Kinetic barriers under steady-state conditions

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It is shown by analysis of a numerical example that kinetic barrier diagrams [Südi (1991) *Biochem. J.* 276; 265–268] are also useful for displaying the phenomenological resistance of an enzymic turnover to net chemical fluxes observed under steady-state conditions. Most importantly, a new additivity rule is revealed by net flux profiles, which refers to limiting ('ideal') conditions. The rule states that the net fluxes that one obtains under initial steady-state conditions are strictly identical with the overall flux in the corresponding equilibrium system. This finding amounts to defining the relation between unidirectional fluxes and net fluxes, and explains why the total 'one-way flux resistance' to the overall reaction at chemical equilibrium on the one hand, and the 'net flux resistance' to both initial steady-state reactions on the other hand, have to be equal. The conclusions are claimed to be generally valid for consecutive chemical reactions.

INTRODUCTION

Since the time of Arrhenius and Van't Hoff we are used to deriving the rate of chemical reactions from the surmounting of some energetic barriers. For the illustration of this idea we draw 'peak-and-valley' type plots. As time has passed, we have been offered a number of quite different interpretations of the physical nature of the peaks and valleys of these diagrams. However, the idea of some sort of an activation energy has survived, and also the notion that this energy requirement can be directly obtained from the numerical value of single ('elementary') rate constants. In any case it is also generally recognized that this conventional approach fails at treating and illustrating sequential reactions [1–4]. The problem is a general one in chemical reaction kinetics, since realistic models of practically all chemical reactions involve the sequence of a number of intermediates.

On the one hand, it is noteworthy that the mathematical analysis, and particularly the numerical simulation, of even complicated multistep reaction sequences presents no difficulties if the concentration of reactants and intermediates is time-independent (i.e. constant), as at chemical equilibrium and in steady states. On the other hand, all kinetic properties of an n -step sequential reaction are exactly described by the numerical values of a set of $2n$ rate constants [5], and one can obviously study the kinetic significance of individual rate constants in a quantitative manner by systematically varying their numerical value. For both reasons it is certainly remarkable that for the visual evaluation of such analytic results no procedure is found in the literature.

The situation is especially paradoxical in the field of enzyme kinetics. In a considerable number of cases (e.g. for lactate dehydrogenase [6–8], ATPase [9], triose-phosphate isomerase [10], alcohol dehydrogenase [11], dihydrofolate reductase [12] and 5-enoylpyruvoylshikimate-3-phosphate synthase [13,14]) a complete set of numerical values has been published, which would allow description and illustration of the turnover reaction in detail. This is especially interesting because the experimental basis of these reports [6–14] is quite different with respect to both methods and assumptions involved. Both could be tested at the numerical level of description by analysing the behaviour of the system under various experimental conditions, such as chemical equilibrium or different steady state situations. Detailed descriptions of concrete kinetic situations should include the concentration of intermediates and their rate of interconversion in individual reaction steps, and further indicate how the overall rate of reaction is related to these features. However, the reports

cited above [6–14] do not contain a single serious attempt to achieve this. Presentation of the results usually culminates in drawing a 'free-energy profile', even though it is admitted that these plots are sketchy and do not refer to any concrete kinetic situation. A recent paper from the laboratory of J. R. Knowles [15] clearly outlines the necessity of looking for new approaches to plotting kinetic results. However, the diagrams of Burbaum *et al.* [15] maintain the main feature of free-energy profiles, in that the kinetic information they yield can only be directly interpreted in terms of rate constants.

I have previously shown [16] that kinetic barrier diagrams provide a much deeper understanding of the equilibrium kinetics of the lactate dehydrogenase turnover if one plots one-way chemical fluxes and intermediate concentrations, instead of just rate constants. The argument that supports this new approach is being extended in the present paper to considering kinetic barriers in the forward and reverse 'initial' steady states of the same five-step reaction sequence. It is shown that very informative plots can also be drawn of net chemical reactions, even though one would expect a fundamental difference with regard to kinetic barriers between (equilibrium) one-way fluxes and (steady-state) net fluxes.

