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Review

Mathematical analysis of enzymic reaction systems using optimization principles

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Glossary of principal symbols. A_i, affinity of a reaction; A_z, amplification factor as to signal Z; $\mathbf{C}_i^J$, flux control coefficient; E, enzyme; E_i, enzyme concentrations; E_0, total concentration of a particular enzyme; ΔG, Gibbs free energy; G, growth rate constant; g_i, osmotic coefficients; H_τ, measure of time hierarchy; J, steady-state flux; k_i, k_{-i}, k_+, k_-, elementary rate constants; k_{cat}, catalytic constant (turnover number); K_m, Michaelis constant; K_{int}, equilibrium constant of EP vs ES; L_{ij}, Onsager coefficients; **M**, Jacobian matrix of a reaction system; M_i, molecular mass of proteins; **N**, stoichiometric matrix; n, number of metabolites, or kinetic order of cooperative enzymes; n_{ij}, stoichiometric coefficients; n_H, Hill coefficient; p_i, kinetic parameters of enzymes; q_i, equilibrium constants; R, unscaled sensitivity, or universal gas constant; $\hat{R}$, scaled sensitivity; r, coupling constant, or number of reactions; S, P, substrate and product of a reaction or reaction pathway; S_i, P_i, metabolite and product concentrations; $S_{0.5}$, half-saturation constant; T, absolute temperature; v, catalytic activity; v_i, reaction rates; V^+, V^-, V_m, maximal activities; V, cellular volume; X_i, thermodynamic forces; Y, state variable of a metabolic system; Z, external signal; β, Brønsted coefficient; η, thermodynamic efficiency; $\varkappa$, rate constant normalized by enzyme concentration; λ, eigenvalue of the Jacobian; μ, Lagrange multiplier; π, osmotic pressure; σ entropy production; σ_i, deviation of concentration S_i from the steady-state value; τ, transient time; Φ, performance function; χ, cost function; Ω, total osmolarity.

Introduction

Mathematical modelling of metabolic networks is nowadays an important subject of theoretical biology. Most studies in this field concern (a) the development of models for the simulation of stationary and time dependent states of metabolic pathways; (b) the analysis of complex dynamic phenomena such as transitions between multiple stationary states, oscillations and even chaotic behaviour; and (c) the quantitative characterization of control properties of metabolic pathways.

In all of these investigations the kinetic parameters of the reactions (enzyme concentrations, maximal activities, Michaelis constants, inhibition constants etc.) are taken from the experiment without attempting to give any theoretical explanation for the observed values. Using these parameters as inputs, 'traditional' modelling aims at the understanding of the behaviour of the system variables, e.g. metabolite concentrations and fluxes. This approach has been successfully applied to elucidate the regulatory features of various cellular metabolic pathways (for recent work cf. [1–10]).

It was stated many years ago that any approach towards the explanation of the kinetic parameters of enzymes and the structural design of metabolic systems must take into account the fact that these systems are, in contrast to chemical systems

of inanimate nature, the outcome of evolution [11–22]. An enormous literature exists on the natural selection and evolution of biological systems ranging from the reconstruction of evolutionary mutation trees up to the study of forces acting on the evolution of whole organisms or populations. In many of these studies evolution is considered as an optimization process. This view is supported by a number of observations, for example by the fact that mutations or other changes in the structure of contemporary enzymes lead in most cases to a worse functioning of cellular metabolism [23]. Much theoretical work has been devoted to enquire whether the structural design and functioning of present-day biochemical systems can be rationalized on the basis of optimization principles. This is the subject of the present review article.

In quantitative investigations the crucial point is the formulation of appropriate performance functions whose maximum (or minimum) value might correspond to the outcome of the evolution of cellular metabolism. In the literature the following optimization principles are mostly considered: (a) maximization of steady-state fluxes; (b) minimization of the concentrations of metabolic intermediates; (c) minimization of transient times; (d) maximization of the sensitivity to external signals; and (e) optimization of thermodynamic efficiencies.

In optimization studies, one has to take into account certain constraints which may be of a different type. There are a number of physical constraints limiting the range of variations of kinetic parameters, for example for the following reasons: (a) any parameter configuration has to meet the thermodynamic equilibrium condition which is independent of the catalyst; (b) there are upper limits for the elementary rate constants of enzymatic reactions due to physico-chemical constraints as, for example, diffusional limitations or steric hindrance imposed by the structural arrangement of the amino acid residues; (c) the stoichiometry of metabolic systems has to fulfil certain physical requirements such as mass conservation.

