of such inconsistencies.

By contrast, as is expected from eq 16, when the sensitivity index of the isotopic step is high (close to 1), a near-maximal isotopic effect is observed (cases 5b and 5c, Figure 2); when the index (for the isotopic step) has an intermediate value, an intermediate effect is observed (cases 6b and 6c); otherwise, either no or essentially no isotopic effect is found (cases 5a and 6a). Hence, if one expects to consistently observe a rate effect when an isotopic bond is broken in the rate-limiting step (and $^{D}k_i - 1 \neq 0$), such a step must be identified as the most sensitive step in a reaction sequence, as in the first paragraph under Results.

Discussion

Results (in conjunction with the Appendix) show that a definition of the rate-limiting step can be formulated which retains the thrust of the traditional concept of such a step and guarantees that *if* there is an isotopic effect on the step so designated, a *related* effect on the overall rate of the reaction also will be observed. This correspondence is not consistently observed for steps designated as rate limiting by other definitions (cf. Northrop, 1981). In one sense, this formulation could be considered trivial since, except for the imposed requirement of not doing violence to the classical concept of the rate-limiting step, the logic of redefining this step tends toward being circular: a derivative, which in reality specifies the magnitude of the isotopic effect, is used as the basis for a definition of the extent to which a step is rate limiting. Nevertheless, it appears that the use of the proposed definition significantly improves one's ability to visualize steady-state relationships (see below). In addition, adopting uniform definitions cannot help but facilitate the exchange of information about such relationships.

A substantial fraction of the Results section deals with the calculation of sensitivity indexes for steps in a completely defined reaction sequence, viz., one in which the rate constants for *all* steps are known. Although the use of defined sequences was necessary to demonstrate the significance of the sensitivity function and the sensitivity index, the experimentalist, of course, faces a much different problem in analyzing a real enzymic reaction. Although $^{D}V - 1$ and $^{D}V/K - 1$ sometimes can be measured, along with $^{D}k_i - 1$ (Northrop, 1975), only infrequently can one assess even a majority of the rate constants for enzymic reactions [on the other hand, see Rose & Iyengar (1982); Albery & Knowles, 1976]. Thus, the value of the present approach does not depend on the actual evaluation of a particular sensitivity index from experimental data. In fact, the *concept* of a sensitivity function and a sensitivity index and the knowledge of their relationship to what is defined here as the rate-limiting step can be as important as the actual value of the index for a given step in a particular reaction. This relationship is especially useful since the most sensitive step is readily identified on Gibbs energy profiles of a reaction that one might sketch to aid in considering various features of that reaction, as is described below.

Gibbs Energy Profiles for V and V/K Systems. Before procedures for identifying the least conductive and the most sensitive steps on the Gibbs energy profile of a reaction are considered, it is necessary to distinguish between those profiles that are appropriate for representing V systems as opposed to those for V/K systems. Actually, it is not strictly correct to use standard Gibbs energy changes to compare equilibria of different molecularity, e.g., $E + A \rightleftharpoons E{\cdot}A$ and $E{\cdot}A \rightleftharpoons E{\cdot}I$. However, if an appropriate allowance for dimensional differences is included, self-consistent comparisons can be made for some purposes. Although there are several different ways to make such an allowance, biochemists frequently do this by converting second-order rate constants into pseudo-first-order constants by multiplying the former by a suitable concentration, e.g.

$$E \underset{k_2}{\overset{k_1[A]}{\rightleftharpoons}} E{\cdot}A$$

This requires the use of the apparent equilibrium constant

[8] Since it is the *relative* values of rate constants that are most important in determining the overall appearance of the Gibbs energy profile for a reaction, unusually large absolute values of rate constants were used to construct the profiles in Figure 2. Scaling profiles in this manner produces a more compact representation because it reduces the heights of peaks relative to the adjacent valleys.

$k_1[A]/k_2$ to calculate the standard Gibbs energy change, $\Delta G°$, for the above process and gives rise to several problems. One of these is that $\Delta G°$ for E + reactants → E + products is not specified by the Gibbs energy profile used to represent the interconversion of the central complexes. Moreover, "$\Delta G°$" for E·P → E + P, calculated from $k_n/(k_{n+1}[P])$, becomes infinitely negative when [P] = 0, a behavior rather different than expected for a "standard" Gibbs energy change.

Although using the above convention to allow for differences in molecularity is convenient for some purposes, it is easier to identify the kinetically important steps defined under Results (see also below) if the required allowance is made in accord with the following criteria: (a) the same standard state is used for all substrates *and* products within a single profile (partly so that $\Delta G°$ for the overall reaction will be the difference between E + S and E + P on the plot used for the interconversion of the various intermediates); (b) the Gibbs energy profile is scaled so that for V systems the Gibbs activation energies for all 2 → 1 processes (bimolecular substrate binding steps) become negligible relative to those for all 1 → 1 processes (interconversions of central complexes) and all 1 → 2 processes (product-dissociation steps); (c) a different scaling is used for V/K systems so that the Gibbs activation energy for all 2 → 1 processes becomes much larger than that for all 1 → 1 and 1 → 2 processes. Since it is the *standard* Gibbs energy that is plotted on such a profile (Boyd, 1978), the above scaling is readily accomplished simply by defining the standard state for the substrate(s) [as well as the product(s)] differently when constructing V as opposed to V/K profiles. Thus, for V profiles a high concentration is used as the standard state [i.e., as the concentration where [A] (or a_A) is equal to unity (Eisenberg & Crothers, 1979)] so that at the standard state concentration, $K_A/[A]$ is small and $\Delta G°_c$ for E + A → E·A not only is negative but also $G^‡_c$ for this process is negligible. The subscript thus indicates that $[a]$ = 1 in a solution that could be more concentrated than a 1 M solution.[9] Similarly, for V/K profiles a low concentration is used as the standard state, so that when [A] = 1, $K_A/[A]$ is large and $\Delta G°_d$ for E + A → E·A not only is positive but also is much larger than $\Delta G^‡$ for any discrete step that subsequently occurs. (The subscript indicates that $[a]$ = 1 in a dilute solution.)

The above conventions are employed in the Gibbs energy profiles of an arbitrary enzymic reaction in Figure 3. In this figure, the V and V/K profiles represent the same reaction and are identical in the region between the transition state for formation of the first E·A complex and the transition state for dissociation of the last E·P complex—as they must be. However, an important difference is apparent when, after dissociation of the product, a unimolecular transformation of the enzyme is required before substrate binding can occur to initiate a second catalytic cycle. As is discussed below, and illustrated in Figure 3, on a V profile the transition state for such a step conceivably could lie higher on the (appropriately scaled) Gibbs energy profile than that for many or all other steps, but this is impossible on a (appropriately scaled) V/K profile.