BASIC CONSIDERATIONS

Equilibrium kinetics

The equilibrium rates considered previously [16] are those of one-way fluxes that maintain the equilibrium concentration of the individual intermediates. The concept of kinetic barriers involved in such processes is firmly based on the idea of a dynamic equilibrium underlying the law of mass action. Accordingly, the kinetics of even complicated multistep reactions appear to be quite simple. Equilibrium kinetic systems are composed of reaction intermediates that are transformed into each other in a fixed sequence of reaction steps, with identical forward and reverse rates. Experimental methods relevant to this type of kinetic processes are, for instance, dynamic n.m.r. spectroscopy and isotope exchange kinetics. Since, in spite of kinetic transformations at the molecular (microscopic) level, the statistical (macroscopic) parameters of an equilibrium system are constant, all reaction steps and overall reactions obey first-order kinetics, independently of their molecularity [17]. For the same reason, the mathematics involved in the treatment of equilibrium reactions is as simple as possible. Equilibrium profiles can be drawn with both logarithmic and arithmetic scales [16], but the latter are preferable because they yield a more easily comprehensible presentation of two additivity rules. These are: (i) the

Fig. 1. Net flux diagram for the forward reaction at low saturation level

The continuous line shows the profile of the initial steady-state reaction in the forward direction, the broken line shows the reference equilibrium profile. The Figure is a representation of the numerical values in Table 1. For both profiles the left-hand ordinate and the lower abscissa refer to the 'valley' levels and yield intermediate concentrations in units of the molar concentration of enzyme active sites (E_i). The right-hand ordinate and the upper abscissa refer to the 'peak' levels, and yield both the net flux resistance (R_{net}) for the steady-state reaction and the five one-way flux resistances (R_1 to R_5) for the equilibrium reaction. Unit resistance is $s \cdot [E]^{-1}$.

Fig. 2. Net flux diagram for the reverse reaction at low saturation level

The continuous line shows the profile of the initial steady-state reaction in the reverse direction. The broken line, and all further details, are the same as in Fig. 1.

emphasize the importance of these two additivity rules, kinetic barrier diagrams may be correctly referred to as state/resistance distribution diagrams.

Net reactions

In contrast with equilibrium systems, one of the opposing one-way fluxes is greater in all non-equilibrium situations, and the resulting net flux is equivalent to the observable change in chemical composition. The literature that attempts to clarify the relation between unidirectional fluxes and net fluxes is almost exclusively concerned with the relationship between kinetics and thermodynamics [18–21]. From the point of view of the present discussion, one should note the following. (i) No attempt can be made at comparing equilibrium and non-equilibrium kinetic situations with reference to transition-state theory. In a kinetic context, transition-state theory is derived from the assumption of equilibrium, and can consequently yield nothing more than an equilibrium approximation to any non-equilibrium phenomenon, such as a net flux. (ii) In a steady state there is always a net flux that is equal in all reaction steps. (iii) A net flux is not necessarily the difference between an overall forward reaction and an overall reverse reaction. In a limiting case there is no opposing overall reaction because of the absence of reaction products ('initial velocity'). (iv) A comparison of unidirectional fluxes and net fluxes referring to some steady-state situation can be done at different levels of description. Chemical reactions can be exactly defined not only with the actual material fluxes, but also with some complex parameters that only refer to rate constants. However, these alternative (algebraic) types of definitions are too complicated even if the consecutive reaction only involves four or five steps. For this reason, algebraic definitions could not as yet pay any significant contribution to clarifying the relation of net fluxes to one-way fluxes.

Steady-state enzyme kinetics

The overwhelming majority of practical and theoretical studies in enzyme kinetics is concerned not with the time evolution of the catalysed reaction, but with its steady-state initial velocity. The mechanistic schemes with which the initial velocity of enzyme-catalysed consecutive reactions is interpreted are very often composed of not more than three types of reaction steps [4,22,23]: (i) the reversible binding of substrates to the enzyme, (ii) the reversible transformation of enzyme-bound substrates into enzyme-bound products, and (iii) the irreversible release of bound products (cf. eqns. 1 and 2 below). The apparent irreversibility of product release follows from the definition of an initial velocity. It further follows from this definition, and thus neither reflects some special feature of the chemical reaction, that all constituent reaction steps follow first-order kinetics.