Furthermore, there are biological constraints which are often called 'cost functions' [24, 25] which are more difficult to express in clear-cut mathematical terms. In the current literature, various cost functions have been proposed, e.g. the total enzyme content of the cell or a given pathway [24, 26, 27] or the total energy utilization [28]. In the concept of 'evolutionary effort' the cost function is related to the number of mutations or other evolutionary events necessary for the attainment of a particular set of kinetic parameters [29, 30]. Mathematically, this type of constraint may be taken into account by use of the method of Lagrange multipliers [31].

Studies on the optimum properties of single enzymes have been made as well as on the mutual interdependence of the enzymes within metabolic pathways. The inclusion of systemic properties into optimization analysis may lead to considerable mathematical difficulties. Even in simulation modelling, analytical solutions are rarely available. Another complicated mathematical problem arises from the nonlinearity of the optimization problems.

Optimization of the catalytic properties of single enzymes

It has often been stressed that evolutionary pressure on enzyme function was mainly directed towards a maximization of catalytic activity [14, 16–19, 32–37],

$$v = v_{max}. \qquad (1)$$

Mostly, the interplay of different enzymes within pathways is neglected, i.e. the enzymes are studied under 'isolated conditions' where the concentrations of substrates and products are considered as fixed parameters.

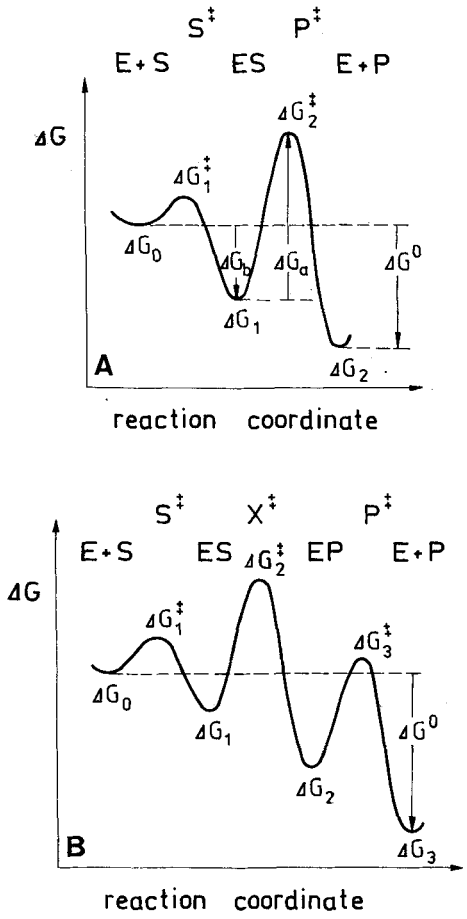

Fig. 1. *Free energy profiles of enzymatic reactions.* (A) Irreversible two-step kinetic mechanism (cf. Scheme 1). ΔG_0, ΔG_1 and ΔG_2 denote the Gibbs free energies of formation of E + S, of the enzyme-substrate complex ES and of E + P, respectively, and $\Delta G_1^{\ddagger}$ and $\Delta G_2^{\ddagger}$ the Gibbs free energies of formation of transition states $S^{\ddagger}$ and $P^{\ddagger}$ (free energies of formation are defined as the free energy change for the reaction by which the substance is formed from its elements under standard conditions, cf. [148]). (B) Reversible three-step kinetic mechanism (cf. Scheme 2). ΔG_0, ΔG_1, ΔG_2 and ΔG_3 denote the free energies of formation of the states E + S, ES, EP and E + P, respectively, and $\Delta G_1^{\ddagger}$, $\Delta G_2^{\ddagger}$ and $\Delta G_3^{\ddagger}$ the free energies of formation of the transition states $S^{\ddagger}$, $X^{\ddagger}$ and $P^{\ddagger}$

Irreversible two-step kinetic mechanism

In pioneering papers [14, 16, 18] evolutionary optimization of enzymatic activity was studied by consideration of the most simple reaction scheme provided by the irreversible two-step kinetic mechanism [38]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P. \qquad \text{(Scheme 1)}$$

The catalytic properties of this reaction were discussed on the basis of free energy profiles [14, 16] (cf. Fig. 1 A). The standard free energy difference $\Delta G^{\circ} = \Delta G_2 - \Delta G_0$ is fixed by the nature of the reaction. It is assumed that ΔG° is low enough for the reaction to be irreversible.