It should be reemphasized that the only reason for introducing the above conventions is to facilitate the use of Gibbs energy profiles to identify steps possessing the properties defined under Results. It also should be pointed out that these properties exist independently of whatever convention is used to aid in identifying them and that the proposed convention

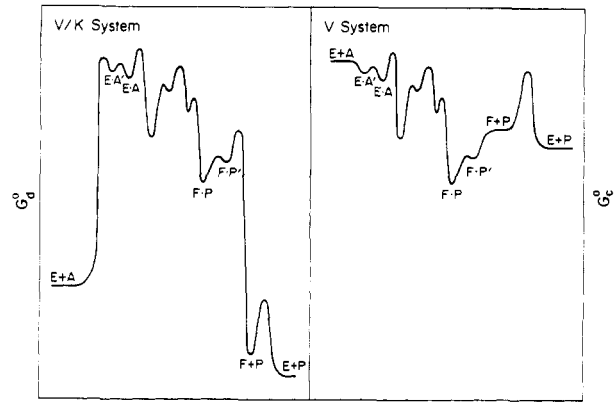


FIGURE 3: Gibbs energy profile showing the difference between a V/K system and a V system for an hypothetical enzymic reaction with eight reaction intermediates: an initially formed enzyme–substrate complex E·A′; a thermodynamically more stable form of this complex, E·A; three enzyme–intermediate complexes; two enzyme–product complexes, FP′ and F·P, which involve a different form of the enzyme, F, than the form E, which binds A. The scaling procedure used in this plot is described under Discussion.

is simply the one that, after adoptation, involves the least complex rules for this identification.

When these conventions are used to represent multiproduct reactions, there are no "breaks" in the Gibbs energy profile corresponding to the "irreversible dissociation of the product at zero concentration" as there are in the profiles that are more widely used by biochemists. This should not detract from the proposed convention since, in general, conditions imposed solely to facilitate measurements (e.g., [P] → 0) should not affect the values of *standard* Gibbs energy changes. On the other hand, if one insists on designating steps that, because of conditions, are unidirectional from a *kinetic* standpoint, this can be done with arrowheads appropriately placed along the profile.

Identifying the Least Conductive Step on a Gibbs Energy Profile. As is indicated under Results, the least conductive step in a (linear) reaction sequence is the forward step with the smallest net rate constant; i.e., the least conductive step originates at the species that is present at the highest concentration in the steady state (Cleland, 1975). This species is referred to herein as the *most abundant intermediate*. Of course, one can readily construct a reaction sequence in which two or more different steps have the same or nearly the same net rate constant (as in case 6b, Figure 2). Such reactions will be considered in a subsequent section; the present section considers how the least conductive step can be identified on a Gibbs energy profile in reactions where one net rate constant is substantially smaller than any other.

If the system were at thermodynamic equilibrium, the species that occupies the lowest point on a Gibbs energy profile would be the most abundant intermediate. However, this is not necessarily true in the steady state.

On a Gibbs energy profile, the valley from which the escape process in the forward direction involves the greatest total difficulty identifies the most abundant intermediate in a steady-state process (unbranched).

This statement is verified for a generalized reaction in Appendix III, both for V and V/K systems. The escape process referred to above can involve one or several steps, but an escape must end in the *first* subsequent valley *lower* than that from which the escape began, or, if no subsequent valley is lower, with the last step in the catalytic cycle (see below). The most difficult escape thus would be that (forward) escape sequence for which the *overall* escape barrier is the highest. Sometimes,

[9] At a sufficiently high concentration, $\Delta G^‡_c$ for the (diffusion-controlled) formation of the E·A encounter complex should approach zero.

the highest proint on the Gibbs energy profile will fix the height of the barrier for the most difficult escape sequence, e.g., the transition state for step 11 in the V system of case 5a, Figure 2, in which the escape sequence

$$\xrightarrow{k_7}\ \xrightarrow{k_9}\ \xrightarrow{k_{11}}$$

ends with the dissociation of the products, or the transition state for step 5, case 6c, in which the escape sequence

$$\xrightarrow{k_3}\ \xrightarrow{k_5}\ \xrightarrow{k_7}\ \xrightarrow{k_9}$$

ends at a lower valley. However, in V systems (as opposed to V/K systems; see below) the highest point on the Gibbs energy diagram need not be involved in the most difficult escape, as in cases 6a and 5b (Figure 2). In case 6a, the most difficult escape is

$$\xrightarrow{k_7}\ \xrightarrow{k_{11}}$$

but the transition state for step 11 is not the highest point on the Gibbs energy diagram; the transition state for step 3 is. Similarly, in case 5b the highest point in the most difficult escape sequence

$$\xrightarrow{k_7}\ \xrightarrow{k_9}$$

is lower than the transition state for a previous step: step 3. Note that the most difficult escape sequence also need not include the most difficult step. Thus, if k_5 in case 5a were 0.025 instead of 10 (see dashed line for case 5a, Figure 1), the step designated by k_5 would be the most difficult step but it would not lie along the most difficult escape sequence:

$$\xrightarrow{k_7}\ \xrightarrow{k_9}\ \xrightarrow{k_{11}}$$

This can be stated in another way.

For a steady-state reaction sequence (unbranched) *in which one net rate constant is substantially smaller than all others, the smallest net rate constant will be associated with the forward reaction of the most abundant intermediate which is the intermediate whose vertical position is farthest from the highest subsequent transition state, where "subsequent" includes all steps up to the first substrate-binding step in the next catalytic cycle.*

A general proof of this statement is given in Appendix III.[10]

Identifying the Most Sensitive Step on a Gibbs Energy Profile. As in the case of the least conductive step, one can design (linear) reaction sequences in which the sensitivity of at least two of the more sensitive steps is the same or essentially the same (cf. case 6b, Figure 2). Such reactions will be considered in the following section. Here, only reaction sequences are considered in which the sensitivity of the most sensitive step is substantially greater than that of any other step.

The most sensitive step in a steady-state reaction will cross the highest point on a Gibbs energy profile along the escape sequence with the greatest total difficulty.

A general proof of the above statement for both V and V/K systems is provided in Appendix IV, although the validity of this statement in V/K systems has been recognized for some time (cf. Jencks, 1969; Stein, 1981). In fact, the derivations in Appendixes III and IV may be summarized as follows: what contributes to the conductivity of a step is the depth of a valley and the relative height of the highest transition state along the escape sequence from that valley; what contributes to the sensitivity of a step is the height of the transition state for that step relative to the depth of the lowest *preceding* valley. When the two contributing factors are appropriately weighted, the least conductive step is characterized by its origin in a low valley that is *followed* by a high transition state while the most sensitive step is characterized by its inclusion of a high transition state that is *preceded* by a low valley, as is illustrated in the following section. (In neither case is "*directly* followed by" or "*directly* preceded by" implied in the above statement.)