If one considers the initial velocity of an enzyme-catalysed reaction at increasing substrate concentrations, it is generally observed that the dependence of the net rate on the concentration of the substrates diminishes. This phenomenon is referred to as increasing saturation of the enzyme with substrates. There are two limiting cases of substrate saturation. In one of these there is a linear dependence of the rate on substrate concentrations, and in the other one the rate is independent of substrate concentrations. The saturation level of an enzyme can be freely manipulated, both experimentally and in numerical simulations, by changing the concentration of substrates. By this, one alters not only the overall net rate, but also the relative rates of the consecutive reaction steps. This variability is, on the one hand, missing from truly monomolecular reaction sequences (cf. Wei & Prater [24] on the three-step isomerization of butene). On the other hand, it is regarded to be one of the most important factors

sum of the intermediate concentrations is equal to the total concentration of enzyme; (ii) the sum of the barrier heights ('resistances') is equal to the barrier to the overall reaction. To

Fig. 3. Net flux diagram for the forward reaction at high saturation level

The continuous line shows the profile of the initial steady-state reaction in the forward direction, the broken line shows the reference equilibrium profile. The Figure is a representation of the numerical values in Table 2. For both profiles the left-hand ordinate and the lower abscissa refer to the 'valley' levels and yield intermediate concentration in units of the molar concentration of enzyme active sites (E_i). The right-hand ordinate and the upper abscissa refer to the 'peak' levels, and yield both the net flux resistance (R_{net}) for the steady-state reaction and the five one-way flux resistances (R_1 to R_5) for the equilibrium reaction. Unit resistance is $s \cdot [E_i]^{-1}$.

Fig. 4. Net flux diagram for the reverse reaction at high saturation level

The continuous line shows the profile of the initial steady-state reaction in the reverse direction. The broken line, and all further details, are the same as in Fig. 3.

when considering the physiologically relevant definition of the catalytic efficiency of enzymes [25–28].

All intermediates to be considered in an enzymic-turnover reaction represent some form of the enzyme. With regard to the

latter, the overall reaction is cyclic. The cycle begins with the free enzyme, passes through a certain number of enzyme–substrate intermediates, and ends by releasing free enzyme. It is an obvious consequence of this type of catalysis that (in principle) the rate of all reaction steps is a linear function of the total concentration of enzyme.

NET FLUX DIAGRAMS

Net flux diagrams are aimed at a numerically exact illustration of the kinetic barriers that are encountered in an initial steady-state reaction. I am going to introduce and evaluate them below with reference to four examples (Figs. 1–4), which refer to the lactate dehydrogenase reaction and the same numerical values of ten rate constants that are given in Table 1(a) of a previous paper [16]. Plots comparable with Figs. 1–4 are not known to me from the literature.

Description of the procedure

Net flux diagrams illustrate initial velocities. These are defined for the lactate dehydrogenase reaction in eqns. (1) and (2):

Forward reaction ($\xrightarrow{v_{\text{net}}}$):

Reverse reaction ($\xleftarrow{v_{\text{net}}}$):

Eqns. (1) and (2) show that the numerical values needed for calculating the concentrations and resistances plotted in Figs. 1–4 comprise in each case eight rate constants and two substrate concentrations. The relevant sets of rate constants are defined in eqns. (1) and (2) and the relevant sets of substrate concentrations are listed in the captions of Tables 1 and 2. The sole difference between Table 1, Figs. 1 and 2 on the one hand, and Table 2, Figs. 3 and 4 on the other hand, is that in the latter case all substrate concentrations are 1000-fold higher.

The simple calculations that yielded the numerical values shown in Tables 1 and 2 were carried out with a pocket calculator, by using conventional algorithms. The three columns in Tables 1 and 2 contain corresponding results for chemical equilibrium and the forward and reverse initial steady-state reactions respectively. The first two sections in each Table show intermediate concentrations and unidirectional fluxes. Unit concentration is the molar concentration of enzyme ($[E_i]$), and the dimension of one-way fluxes is $[E_i] \cdot s^{-1}$. One-way fluxes in the forward and reverse directions are distinguished by a + and a – sign respectively, according to the usual convention. The third section in Tables 1 and 2 contains numerical results for some five-step overall reactions. These are total resistance (R_i) and the corresponding overall flux (F_i) for the reaction at chemical equilibrium [16], and the net flux (F_{net}) and the corresponding net flux resistance (R_{net}) for both initial steady-state reactions. One

Table 1. Intermediate concentrations, one-way fluxes, and kinetic barriers for the five-step lactate dehydrogenase reaction at low substrate saturation

The forward and reverse reactions are steady-state initial velocities; the concentration of substrates (if present) is the same as at the reference equilibrium (column 2). Forward reaction with $[\text{NAD}^+] = 1.6142 \times 10^{-8} \text{ M}$, $[\text{lactate}] = 1.3437 \times 10^{-4} \text{ M}$; reverse reaction with $[\text{NADH}] = 3.8824 \times 10^{-8} \text{ M}$, $[\text{pyruvate}] = 1.3223 \times 10^{-7} \text{ M}$.