According to transition state theory the catalytic constant of the enzyme may be written as follows

$$k_2 = k_{cat} = k_m \exp\left(-\frac{\Delta G_2^{\ddagger} - \Delta G_1}{RT}\right) \quad (2)$$

where k_m is the maximal value of the first-order rate constant k_2. If $k_2 \ll k_{-1}$ the Michaelis constant may be approximated by the dissociation constant of the enzyme-substrate complex,

$$K_m = \frac{k_{-1} + k_2}{k_1} \approx \frac{k_r}{k_d} \exp\left(\frac{\Delta G_1 - \Delta G_0}{RT}\right) \quad (3)$$

where k_d and k_r are the maximal values for the rate constants k_1 and k_{-1}, respectively. For enzymatic reactions detailed investigations on the pre-exponential factors of kinetic constants were carried out [39–43]. It was shown that these factors (denoted here by k_d, k_m and k_r) depend on the diffusion coefficient of the ligand and on the viscosity of the medium.

Inserting Eqns (2) and (3) into the Michaelis-Menten equation, one obtains [18]

$$v = \frac{V_m S}{K_m + S} = \frac{S E_0 k_m \exp\left(-\frac{\Delta G_2^{\ddagger} - \Delta G_0}{RT}\right)}{\frac{k_r}{k_d} \exp\left(\frac{\Delta G_1 - \Delta G_0}{RT}\right) + S}. \quad (4)$$

From this equation, one derives

$$\frac{\partial v}{\partial \Delta G_1} > 0, \frac{\partial v}{\partial \Delta G_2^{\ddagger}} < 0 \quad (5)$$

which indicates that evolution should increase ΔG_1 and decrease $\Delta G_2^{\ddagger}$. This is equivalent to an enhancement of the Michaelis constant as well as of the quantity $\varkappa$ defined by

$$\varkappa = \frac{k_{cat}}{K_m} = \frac{k_m k_d}{k_r} \exp\left(-\frac{\Delta G_2^{\ddagger} - \Delta G_0}{RT}\right). \quad (6)$$

On the basis of such calculations it was stated that high K_m values, i.e. $K_m > S$, are catalytically advantageous [14]. The same conclusion was derived on the basis of a reversible three-step kinetic mechanism [19].

It was objected, however, that the free energies ΔG_1 and $\Delta G_2^{\ddagger}$ cannot be considered to be really independent of each other, since structural changes of the enzyme which affect ΔG_1 will to a certain degree also change the free energy $\Delta G_2^{\ddagger}$ [18]. The following Ansatz was made [18]

$$\Delta G_1 = \Delta G_{1,0} + \gamma, \quad \Delta G_2^{\ddagger} = \Delta G_{2,0}^{\ddagger} + r\gamma \quad (7)$$

where r is a coupling constant ($0 \leq r \leq 1$) and the subscript 0 refers to a reference state. γ is a free-energy change that may be adjusted under evolutionary pressure. Using Eqn (7), the binding energy of the ES complex ΔG_b and the activation energy ΔG_a for product formation (cf. Fig. 1 A) may be expressed by the parameter γ and the coupling constant r,

$$\Delta G_b = \Delta G_{1,0} - \Delta G_0 + \gamma \quad (8a)$$

$$\Delta G_a = \Delta G_{2,0}^{\ddagger} - \Delta G_{1,0} + (r-1)\gamma. \quad (8b)$$

From Eqns (4) and (8) one derives [18]

$$\frac{\partial v}{\partial \gamma} = \frac{v[S(1-r) - r K_m]}{RT(K_m + S)}. \quad (9)$$

Two extreme cases may be considered.

First, $r = 0$. The free energy of the activated complex remains unchanged by mutations; variations of the parameter γ will affect equally the binding energy and the activation energy.

The derivative Eqn (9) is positive, i.e. an improved binding of the substrate (expressed by a lowering of γ) will lead to a worse functioning of the catalyst (cf. [14]).