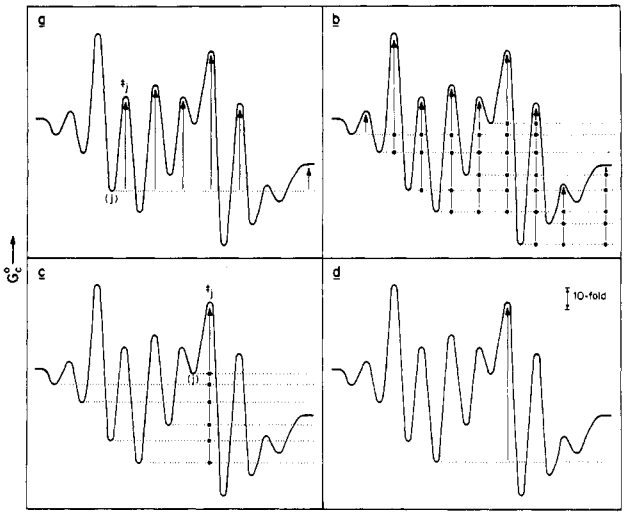


FIGURE 4: Gibbs energy profiles of a hypothetical reaction with nine reaction intermediates showing (a) a representation of the terms that contribute to the net resistance, $1/k_j'$, of the step designated by k_j (fourth step), (b) a representation of the net resistance of the overall reaction, (c) a representation of those terms that contribute to the sensitivity function for the step designated by k_j (seventh step), i.e., ${}^{V}SF_j$, and (d) a simplified representation (vertical arrow) of the net resistance of the least conductive step and the sensitivity function for the most sensitive step if the distance specified by the double-headed arrow is equivalent to a factor of 10-fold.

The reasons why the most sensitive step can be different in V and V/K systems can be seen by examining the Gibbs energy diagrams in Figure 3. In V/K systems the first step (the substrate-binding step) *always* will be the least conductive step (as well as the most difficult step). Thus, *all* transition states that represent the interconversions of the central complexes must *follow* the least conductive step and lie along the most difficult escape sequence. Hence, as noted above, the most sensitive step is simply the step that passes through the highest point on the Gibbs energy profile. By contrast, in V systems, the most difficult escape sequence may not include all transition states and thus may not include the transition state with the highest energy on a Gibbs energy profile, as in the reaction illustrated in Figure 3, where the third step is the most sensitive (rate-limiting) step in the V/K system, but not in the V system.[11]

A second reason for a difference in the most sensitive steps for V and V/K systems is that the release of a product affects the Gibbs energy profile for V and V/K systems in a different manner. Thus, while the transition state for a unimolecular process such as the isomerization of an enzymic species or the

[10] I am grateful to Professor W. W. Cleland for suggesting that the least conductive step involves the "steepest ascent" on a Gibbs energy profile.

[11] Another way of describing the basic difference between steady-state V and V/K systems is in terms of the cycling of the catalyst. This is required in V but not V/K systems since, as long as both $[E_0]$ and $[S_0]$ are much smaller than K_m, $[E_0]$ may be much larger than $[S_0]$, thus obviating any necessity for cycling and ensuring that no significant fraction of the enzyme accumulates in a kinetic as opposed to a thermodynamic well. Because of this, V/K systems behave in a manner more akin to simple, bimolecular reactions than do V systems.

release of a second product may or may not lie higher on a Gibbs energy profile of a V system than the transition states for the interconversion of the various central complexes, the transition state for any process that *follows* the release of the first product *never* can even approach the heights of the transition states for interconversion of the central complexes on an *appropriately scaled* Gibbs energy profile for a V/K system—because $\Delta G°_d$ for the release of the first product will be such a large negative number (see Figure 3). Thus, *no* step in a V/K system that follows a product release step can qualify as the most sensitive step, although such a step could well represent the most sensitive step in a V system. In fact the procedure described by eq 22, Appendix I, for formulating sensitivity indexes specifies a sensitivity index of zero for *all steps subsequent to a product release step* in a V/K system (but not a V system).[12]

A final generalization can be made about the relationship between the least conductive and most sensitive steps, although this relationship seems fairly obvious. If the highest point on the most difficult escape sequence is attained in a single step, that step will be both the least conductive as well as the most sensitive step, and also will be the most difficult (single) step.[13] However, if the highest point in the above sequence requires more than one step, the least conductive and most sensitive steps *cannot* be the same. Moreover, in such a case additional information will be required to specify the most difficult step, which may correspond with the least conductive step, or the most sensitive step, or neither. In cases where the most sensitive and least conductive steps are different, it seems appropriate to define as the "bottleneck" the series of steps bounded on one side by the least conductive step and on the other by the most sensitive step. In such a case, for an endergonic reaction such as the reverse of the reaction in case 6b, Figure 2, the entire reaction constitutes the bottleneck for the reverse process. However, it is important to note that steps other than the bottleneck steps can contribute to the overall rate limitation, as in case 6c, where the bottleneck is represented by

$$\xrightarrow{k_3}\ \xrightarrow{k_5}$$

but step 7, with its nonzero sensitivity index (see Figure 2), also contributes to the overall rate limitation (see following section).

General Representations of Sensitivity and Conductance. In the preceding sections procedures were described for identifying the least conductive and most sensitive steps on a Gibbs energy profile for unbranched sequential reactions where the conductance of one step is substantially lower than that of any other and where the sensitivity index for the most sensitive step is substantially greater than that of any other step. This section deals with generalizations about conductance and sensitivity without regard to these restrictions—particularly in V systems. In a subsequent section, reactions are considered in terms of these generalized concepts, again without the above restrictions. In these sections, it is convenient to abandon the system of designating forward and reverse rate constants for a step as k_j and $k_{(j+1)}$, respectively (Cleland, 1963), that has been used up to this point (see eq 2). Thus, in order to relate a generalized rate constant, k_j, to the species that reacts in the forward direction via the step specified by k_j, the following convention is used both in the remainder of the Discussion and in the Appendix:

$$\rightleftharpoons j \rightleftharpoons (\ddagger_j) \rightleftharpoons j+1 \rightleftharpoons \quad \left(k_j \text{ forward}, k_{-j} \text{ reverse}\right)$$

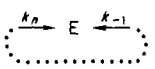
Here, $K_j = k_j/k_{-j}$.