	Equilibrium	Steady state	
		Forward	Reverse
Concentration ($1 = [\text{E}_i]$)			
E	0.990997	0.999693	0.991299
E^{NAD^+}	2.655061×10^{-4}	2.661682×10^{-4}	1.094213×10^{-6}
E^{NAD^+} Lactate	1.804614×10^{-6}	1.326743×10^{-6}	4.796300×10^{-7}
E^{NADH} Pyruvate	7.350500×10^{-6}	3.049049×10^{-6}	4.293273×10^{-6}
E^{NADH}	8.728812×10^{-3}	3.620726×10^{-5}	8.695579×10^{-3}
One-way fluxes ($1 = [\text{E}_i] \cdot \text{s}^{-1}$)*			
F_1	0.13966	+0.140584/−0.140004	−0.000576/+0
F_2	0.00216	+0.002171/−0.001592	−0.000576/+0
F_3	0.00181	+0.001329/−0.000750	−0.001056/+0.000481
F_4	0.00140	+0.000579/−0	−0.001391/+0.000816
F_5	0.13966	+0.000579/−0	−0.139705/+0.139129
Overall resistance†	$R_t = 1745.16 \text{ s} \cdot [\text{E}_i]^{-1}$	—	—
'Equilibrium flux'‡§	$F_t = 0.000573 [\text{E}_i] \cdot \text{s}^{-1}$	—	—
Net flux§	—	$\vec{F}_{\text{net}} = 0.000579 [\text{E}_i] \cdot \text{s}^{-1}$	$\vec{F}_{\text{net}} = -0.000576 [\text{E}_i] \cdot \text{s}^{-1}$
'Net flux resistance'	—	$\vec{R}_{\text{net}} = 1726 \text{ s} \cdot [\text{E}_i]^{-1}$	$\vec{R}_{\text{net}} = -1736 \text{ s} \cdot [\text{E}_i]^{-1}$

* At equilibrium equal in both directions; in steady states the opposing directions are denoted by + and −.

† The sum of the five one-way resistances; $R_t = \sum_{i=1}^5 (F_i)^{-1}$.

‡ $F_t = (R_t)^{-1}$.

§ Under the limiting conditions defined in eqn. (3) $F_t = \vec{F}_{\text{net}} = -\vec{F}_{\text{net}}$ ('limiting case 1').

|| The difference between two opposing one-way fluxes.

should note that it is reciprocal flux that I refer to as resistance ($R = 1/F$).

Results of the calculations in Tables 1 and 2 are plotted in Figs. 1–4. Each of the four Figures compares a steady-state kinetic profile (solid line) with the corresponding reference equilibrium profile (broken line). Note that the reference equilibrium profiles are calculated as in the previous paper [16] and involve the complete set of ten rate constants and four reactant concentrations. For the sake of clarity, net flux profiles are uniformly shifted to the right (forward) and to the left (reverse) respectively. Because of a more than 10^4 -fold difference in the extreme values of enzyme–substrate intermediate concentrations, all concentrations are plotted in Figs. 1 and 2 on a logarithmic scale. Note that Figs. 3 and 4 could be drawn with arithmetic scales. The second ordinate denoted as 'resistance' was used for plotting in each diagram both the 'net flux resistances' of the steady-state profile and the 'one-way flux resistances' of the corresponding equilibrium profile (cf. Tables 1 and 2).

It is shown below that all 'distortions' of the kinetic profile that are experienced under non-equilibrium conditions can be depicted exactly, and in a self-consistent manner. Indeed, the profiles even provide additional insight into the nature of multi-step equilibrium reactions. The following discussion of these points is centred around the effects of substrate saturation level and the relationship between the forward and reverse net reactions.