Second, $r = 1$. The effect of γ on the binding energy ΔG_b equals its effect on the free energy of the activated complex, so that the activation energy ΔG_a remains unchanged. In this case the derivative Eqn (9) is negative, so that an improved binding of the substrate to the enzyme will enhance catalytic effectiveness. For intermediate values of r, states of maximal activity are obtained if $\partial v/\partial \gamma = 0$, i.e. if the condition

$$\frac{S}{K_m} = \frac{r}{1-r} \quad (10)$$

is fulfilled. From Eqn (10) it follows that maximization of enzymatic activity will result in Michaelis constants which are comparable with the substrate concentration provided that the coupling constant r is neither close to zero nor close to unity [18].

Expressing the Michaelis-Menten equation in terms of the elementary rate constants, one arrives at the inequalities

$$k_1 \frac{\partial v}{\partial k_1} > k_2 \frac{\partial v}{\partial k_2} \quad \text{for } k_1 S < k_2 \quad (11a)$$

$$k_1 \frac{\partial v}{\partial k_1} < k_2 \frac{\partial v}{\partial k_2} \quad \text{for } k_1 S > k_2 \quad (11b)$$

which were used to propose an optimal strategy for increasing v by changing the kinetic constants k_1 and k_2 [16]. Under the assumption that, on the evolutionary time scale, fractional rather than absolute changes of k_1 and k_2 are relevant, formulae (11 a) and (11 b) indicate that, at low values of k_1, it is more advantageous to increase k_1, while for $k_1 S > k_2$ it is better to increase k_2. In both cases one arrives eventually at states which are characterized by the condition

$$k_1 S = k_2 \quad (12)$$

where relative changes of k_1 and k_2 have the same effect on the enzymatic activity. Further on, the optimal strategy consists in an increase of both parameters in a way that relation (12) remains fulfilled (cf. Fig. 2).

From Eqn (12) it follows that

$$\frac{S}{K_m} = \frac{k_1 S}{k_{-1} + k_2} = \frac{k_1 S}{k_{-1} + k_1 S}. \quad (13)$$

Thus, the ratio between the substrate concentration and the Michaelis constant becomes closer to unity as k_1 increases [16].

Reversible three-step kinetic mechanism

In [17, 19] and in more recent papers [32–37] the evolutionary optimization of the catalytic properties of enzymes was considered on the basis of the reversible three-step kinetic mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P \quad \text{(Scheme 2)}$$

originally proposed by Haldane [44]. The steady-state rate equation for this mechanism is [45]

$$v = \frac{E_0(k_1 k_2 k_3 S - k_{-1} k_{-2} k_{-3} P)}{k_{-1}k_{-2} + k_{-1}k_3 + k_2 k_3 + (k_1 k_{-2} + k_1 k_3 + k_1 k_2)S + (k_{-1}k_{-3} + k_2 k_{-3} + k_{-2}k_{-3})P} \quad (14)$$

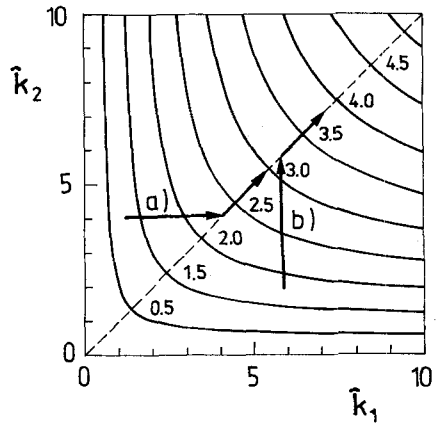

Fig. 2. *Optimal strategies for the increase of the activity of an enzyme obeying Michaelis-Menten kinetics for two different initial conditions.* Conditions: (a) $k_1S < k_2$; (b) $k_1S > k_2$. The solid lines connect points of equal values of the normalized reaction rate $\hat{v} = v/k_{-1}E_0$ and are calculated according to the formula $\hat{k}_2 = \hat{v}\,(1+\hat{k}_1)/(\hat{k}_1 - \hat{v})$ with $\hat{k}_2 = k_2/k_{-1}$, $\hat{k}_1 = k_1S/k_{-1}$

which may be reformulated by introducing maximal activities and Michaelis constants

$$v = \frac{\frac{V^+}{K_m^+}S - \frac{V^-}{K_m^-}P}{1 + \frac{S}{K_m^+} + \frac{P}{K_m^-}} \tag{15}$$

with

$$V^+ = \frac{k_2k_3}{k_2 + k_3 + k_{-2}},\quad V^- = \frac{k_{-1}k_{-2}}{k_2 + k_{-1} + k_{-2}} \tag{16}$$

and

$$K_m^+ = \frac{k_2k_3 + k_{-1}k_3 + k_{-1}k_{-2}}{k_1(k_2 + k_3 + k_{-2})} \tag{17a}$$

$$K_m^- = \frac{k_2k_3 + k_{-1}k_3 + k_{-1}k_{-2}}{k_{-3}(k_2 + k_{-1} + k_{-2})}. \tag{17b}$$