If the net (effective) conductance of a step (in a sequence of steps) is taken as k_j', $1/k_j'$ becomes the net resistance of the step, by analogy with electrical circuitry (Cleland, 1975). Here, net resistances are considered rather than net conductances (a) because the net resistance of a *series* of steps is simpler to represent than its net conductance (since resistances rather than conductances are additive) and (b) because the net resistance of a *single* step is given by the sum of a series of terms, so constituted that each can be represented in a unique manner on the Gibbs energy profile for the overall reaction (eq 35, Appendix III). This representation is illustrated in Figure 4a for a V system in which the third intermediate of the nine indicated is referred to as intermediate j. On this profile, a dotted line has been constructed that proceeds from the valley designating (j) to the right-hand side of the scheme. According to eq 35, the sum of the terms comprising $1/k_j'$ is related to the distances from the dotted line originating at the valley which represents (j) to each transition state *subsequent* to (j), i.e., $\ddagger_{(j)}$, $\ddagger_{(j+1)}$, etc. In fact, if the ordinate is expressed in units of $G°_c/RT$

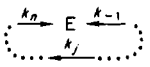
$$1/k_j' \propto \exp(C_j) + \exp(C_{j+1}) + \exp(C_{j+2}) + \ldots + \exp(C_n) \tag{18}$$

where C_j is the distance specified by the length of the arrow from the dotted line to $\ddagger_j$. (An upward arrow designates a positive exponent.) Because $1/k_j'$ is the sum of exponential terms, in many, but not all cases, the value of $1/k_j'$ will be determined by $\exp(C_{\max})_j$, where $(C_{\max})_j$ is the distance specified by the longest arrow produced by the above construction. It is the length of this arrow that is considered in the procedure described in a preceding section for selecting the least conductive step in systems where one step is substantially less conductive than any other. Problems can arise

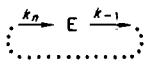
[12] The following argument is based on the approach used in Appendix I to formulate expressions for sensitivity indexes. In the King–Altman terminology (1956), a network involves a sequence of steps which passes through all species in the reaction. The networks which comprise const (cf. Cleland, 1963) are based on the cycle



where the arrows designate steps whose direction is specified, the dots represent steps for which either forward or reverse processes can be considered, depending on where the individual network terminates. All bona fide networks for a single cycle will omit the forward and reverse phases (where applicable) of a single step in this cycle, and all feasible omissions of this type are considered. However, a product release step will incorporate a second arrow into such a cycle, e.g.



Because of the added arrow, networks cannot involve omission of any step subsequent to step j. Hence, there will be no networks comprising const that contain neither k_k nor k_{-k}, where the steps so designated occur after step j; i.e., there will be no "e terms" (see eq 22, Appendix I) for steps subsequent to step j. By contrast the networks which comprise coeff A are based on the cycle.



Hence, the addition of arrows representing one-way product release does not restrict the networks comprising coeff A nearly so much as above, and at least one network containing neither k_j nor k_{-j} will be found for all values of j; i.e., there will be an e term for all steps.

[13] This situation can occur in an exergonic reaction where the standard Gibbs energy for the sequence of intermediates decreases monotonically throughout the reaction, e.g., case 6b, Figure 2, *if* the isotopic step were the most difficult.

when this approach is used for systems in which the net conductance of additional steps is equal or close to that of the least conductive step; these are considered in the following section.

Cleland shows that the maximum velocity of a reaction is a function of all of the net rate constants for that reaction: $1/V = (\sum_2^n 1/k_j')^{-1}$. This means that the net resistance of the overall reaction, $1/V$, is the sum of the net resistance of each step in the reaction, by analogy with the resistance provided by a series of electrical resistors. Thus, the net resistance of the entire reaction can be represented by applying the above procedure for representing the net resistance of step j to all steps in the reaction. This is illustrated in Figure 4b, where a dotted line has been constructed from *each* intermediate and extended to the extreme right of the scheme and the appropriate arrows have been added according to the procedure used to construct Figure 4a. Here, the *origins* of the various coaxial arrows are represented by solid circles. The representation of net resistance of the overall reaction in Figure 4b is essentially identical with that used by Albery & Knowles (1976) to represent $1/V$, although in a somewhat different context.[14]

${}^{V}SF_j$, the sensitivity function for step j, can be represented on a Gibbs energy profile in a somewhat analogous manner, as is shown by eq 39, Appendix IV. Such a representation utilizes not one but a series of dotted lines originating at intermediate (j) as well as at all *previous* intermediates. In fact, ${}^{V}SF_j$ (and thus ${}^{V}SI_j$) is related to the series of *coaxial* arrows constructed from all dotted lines that pass beneath transition state, $\ddagger_j$, upward to $\ddagger_j$, i.e., from dotted lines originating at previous intermediates, as is illustrated in Figure 4c, where the sixth intermediate is designated as j. Thus, the sensitivity of the overall rate to changes in k_j is proportional to $\sum\exp(S_j)$, where S_j is the (positive) distance specified by the length of the various arrows. Again, as in the case of the least conductive step, the procedure suggested in the previous section for identifying the most sensitive step in a system where the sensitivity index for one step is substantially larger than that for any other step depends on the extent to which SF_j is determined by $\exp(S_{max})_j$.

Since $\sum {}^{V}SF_j = 1/V$ (see Appendix IV), the sum of the sensitivity functions for all steps also is related to the sum of the net resistances for those steps.

$$\sum {}^{V}SF_j = \sum 1/k_j' \tag{19}$$

Thus, the same result is obtained if the net resistance of the overall reaction is represented in terms of the sum of $1/k_j'$ or the sum of ${}^{V}SF_j$, and Figure 4b is generated either from part a or c of Figure 4 when the contributions of all steps in the reaction are taken into account. However, for a single step

$${}^{V}SF_j \neq 1/k_j' \tag{20}$$

just as Figure 4a is not the same as 4c. On the other hand, the sensitivity function for the most sensitive step is directly related to the net resistance of the least conductive step in systems where a clear identification of these is feasible.

V/K systems are easier to analyze than V systems. This is primarily because V/K systems share numerous similarities with the simple, more familiar nonenzymic reactions where the cycling of a catalyst is not involved.[11] However, it seems reasonable to point out that $1/k_j'$ can be represented on the Gibbs energy profile for a V/K system in the same way as described above, for a V system. The only difference is that in a V/K system the net resistance of all steps subsequent to the first step is so small that *only* the net resistance of the first step (which is related to the distance from E + S to the various transition states) contributes significantly to the net resistance of the overall reaction. Correspondingly, only one distance contributes to each value of ${}^{V/K}SF_j$: the distance from E + S to $\ddagger_j$. In both cases, the net resistance of the least conductive step corresponds with the net resistance of the bottleneck.

Limitations on the Concepts of the Least Conductive and the Rate-Limiting Steps. The objections cited by Northrop (1981, 1982) to the continued use of the concept of a rate-limiting step for describing enzymic reactions can be separated into two groups: those arising from uncertainty about the appropriate definition for the rate-limiting step (if any) and those arising from the occurrence of one or more steps with sensitivity indexes equal, or approximately equal, to that of the most sensitive step. When the rate-limiting step is equated with the most sensitive step, the present approach eliminates what appears to be the larger group of objections: those in the first group. Thus, the definitions posed herein are readily applied in a consistent manner to the systems in Figure 2 that were considered by Northrop. On the other hand, it is not the purpose of this paper to suggest that one can identify a unique, rate-limiting step in every sequential reaction. Clearly it is possible to construct a reaction sequence containing a series of steps that have either identical or nearly identical sensitivity functions (as well as identical or near identical net conductances). In fact, such situations are particularly likely in enzymic systems subject to the type of evolutionary pressure posed by Albery & Knowles (1976). In these cases, the most sensitive step might be referred to as the most nearly rate-limiting step, although alternative terms may seem more appropriate to others. On the other hand, specifying that a scheme contains steps with ${}^{V}SI_j$ values of 0.1, 0.15, 0.25, and 0.5, for example, provides a precise and easily grasped description of the system. Thus, although such a description may not prevent disagreement about whether or not the last of the above steps should be designated as rate limiting or most nearly rate limiting, it does provide a clear-cut description upon which arguments can be based.