Substrate saturation of the enzyme

There is a 1000-fold difference between Tables 1 and 2 regarding the concentration of all substrates, so that Figs. 1–4 allow an evaluation of the consequences of substrate saturation. A strictly valid definition of the two limiting cases defined by Michaelis–Menten-type saturation curves is given in eqns. (3) and (4): Stoichiometric dependence on substrate concentration ($\hat{=}$ 'limiting case 1' in Table 1):

$$[\text{E}] \approx [\text{E}_i], \text{ i.e. } [\text{E}_a] \ll [\text{E}_i], \text{ but also } v_E = k_E [\text{S}_1] \dots [\text{S}_n] [\text{E}_i] \quad (3)$$

Substrate saturation of the enzyme ($\hat{=}$ 'limiting case 2' in Table 2):

$$[\text{E}] \ll [\text{E}_i], \text{ i.e. } [\text{E}_a] \approx [\text{E}_i], \text{ but also } v_{\text{max.}} = k_{\text{max.}} [\text{E}_i] \quad (4)$$

In these equations E and E_a stand for free enzyme, and liganded ('active') enzyme respectively, and the sum of the concentrations of these two collective forms is equal to the total concentration of enzyme ($[\text{E}_i]$). In the kinetic equations both v_E and $v_{\text{max.}}$ denote steady state initial velocities, i.e. net fluxes. The catalytic constants k_E and $k_{\text{max.}}$ have different kinetic orders, the $(n+1)$ th and first respectively. It is seen from Tables 1 and 2 and Figs. 1–4 that with the substrate concentrations chosen one does obtain useful approximations of the limiting cases defined in eqns. (3) and (4). Accordingly, the net flux profiles presented yield useful illustrations of the situation at very low and very high substrate concentrations. As already noted as one of the basic con-

Table 2. Intermediate concentrations, one-way fluxes and kinetic barriers for the five-step lactate dehydrogenase reaction at high substrate saturation

The forward and reverse reactions are steady-state initial velocities; the concentration of substrates (if present) is the same as at the reference equilibrium (column 2). Forward reaction with $[\text{NAD}^+] = 1.6142 \times 10^{-6} \text{ M}$, $[\text{lactate}] = 0.1344 \text{ M}$; reverse reaction with $[\text{NADH}] = 3.8824 \times 10^{-6} \text{ M}$, $[\text{pyruvate}] = 1.3223 \times 10^{-4} \text{ M}$.

	Equilibrium	Steady state	
		Forward	Reverse
Concentration ($l = [\text{E}_l]$)			
E	0.051775	0.111080	0.258287
E^{NAD^+}	0.013871	0.005793	0.055731
E^{NAD^+}			
Lactate	0.094281	0.028871	0.024429
E^{NADH}			
Pyruvate	0.384031	0.066350	0.218667
E^{NADH}	0.456042	0.787906	0.442887
One-way fluxes ($l = [\text{E}_l] \cdot \text{s}^{-1}$)*			
F1	7.296	+15.653767/−3.047268	−29.314454/+0
F2	113.135	+47.251527/−34.645028	−29.314454/+0
F3	94.470	+28.928598/−16.322099	−53.792023/+24.477569
F4	72.966	+12.606499/−0	−70.861139/+41.546685
F5	7.296	+12.606499/−0	−36.400643/+7.086189
Overall resistance†	$R_t = 0.307234 \text{ s} \cdot [\text{E}_l]^{-1}$	—	—
'Equilibrium flux'‡§	$F_t = 3.254848 [\text{E}_l] \cdot \text{s}^{-1}$	—	—
Net flux§§	—	$\vec{F}_{\text{net}} = 12.6065 [\text{E}_l] \cdot \text{s}^{-1}$	$\vec{F}_{\text{net}} = -29.3145 [\text{E}_l] \cdot \text{s}^{-1}$
'Net flux resistance'	—	$\vec{R}_{\text{net}} = 0.07932 \text{ s} \cdot [\text{E}_l]^{-1}$	$\vec{R}_{\text{net}} = -0.03411 \text{ s} \cdot [\text{E}_l]^{-1}$

* At equilibrium equal in both directions; in steady states the opposing directions are denoted by + and −.

† The sum of the five one-way resistances; $R_t = \sum_{i=1}^5 (F_i)^{-1}$.

‡ $F_t = (R_t)^{-1}$.

§ The limiting conditions defined in eqn. (4) are, with reference to the equilibrium flux treated here, equivocal and in no respect useful [30].

On the contrary, for both net fluxes there is an unequivocally defined 'limiting case 2'. In limiting case 2 one obtains $\vec{F}_{\text{net}} = \vec{V}_{\text{max.}} = 14.274842 [\text{E}_l] \text{ s}^{-1}$ and $-\vec{F}_{\text{net}} = \vec{V}_{\text{max.}} = 98.098618 [\text{E}_l] \text{ s}^{-1}$.

|| The difference between two opposing one-way fluxes.

siderations, kinetic barrier diagrams are in principle independent of the total concentration of enzyme. This should be noted because the steady-state assumption involves the inequality $[\text{E}] \ll [\text{S}]$ (or, rather, $[\text{E}_l] \ll [\text{S}]$; cf. eqn. 3), and Figs. 1 and 2 refer to very low substrate concentrations (e.g. $[\text{NADH}] = 4 \times 10^{-9} \text{ M}$; Fig. 2). Accordingly, an experimental reproduction of the conditions defined in Figs. 1 and 2 would require unrealistically low enzyme concentrations. However, since the Figures are aimed at clarifying the situation under limiting ('ideal') conditions, this point does not affect the main line of argument.