In addition, the present approach provides a rationale for designating the kinetic complexity of a reaction from Gibbs energy diagram. For a V system, the number of denominator terms in an initial velocity equation which takes each intermediate into consideration increases rapidly with the number of intermediates. In Figure 4b each arrow represents one of the denominator terms for an arbitrary reaction with nine intermediates. However, since the extent to which a step is either least conductive or most sensitive is given by an expression analogous to $\exp(C_1) + \exp(C_2) + \ldots$, many of the terms in such expressions may be insignificant with respect to others and can be disregarded. To evaluate kinetic complexity and to identify steps that might be candidates for the above labels, one can delete arrows that are not essential to the combinations of terms whose sum is equal to $1/k_j'$ or ${}^{V}SF_j$ and subsequently delete various arrows representing $1/k_j'$ or ${}^{V}SF_j$ that do not contribute substantially to V. No rules for this type of procedure are posed other than the exercise of due caution. However, in Figure 4d [which admittedly was carefully crafted to ensure the absence of long arrows (large exponential terms) of nearly equal length within various combinations of arrows] the result of such a procedure readily

[14] In their analysis Albery and Knowles refer to "kinetically significant intermediates" and "kinetically significant transition states" without offering a quantitative definition of these. However, their usage suggests that in the present terminology, the "most significant" intermediate, kinetically, is the intermediate that precedes the least conductive step and that the "most significant" transition state is the transition state for the most sensitive step.

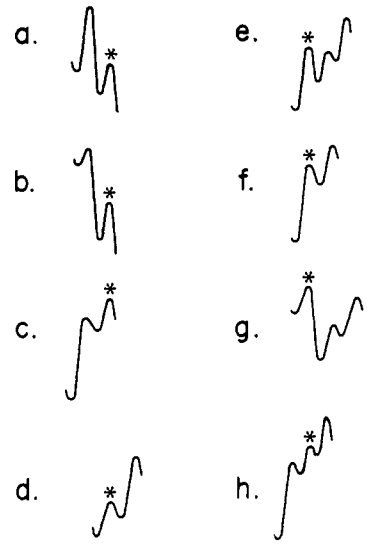


FIGURE 5: Isolated patterns from Gibbs energy profiles of V systems and their effect on R_f/E_f. The isotopic step (designated by asterisks) is shown together with adjacent steps. Unless other steps affect the overall picture, these patterns would produce values of R_f/E_f in the following ranges: a, $\gg 1$; b, $\ll 1$; c, $\ll 1$; d, $\gg 1$; e, $\gg 1$; f, $\ll 1$; g, $\gg 1$; h, $\ll 1$.

allows one to identify steps which reasonably qualify for the labels, least conductive and most sensitive, and the single remaining arrow approximates both the reciprocal net rate constant for the least conductive step and the sensitivity function for the most sensitive step. The kinetics of the reaction scheme described by the Gibbs energy profile in Figure 4 thus is not as complex, kinetically, as it might at first seem; it only has the trappings of complexity.

It should be pointed out that the procedure for identifying the least conductive and most sensitive steps in a reaction where these can be uniquely identified (second and third sections under Discussion) is a sort of "quick and dirty" procedure and will lead to inconsistent conclusions when applied to systems of the type described above. Thus, when one or more arrows as long or nearly as long as the longest arrow are found during an analysis of a Gibbs energy profile by the quick and dirty procedure, the more straightforward procedure described in this section should be used. In fact, in using the latter procedure one can lengthen a given arrow somewhat to appropriately compensate for eliminating a different arrow whose length is comparable to the arrow retained. Of course, the next level of accuracy would involve simply calculating all denominator terms from the profile and analyzing these directly.

Relationships between the Sensitivity Index for the Isotopic Step and General Features of Gibbs Energy Profiles. Previous sections have dealt with procedures that can be used to estimate whether the sensitivity index of an isotopic step is high (close to 1) or low (close to 0) on the basis of a Gibbs energy profile for the reaction. It is the purpose of Appendix V to point out what, to a first approximation, are the general features of such profiles associated with low or high values of the sensitivity index for the isotopic step, especially for profiles of V systems. This is useful because there is a relationship between these features and the various commitment factors that determine the sensitivity index. These commitment factors, which are experimentally accessible in some cases, in turn specify the extent to which a determination of SI_f will be substantially influenced by an isotopic effect on the overall equilibrium constant for the reaction, if appreciable, instead of depending solely on the intrinsic effect on the forward rate constant for the isotopic step, i.e., whether or not Northrop's procedure for determining the intrinsic isotope effect can be successfully applied (Northrop, 1977). Within this context, it seems reasonable to restate the relationship between the magnitude of the isotopic rate effect and the sensitivity index for the isotopic step: a near maximal isotopic rate effect is both necessary and sufficient to produce a sensitivity index for the isotopic step close to 1; a sensitivity index close to 1 is necessary but not sufficient to produce a near maximal isotopic rate effect (see Results).

Other Treatments. The analysis described herein was developed for V systems. In some respects its application to V/K systems does not differ greatly from other treatments of such systems, e.g., Stein (1981), and V/K systems were considered partly to demonstrate generality and to provide a contrast with V systems. Actually, the basic differences between V and V/K systems are such that analyses developed for the latter are unlikely to be generally applicable to the former, although the converse statement does not hold.

Finally, no attempt has been made to describe all of the insights into steady-state V systems that the current approach seems capable of representing in a relatively simple manner. Hopefully, even more will be uncovered than have occurred to the author.[15]

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Appendix

(I) A Simple Algorithm for Formulating Sensitivity Indexes. Sensitivity indexes can be formulated by inspecting the expressions for V or V/K when these are arranged in the manner described by Cleland (1963). This procedure is illustrated first for a V system, where $V = \text{num}_1/\text{coeff A}$. In Cleland's nomenclature, coeff A is a collection of terms that may be separated into a group that contains any given forward rate constant, k_j, a group that contains the rate constant for the reverse of this step, k_{-j}, and a group that contains neither. Hence, the reciprocal of the above expression can be written as

$$\frac{1}{V} = \frac{\text{coeff A}}{\text{num}_1} = \frac{bk_j + ck_{-j} + e_j}{ak_j} \qquad (21)$$

where $ak_j = \text{num}_1$, bk_j is the sum of the terms in coeff A that contain k_j, ck_{-j} is the sum of the terms containing k_{-j}, and e_j is the sum of the remaining terms, i.e., those that contain

[15] For example, in a double-label study of a V/K system, the effect of one isotopic substitution on the observed rate effect produced by a second isotopic substitution in a *subsequent* step is described by eq 10 of Hermes et al. (1982). This equation can be recast in a much simpler manner: ${}^{D}SF_{C13} = {}^{D}K_e^{-1}$. The left-hand side of this expression specifies the effect of the isotopic substitution (deuterium) in the first isotopically sensitive step on the V/K sensitivity factor (superscript V/K omitted, above) for the second isotopically sensitive step, i.e., the ^{13}C-sensitive step. This effect, which experimentally is equal to $[{}^{13}(V/K)_H - 1]/{}^{D}(V/K)[{}^{13}(V/K)_D - 1]$ (see above reference) is equal to the reciprocal effect on K_e produced by the isotopic substitution in the first step. This recast relationship is readily verified by examining Gibbs energy profiles of V/K systems. Other relationships in the above paper can be illustrated in an analogous manner, but only when $C_t({}^{i}K_e - 1) \approx 0$, either because the reverse commitment is essentially zero or there is essentially no isotopic effect on the overall equilibrium constant. Under such conditions, eq 16 becomes ${}^{D}SF_{C13} = 1$; i.e., the isotopic effect in the first isotopically sensitive step is not altered by isotopic substitution in a subsequent step.

neither k_j nor k_{-j}. (Note, however, that the identity of the "constants" a, b, c, and e_j will change when j is changed, although only the latter term is so designated.) After differentiation in which ck_{-j}/ak_j is treated as a constant (see Results) and rearrangement

$$\frac{\partial(1/V)}{1/V} \Big/ \frac{\partial(1/k_j)}{1/k_j} = {}^{V}\mathrm{SI}_j = \frac{e_j}{\text{coeff A}} \qquad (22)$$

The sensitivity index for the forward step, j, in a V system is a ratio; the numerator of the ratio is the sum of all terms in coeff A that do not contain either k_j or k_{-j}; the denominator is coeff A.

Thus, since the sensitivity factor is related to the corresponding sensitive index by $1/V$

$$^{V}\mathrm{SF}_j = e_j/\mathrm{num}_1 \qquad (23)$$

A similar approach can be used for a V/K system, where const/coeff A = K_A.

In a V/K system, the numerator of the sensitivity index for step j is the sum of all terms in const that contain neither k_j nor k_{-j}; the denominator is const.

When the various networks which give rise to the terms in const or coeff A are examined, it becomes obvious that each term in const and each term in coeff A will be designated as e_j in one and only one senstivity index for a particular type of system so that the sum of the e_j values for the sensitivity indexes of all forward steps is equal to const or coeff A, respectively.

Sensitivity indexes also can be written in terms of Northrop's commitment factors (1981). Thus

$$^{V/K}\mathrm{SI}_j = \left(\frac{b}{e_j}k_j + \frac{c}{e_j}k_{-j} + 1\right)^{-1} \qquad (24)$$

The term bk_j/e_j is a generalized form of the forward commitment to catalysis factor, C_f, and becomes equal to C_f when k_j refers to the isotopic step and is designated by k_i. A similar relationship holds between ck_{-j}/e_j and C_r. Hence, for the isotopic step

$$^{V/K}\mathrm{SI}_i = (1 + C_f + C_r)^{-1} \qquad (25)$$

In addition

$$^{V}\mathrm{SI}_i = (1 + R_f/E_f + C_r)^{-1} \qquad (26)$$

Thus, $bk_H/e_i = R_f/E_f$ and $ck_{-H}/e_i = C_r$. (Although constants b, c, and e_i from coeff A are not the same as constants b, c, and e_i from const, the ratio ck_{-H}/e is the same in both cases and is equal to C_r.)

(II) Relationship between the Deuterium Isotopic Effect on V or V/K and the Sensitivity Index for the Isotopic Step. The deuterium isotope effect on V can be expressed as follows, where the subscripts, H and D, refer to parameters for the normal and deuterated substrate.

$$^{D}V = \frac{(\mathrm{num}_1/\text{coeff A})_H}{(\mathrm{num}_1/\text{coeff A})_D} \qquad (27)$$

Since coeff A can be separated into three different sets of terms in the manner described in Appendix I

$$^{D}V = \frac{k_H(k_D b + k_{-D}c + e_i)}{k_D(k_H b + k_{-H}c + e_i)} \qquad (28)$$

where k_Hb, k_Db, k_Hc, k_Dc, and e_i have the same general significance as in eq 21, except that the isotopic step (subscript "i" instead of "j") is designated. If $(^{D}K_e - 1) = 0$, $k_H/k_{-H} = k_D/k_{-D}$;[6] hence, rearranging eq 28 gives

$$(^{D}V - 1) = \frac{(k_H/k_D - 1)e_i}{k_H b + k_{-H}c + e_i} = (^{D}k_i - 1)^{V}\mathrm{SI}_i \qquad (29)$$

If $^{D}(K_e - 1) \neq 0$

$$(^{D}V - 1) = [^{D}k_i - 1 + C_r(^{D}K_e - 1)]^{V}\mathrm{SI}_i \qquad (30)$$

The difference between eq 29 and eq 30 is considered under Discussion.

Analogous equations for $^{V/K}\mathrm{SI}_i$ can be derived by using the same procedure as used above for $^{V}\mathrm{SI}_i$, and the significance of the various concepts is parallel for the two cases. In fact, a simple relationship for the ratio $^{V}\mathrm{SI}_i/^{V/K}\mathrm{SI}_i$ [or the ratio $(1 + C_f + C_r)/(1 + R_f/E_f + C_r)$] can be derived by using this approach (not shown).

(III) Relationship between the Conductivity of a Step and Features of the Gibbs Energy Profile for the Reaction. Most rate constants for fundamental steps in chemical reactions can be represented as in eq 31.

$$k_j \propto \exp[-\Delta G^{\ddagger}_j/(RT)] \qquad (31)$$

Net rate constants also can be represented similarly:

$$k_j' \propto \exp[\Delta G^{\ddagger}_j'/(RT)] \qquad (32)$$

Moreover, $\Delta G^{\ddagger}_j'$ can be expressed in terms of changes in the Gibbs function that accompany fundamental steps after appropriately factoring the expression for k_j'. To do this, it is convenient to represent the enzymic reaction in the following general way.

$$\mathrm{E} + \mathrm{A} \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} (1) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (2) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \cdots \underset{k_{-(j-1)}}{\overset{k_{(j-1)}}{\rightleftharpoons}} (j) \underset{k_{-j}}{\overset{k_j}{\rightleftharpoons}} \cdots \underset{k_{-(n-1)}}{\overset{k_{(n-1)}}{\rightleftharpoons}} (n) \overset{k_n}{\rightleftharpoons} \mathrm{E} + \mathrm{P} \qquad (33)$$

Here, the various intermediates are numbered, and both transition states and equilibrium constants *leading from* these intermediates toward products are designated by the numbers assigned to the respective intermediates. Thus, the transition state by which intermediate (j) reacts to give $(j + 1)$ is $\ddagger_j$, and the rate constant ratio, k_j/k_{-j}, which specifies the equilibrium constant, $(j + 1)/j$, is designated by K_j. In addition, the partition ratio, $k_j/k_{-(j-1)}$, which is equivalent to the transition-state equilibrium constant, $[\ddagger_j]/[\ddagger_{j-1}]$, is designated by K_j^*. Thus, K_j^* is the transition-state equilibrium constant leading from $\ddagger_{j-1}$ through j to $\ddagger_j$. [This means that $\ddagger_n$ is the transition state for the process E·P → E + P, where E·P = (n).]