The equilibrium profiles plotted with broken lines in all four Figures show that substrate concentrations have a marked influence even on equilibrium kinetics. This is shown by both the concentration of intermediates and the magnitude of consecutive one-way fluxes. In this context I should only like to address two points. The first one is concerned with the marked difference between kinetic barriers for the binding for both NAD^+ and NADH at low and at high substrate concentrations respectively. Compared with the three central barriers, barriers R_1 and R_5 are very low at low substrate concentrations (Fig. 1 or Fig. 2) and very high at high substrate concentrations (Fig. 3 or Fig. 4). The reason for this is quite simple if one recalls that the fluxes F_1 and $F(-5) = F_5$ (which define barriers R_1 and R_5 as $1/F_1$ and $1/F_5$ respectively; Tables 1 and 2) are obtained as the product of an intermediate concentration and a pseudo-first-order rate constant. For instance, if substrate concentrations are low, $[\text{E}]$ is

practically invariable, whereas $[\text{E}^{\text{NAD}^+}]$ increases linearly with the concentration of NAD^+ . On the contrary, if substrate concentrations are high, it is $[\text{E}^{\text{NAD}^+}]$ that is practically invariable, whereas $[\text{E}]$ decreases linearly when the concentration of NAD^+ is increased further. There is full agreement between this feature of Figs. 1–4 and the experimental observations made by Boyer and co-workers [29,30], who have reported severe inhibition of the rate of equilibrium isotope exchange between NAD^+ and NADH when increasing the concentrations of lactate and pyruvate. The explanation for the fact that the height of barriers R_1 and R_5 in Figs. 1–4 are identical can be derived [16] from the constant ratio of the concentration of NAD^+ and NADH .

The second point that I should like to address concerns the height of the three central barriers of the equilibrium profiles. As seen from the four Figures, the heights of these barriers are quite similar, and the small deviations among the three remain conserved when substrate concentrations are varied. The reason why the ratios of these three equilibrium concentrations are constant lies in the fact [16] that the three central one-way fluxes $F(-2)$, F_3 and F_4 are coupled by 1st order rate constants [note that $F_2 = F(-2)$].

Next we should consider the dependence of net fluxes on substrate saturation levels by comparing Fig. 1 with Fig. 3 and Fig. 2 with Fig. 4. It is seen that the actual information content of the profiles lies more in the pattern of intermediate concentra-

tions than in the magnitude of the net flux. Note that the net flux is a property of the complete steady-state system and has, by definition, the same value in all reaction steps of the same Figure. On the other hand, there is a different net flux characteristic of each steady state (each Figure). The fact that in the special case of Figs. 1 and 2 the forward and reverse fluxes are very similar (cf. Table 1) follows from approaching the limiting case defined in eqn. (3), and will be discussed in the next section. It is certainly the most striking difference between the profiles at low and at high saturation levels respectively that at high levels the concentrations of the intermediates are more closely similar to one another. This observation is valid for both equilibrium and steady-state situations (note the difference in scales between the Figures). Less conspicuous but much more important is a second observation that refers to a comparison of the equilibrium and steady-state concentration of each intermediate. Figs. 1 and 2 show that if the saturation level is low (i) the concentration of all liganded forms of the enzyme is lower under initial steady-state conditions than at equilibrium, and (ii) the difference between the equilibrium and steady-state concentrations of the liganded intermediates is increasing with each successive reaction step. Both of these regularities disappear as the concentration of substrates is increased. Individual steady-state concentrations are in some cases even higher than the reference equilibrium concentration (see E^{NADH} in Fig. 3 and E^{NAD^+} in Fig. 4). It will be shown in the next section that all these effects of substrate concentration can be easily explained by competition of substrates for the enzyme.