Cleland's algorithm (Cleland, 1975) provides the following expression for k_j' in eq 33.

$$k_{(j)}' = \frac{K_n K_{n-1} K_{n-2} \cdots K_j}{1 + K_n^* + K_n^* K_{n-1}^* + \cdots + (K_n^* K_{n-1}^* \cdots K_{j+1}^*)} \qquad (34)$$

On a Gibbs energy profile, it is easier to represent $1/k_j'$ than k_j'. Thus, when the reciprocal of eq 34 is taken and the terms are rearranged, eq 35 is obtained.

$$(k_j')^{-1} = k_j^{-1} + (k_{(j+1)}K_j)^{-1} + (k_{(j+2)}K_jK_{j+1})^{-1} + \cdots + (k_nK_jK_{j+1}\cdots K_{n-1})^{-1} \qquad (35)$$

The terms in eq 35 represent the respective reciprocal equilibrium constants for conversion of (j) to $\ddagger_j$, $\ddagger_{j+1}$, $\ddagger_{j+2}$, ..., and $\ddagger_n$, i.e., to a transition state *subsequent* to (j).

(IV) Relationship between the Sensitivity Function for a Step and Features of the Gibbs Energy Profile for the Reaction. An analysis of expressions for $^{V/K}\mathrm{SF}_j$ and $^{V}\mathrm{SF}_j$ is given in terms of the generalized uni-uni reaction scheme of eq 33

plus the conventions specified immediately after that equation (see Appendix III). When the King–Altman procedure (King & Altman, 1956) plus eq 22 is used, the following expression can be written for ${}^{V/K}SF_j$:

$${}^{V/K}SF_j = k_{-0}k_{-1}k_{-2}...k_{-(j-1)}k_{(j+1)}...k_n/\text{num}_1 \quad (36)$$

Since constants with subscripts equal to or larger than $j + 1$ will cancel

$${}^{V/K}SF_j = (k_jK_0K_1K_2...K_{j-1})^{-1} \quad (37)$$

Thus, the right-hand side of eq 37, is simply the reciprocal equilibrium constant for the process E + A $\rightarrow$ $\ddagger_j$; i.e., the sensitivity function for step j is related to the height of $\ddagger_j$ on a Gibbs energy profile for a V/K system, relative to that of E + A. The step with the largest sensitivity function and thus the largest sensitivity index in such a system is the step that passes through the highest point on a Gibbs energy profile.

Although the expression for ${}^{V}SF_j$ is more complex than that for ${}^{V/K}SF_j$, it can be formulated in an analogous manner. Thus, when the King–Altman procedure and eq 22 are used

$$\begin{aligned}{}^{V}SF_j = {} & k_0k_{-1}k_{-2}k_{-3}...k_{-(j-1)}k_{(j+1)}...k_nk_{(n+1)}/\text{num}_1 + \\ & k_0k_1k_{-2}k_{-3}...k_{-(j-1)}k_{(j+1)}...k_nk_{(n+1)}/\text{num}_1 + \\ & k_0k_1k_2k_{-3}...k_{-(j-1)}k_{(j+1)}...k_nk_{(n+1)}/\text{num}_1 + \\ & k_0k_1k_2k_3...k_{-(j-1)}k_{(j+1)}...k_nk_{(n+1)}/\text{num}_1 + ... + \\ & k_0k_1k_2k_3...k_{(j-1)}k_{(j+1)}...k_nk_{(n+1)}/\text{num}_1 \quad (38)\end{aligned}$$

After appropriate factoring

$$\begin{aligned}{}^{V}SF_j = {} & k_j^{-1} + (k_jK_{j-1})^{-1} + (k_jK_{j-1}K_{j-2})^{-1} + ... + \\ & (k_jK_{j-1}K_{j-2}...K_1)^{-1} \quad (39)\end{aligned}$$

Thus, the terms on the right-hand side of eq 39 represent the respective reciprocal equilibrium constants for conversion of $\ddagger_j$ to (j), $(j-1)$, $(j-2)$, ..., and the first intermediate, (1), i.e., to an intermediate *prior* to $\ddagger_j$. A comparison of this equation with that given in Appendix III for identifying the least conductive step in a reaction shows that the most sensitive step never can precede the least conductive step and that the most sensitive step passes through the point of highest energy subsequent to the valley from which the least conductive step originates.

According to eq 22

$${}^{V}SF_j = e_j/\text{num}_1$$

where e_j is the term or sum of terms in coeff A that contains neither k_j nor k_{-j}. Since, $\sum_2^n e_j$ = coeff A

$$\sum_2^n {}^{V}SF_j = \text{coeff A}/\text{num}_1 = 1/V \quad (40)$$

(V) The Sensitivity Index, Commitment Factors, and General Features of Gibbs Energy Profiles. Appendix II shows that the sensitivity index for the isotopic step in a V/K system is equal to $(1 + C_f + C_r)^{-1}$, where C_f and C_r are commitment factors for the forward and reverse isotopic steps. Thus, ${}^{V/K}SI_i$ is equivalent to Northrop's parameter, $f_{V/K}$ (Northrop, 1975, 1977), although the sensitivity index was obtained by an entirely different line of reasoning. Since $C_f = k_i/k_{-(i-1)}'$ and $C_r = k_{-i}/k_{(i+1)}'$, the denominators of C_f and C_r refer to preisotopic and postisotopic steps. According to the algorithm suggested in a preceding section for estimating the relative magnitudes of net rate constants from a Gibbs energy profile, if a *prior* transition state lies substantially higher on the profile than the transition state for the isotopic step, C_f will be substantially larger than 1, and ${}^{V/K}SI_i$ thus will be small; if a subsequent transition state lies substantially higher, C_r will be substantially larger than 1, and ${}^{V/K}SI_i$ again will be small. If the transition state for the isotopic step is (significantly) higher than all others, both C_f and C_r will approach zero, SI_i will approach 1, and a near-maximal isotopic rate effect will be observed.