Forward and reverse reactions

Corresponding net flux profiles for the forward and reverse reactions compare quite differently at low and at high substrate saturation levels. At low substrate concentrations the forward and reverse net fluxes are very similar not only to each other, but also when compared with the equilibrium flux F_i (Figs. 1 and 2 and Table 1). On the other hand, at 1000-fold higher substrate concentrations the net flux of the reverse reaction (pyruvate oxidation) is more than twice as high as the net flux in the forward direction (lactate oxidation), and about 10 times higher than the equilibrium flux F_i (Figs. 3 and 4 and Table 2).

Marked differences are also apparent in the concentration of intermediates. Figs. 1 and 2 show that for each individual enzyme-substrate intermediate the sum of both steady-state concentrations equals the reference equilibrium concentration in a remarkably good approximation (cf. Table 1). These relationships no longer hold at high substrate concentrations (Figs. 3 and 4). As already pointed out above, at high substrate levels some of the intermediate concentrations can even be considerably higher under initial steady-state conditions than at chemical equilibrium.

For a better understanding of these special features of Figs. 1–4 one has to have a clear idea of the way in which the forward and reverse reactions are related to the reference equilibrium. Both volume and direction of a net flux are determined by the choice of substrate concentrations. In this paper I derive forward and reverse net fluxes from a common reference equilibrium by an unusual sort of perturbation, by the ‘instantaneous and complete’ removal of the free reaction products. It is to be noted that the time evolution of the forward and reverse net fluxes derived in this manner would not lead to the same equilibrium situation. Instead, I am considering those initial steady-state velocities that one would obtain with the same substrate concentrations as at the reference equilibrium, but in the absence of reaction products.

As early as 1959 P. D. Boyer [31] described a very interesting derivation of the rate of isotope exchange reactions at chemical equilibrium. His reasoning is based on considering that the

equilibrium concentration of all intermediates of a consecutive reaction is maintained by two opposing fluxes, originating at one or the other ‘end’ of the overall reaction sequence. Obviously, the equilibrium concentrations necessarily correspond to the sum of these two partial concentrations, whenever equilibrium prevails. It is similarly clear that, in principle, there must be a competition between the forward and reverse reactions for the enzyme. Although the competition has to be especially keen at substrate saturation, it must completely disappear in the limiting case of very low substrate concentrations (eqn. 3). Now the point is that, if competition disappears, the forward and reverse net fluxes must become fully independent reactions, which take place in exactly the same manner whether there is an opposing flux or not. There are some far reaching consequences of this conclusion, which can be formulated in different ways. For instance: 1. the forward and reverse net fluxes are equal; 2. the net fluxes are also equal to the rate of isotope exchange reaction at chemical equilibrium; 3. the net fluxes that one obtains under initial steady-state conditions are actually identical with the overall flux in the corresponding equilibrium system (see ‘Note added in proof’ below).

One should note that these three ‘rules’ refer to initial steady-state velocities, and are only valid under the ‘ideal conditions’ defined in eqn. (3). The first one of them is one of the basic principles of steady-state enzyme kinetics, and the rate equations that refer to the limiting conditions defined in eqn. (3) play an important role in this discipline under the name ‘Haldanes’ or ‘Haldane relations’. The relationship between net fluxes obtained under initial steady-state conditions on the one hand, and isotope exchange reactions at chemical equilibrium on the other hand, has received hardly any attention, at least in connection with the limiting conditions defined in eqn. (3). Indeed, one can even meet the opinion that isotope exchange at equilibrium is no true equilibrium reaction, because ‘elementary reactions involving labelled molecules are (in fact) not proceeding at equilibrium rates’ [32]. Although according to this latter view there is no common denominator for a net flux and an ‘equilibrium rate process’, the third formulation above states that the net fluxes that one obtains under initial steady-state conditions are actually identical with the overall flux in the corresponding equilibrium system (F_i in Tables 1 and 2). It should be noted that this conclusion is the most important one that can be drawn from net flux diagrams such as Figs. 1 and 2. Obviously rule 3 not only implies but also explains rules 1 and 2.