In V systems, the sensitivity index for the isotopic step is equal to $(1 + R_f/E_f + C_r)^{-1}$, i.e., is equivalent to Northrop's f_V parameter (see Appendix II). Here, C_r has the same significance as in V/K systems and (defined) reactions in which ${}^{V}SI_i$ is low because postisotopic transition states have higher Gibbs energies than the isotopic transition state ($C_r \gg 1$) can be readily identified from their Gibbs energy profiles. By contrast the R_f/E_f parameter relates the *difficulty* of *both* pre- and postisotopic steps to that of the isotopic step and is more complex than the commitment factors. Because of this, several isolated patterns from Gibbs energy profiles are sketched in Figure 5. These show the isotopic step and adjacent steps that, *unless influenced by other steps*, would produce high or low values of R_f/E_f (relative to 1), where the isotopic step is indicated by an asterisk. A preisotopic step contributes substantially to a large value of R_f/E_f *only* when it is more difficult *and* involves a higher lying transition state (as in Figure 5a, but not 5b). Thus, a lower lying transition state of a more difficult step does not contribute substantially to R_f/E_f (Figure 5c). A single postisotopic step or sequence of (postisotopic) steps can contribute to a large R_f/E_f when the difficulty of that step, alone (Figure 5d), or the overall difficulty of that sequence of steps (Figure 5e) is greater than the difficulty of the isotopic step, even when a higher lying transition state is not involved, as in Figure 5g. A less difficult step with a higher lying transition state (Figure 5f) does not contribute to a large value of R_f/E_f (although such a step does produce a large value of C_r, as do the patterns in Figure 5d,e). However, the effect on R_f/E_f of difficult postisotopic steps will be reduced by endergonic steps preceding the isotopic step, and unless the overall "kinetic difficulty" of postisotopic steps exceeds the "equilibrium difficulty" (thermodynamic difficulty) of preisotopic steps, postisotopic steps will not produce a large R_f/E_f (as in Figure 5h).

The above generalizations represent an extention of the quick and dirty approach described earlier. Thus, unless the above differences are substantial, conclusions should be checked by comparing the lengths of the relevant arrows, especially in V systems. Rules for constructing such arrows can be deduced from eq 19 and 20 of Northrop (1981) and the approach described in preceding sections.

The significance of C_f, C_r, and R_f/E_f in isotopic experiments has been discussed [see Northrop (1981) and references therein] and will not be further considered, here. However, an alternative statement of some of the conclusions in these treatments seems worthwhile: systems are most tractable when the isotopic step is the rate-limiting step; when the rate-limiting step *follows* the isotopic step, one must contend with the isotopic effect on the equilibrium constant of the overall reaction in experiments designed to assess the intrinsic isotope effect; when the rate-limiting step *precedes* the isotopic step, the isotopic effect of the overall equilibrium constant will produce fewer problems, other factors being equal.

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Kinetic Mechanism of the Reaction Catalyzed by Nuclear Histone Acetyltransferase from Calf Thymus†

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ABSTRACT: The kinetic mechanism for calf thymus histone acetyltransferase A has been determined from the initial velocity studies. The kinetic patterns at low substrate concentrations suggest that the reaction proceeds via two half-reactions as in a ping-pong pathway with the formation of an acetyl-enzyme intermediate. Such acetyl-enzyme has been isolated and found to be chemically competent. In addition, product inhibition patterns by coenzyme A are consistent with a hybrid ping-pong mechanism. These findings indicate that the acetyltransferase A from calf thymus has two separate and independent binding sites, one for each of the two substrates. Consequently, the mechanism constructed for the acetyltransferase A catalyzed reaction may be described as a double-displacement, two-site ping-pong mechanism.

Nuclear histone (acetyltransferase A) (EC 2.3.1.48) catalyzes the transfer of acetyl group from acetyl-CoA[1] to histones according to the following equation:

$$\text{acetyl-CoA} + \text{histone} \rightleftharpoons \text{acetylhistone} + \text{CoA}$$

The acetylation takes place at the ϵ-amino groups of lysines located within the first 25 amino acid residues of the N-termini of the histones (DeLange & Smith, 1975; Allfrey, 1977). As a result of the modification, the positive charge density of the histones is drastically reduced and thus their ionic interactions with DNA. Such change of interaction between histones and DNA is important in the control of structure and function of chromatin, which in the eukaryotic cell is composed of repeating units of nucleosomes, each of which consists of two each of the four histones H2a, H2b, H3, and H4, with 146 base pairs of DNA wrapped around the octameric histone core particle. The nucleosomes are linked together by 40–60 base pairs of DNA with the association of one molecule of histone H1 (Kornberg, 1977; Felsenfeld, 1978). The amino acid sequences of the four nucleosomal histones have been highly conserved during evolution (DeLange & Smith, 1975; Isenberg, 1979). However, extensive postsynthetic chemical modifications such as acetylation, phosphorylation, methylation, and ADP-ribosylation have been noted (Allfrey, 1977). The degree and extent of modification on the histones would certainly change the ionic interactions between the histones and DNA in the nucleosomes, thus providing a means of control of chromatin structure and function (Allfrey, 1977; Alberts et al., 1977; Dixon et al., 1975). In particular, acetylation has been speculated to correlate with increased transcriptional activity of genetic codon by RNA polymerase (Allfrey, 1977; Chahal et al., 1980). Such speculation has been supported by the observations that histones associated with template-active chromatin are preferentially acetylated (Wong & Alberts, 1977; Levy-Wilson et al., 1977; Davie & Candido, 1978; Simpson, 1978; Nelson et al., 1978; Vidali et al., 1978).

The enzyme system involved in the important acetylation process has not been fully investigated in detail because of its minute amount in the cell and its unstability on purification. Recently, nuclear acetyltransferase has been purified to a high degree from calf thymus (Belikoff et al., 1980) and rat liver (Wiktorowicz & Bonner, 1982), and some general properties have been investigated. We present here detailed kinetic studies on the reaction mechanism in an attempt to understand the regulation and involvement of the enzyme in gene expression (Wong, 1980). This report will show that the reaction follows a double-displacement pathway.

Materials and Methods

Materials. Calf thymus histones (a mixture of H1, H2a, H2b, H3, and H4), CoA, acetyl-CoA, N^{ϵ}-acetyllysine, and bovine serum albumin were purchased from Sigma Chemical Co. Sea urchin sperm histones were prepared as described previously (Wong, 1980). P-81 phosphocellulose paper was obtained from Whatman while [^{3}H]acetyl-CoA (10 Ci/mmol) was from ICN. All other chemicals used were of reagent grade.

Enzymes. Nuclear histone acetyltransferase was purified from calf thymus according to either the procedure published earlier (Belikoff et al., 1980) or that modified as follows. Extract II after poly(ethylene glycol) fractionation was chromatographed on a DEAE-cellulose followed by a phos-

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[1] Abbreviations: CoA, coenzyme A; acetyl-CoA, acetyl coenzyme A; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

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