It is known from both experiment and numerical simulation that initial steady-state velocities and equilibrium exchange rates do not remain equal if the concentration of substrates is increased (Figs. 3 and 4). The reason for this is competition among the substrates. If the total amount of enzyme is liganded, and is so not only in the presence of all four reactants (equilibrium) but in the absence of pyruvate and NADH (forward reaction) as well as in the absence of lactate and NAD^+ (reverse reaction), the limiting case of substrate saturation (eqn. 4) has been attained. One should note that in this limiting case the sum total of the concentrations of all liganded forms of the enzyme in the forward plus reverse reactions is $2[E_t]$. A single look at eqns. (1) and (2) clearly shows that the ‘concentration profiles’ for the forward and reverse steady-state reactions must be different from one another (and also from that for the reference equilibrium), even if the enzyme is fully saturated with substrates. Figs. 3 and 4 confirm this expectation, and show up to 6-fold deviations between corresponding steady-state and equilibrium concentrations. Comparison of the profiles for the forward and reverse reactions (Figs. 3 and 4) shows that the deviation from the equilibrium situation is much less ‘symmetrical’ than at low substrate concentrations (Figs. 1 and 2).

GENERAL DISCUSSION

As stated by Denbigh ([33], chapter iii) it is general practise in natural sciences to define and investigate models for describing real systems under limiting conditions. Certainly the law of mass action refers to the most frequently used model of this kind in chemical kinetics. In spite of this, there appears to be general agreement that the rate expression describing a complex reaction, made up of a sequence of chemical (and even 'physical') events will, in general, not have mass-action form [34]. There is a far-reaching analogy in the arguments applied to widely different levels of description, ranging from the stochastic treatment of a thermally induced isomerization reaction (e.g. ref. [19]) to the formal kinetics of elementary reaction steps in an enzymic or surface-catalysed reaction (e.g. ref. [35]).

In eqn. (3) I am defining limiting ('ideal') conditions under which mass-action kinetics exactly applies to the net flux coefficient of a typical consecutive enzyme reaction. I should like to point out that this enzyme model yields conclusions that generally refute the above objections to mass-action kinetics. The crux of the argument can be summarized in two points. (i) There is every reason to expect that limiting conditions, under which mass-action kinetics does apply, can be easily found for each reversible consecutive reaction. The enzyme model discussed in this paper shows that it is essential to ensure independence of the forward and the reverse reactions (comp. Hammett [36], chapter 4.26). It is shown above that for a catalytic reaction this can only be realized if there is no competition between the forward and the reverse reactions for the catalyst. One should note that a number of additional factors may also lead to an interdependence of the forward and reverse reactions under non-ideal conditions. (ii) If mass-action kinetics exactly applies to a consecutive reaction, the overall 'initial reaction' is strictly identical with the overall 'equilibrium reaction'. In other words, and contrary to the apparently self-evident conventional view, for a sequential overall reaction there is no 'equilibrium rate process' that is different from the 'initial steady-state flux' (for the conventional view see Fleck [32], as cited above).

The presented evidence heavily relies on the choice of the reference equilibrium. When discussing the forward and reverse rate processes of a reversible chemical reaction, one normally refers to that equilibrium to which the time evolution of the reaction system would lead. In contrast, I have only regarded time-independent reaction rates, and have also used a reference equilibrium that has got nothing to do with the eventual time evolution of the system. In order to follow the presented line of reasoning, it is important to distinguish very clearly among three different kinds of net chemical fluxes. These are: (i) the difference between a forward flux and a reverse flux in a single (elementary) reaction step; (ii) the difference between an overall forward flux and an overall reverse flux in a multistep (consecutive) reaction; (iii) the net flux of an overall reaction with no opposing overall flux ('initial rate process'). I suggest that type (i) and (ii) fluxes are denoted as Δ fluxes, in contradistinction to net fluxes of type (iii). Although the time evolution of (reversible) chemical reactions necessarily involves Δ fluxes, the kinetic barrier diagrams discussed in this paper can only be applied to net fluxes of type (iii). One should note that it is this last type of chemical flux that is directly relevant to most theoretical models of chemical reaction kinetics, including mass-action kinetics and transition-state theory.

Kinetic barrier diagrams apply to consecutive reactions irrespective of the nature of the molecular events that are considered to constitute the reaction steps. This is important because

different types of chemical reactions and usually even consecutive steps of the same overall reaction are widely different in this respect. Generally applicable treatments of this diversity have to be of a formal kinetic nature.

Note added in proof (received 24 February 1992)

Whereas eqns. (1) and (2) refer to a reaction sequence involving two irreversible steps, the $\text{NAD}^+ \rightleftharpoons \text{NADH}$ isotope exchange reaction defines (because of chemical equilibrium) another steady state with just one irreversible step. This distinction has been overlooked in the present paper. However, none of the main conclusions are affected, since (i) there is no discernible distinction between the two processes under ideal conditions, and (ii) the analogous steady states are under all conditions strictly identical if the reaction only involves a single reaction product.

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