The six elementary rate constants are not fully independent but related to the equilibrium constant

$$q = \frac{k_1k_2k_3}{k_{-1}k_{-2}k_{-3}} \tag{18}$$

which cannot be changed by alteration of the catalytic properties of the enzyme. By elimination of k_{-3} rate equation (Eqn 14) may be written as follows:

$$v = \frac{E_0}{N}(Sq - P) \tag{19a}$$

with

$$N = q\left(\frac{k_{-1}k_{-2}}{k_1k_2k_3} + \frac{k_{-1}}{k_1k_2} + \frac{1}{k_1}\right) + Sq\left(\frac{k_{-2}}{k_2k_3} + \frac{1}{k_2} + \frac{1}{k_3}\right) + P\left(\frac{k_2}{k_{-1}k_{-2}} + \frac{1}{k_{-1}} + \frac{1}{k_{-2}}\right). \tag{19b}$$

Obviously, at fixed values of the concentrations S and P the reaction rate v becomes maximum for minimal values of the denominator N.

It is often assumed that the second-order rate constants k_1 and k_{-3} are not subject to evolutionary variation but are fixed by diffusional constraints [15, 17, 19, 32, 35, 36] so that

$$k_1 = k_{-3} = k_d = \text{const.} \tag{20}$$

Typical values for k_d are in the range $10^8 - 10^{10}$ M^{-1} s^{-1} [36, 45, 46]. With Eqn (20) the equilibrium relation (Eqn 18) simplifies to

$$q = \frac{k_2k_3}{k_{-1}k_{-2}} \tag{21}$$

and only three rate constants, rather then five, may be treated as variables. If it is further assumed that all first-order rate constants may be infinitely high, the minimal value of the denominator N reads as follows [17, 35, 36]

$$N_{min} = \frac{q}{k_1} + \frac{1}{k_{-3}} = \frac{(1+q)}{k_d}. \tag{22}$$

The following measure for the catalytic efficiency was introduced [17, 35]

$$\Phi = \frac{N_{min}}{N}. \tag{23}$$

According to Eqn (23), the principle $N = N_{min}$ is equivalent to $\Phi = \Phi_{max}$. Similarly as for the simple Michaelian enzyme (see previous section), the reversible three-step kinetic mechanism may be discussed in terms of free energy diagrams [17, 32–35] (cf. Fig. 1B). The free energies are not fully independent since

$$\Delta G_3 - \Delta G_0 = \Delta G^\circ = -RT\ln q = \text{const.} \tag{24}$$

Since according to the assumption of Eqn (20), the activation energies of the two binding processes are also fixed, there remain only three degrees of freedom which refer to changes of the free energies ΔG_1, $\Delta G_2^\ddagger$ and ΔG_2. It was proposed that variations of these free energies due to mutations may be understood in terms of three new variables corresponding to different classes of binding interactions [17, 32, 35].

a) Uniform binding. This corresponds to equal changes of free energies of the states ES, EP and transition state $X^\ddagger$. Such a process changes k_{-1} and k_3 by the same factor while k_2 and k_{-2} remain constant.

b) Differential binding. This is connected with relative changes of the free energies of EP and ES. The process is characterized by variations of the 'internal equilibrium constant'

$$K_{int} = \frac{k_2}{k_{-2}}. \tag{25}$$

c) Catalysis of an elementary step. This changes the free energy of transition state $X^\ddagger$. The process is characterized by equal fractional changes of rate constants k_2 and k_{-2}. According to this classification of mutational processes, the quantities k_{-1}, K_{int} and k_2 are treated as independent parameters. It must be doubted, however, whether it is typical that these parameters are affected independently by mutations.

One of the conclusions derived from the principle $\Phi = N_{min}/N = \Phi_{max}$ concerns the optimal value of the internal equilibrium constant. From $\partial\Phi/\partial k_{-1} = 0$ and $\partial\Phi/\partial K_{int} = 0$ the following relationship was obtained [35]

$$K_{int} = \frac{\beta}{1-\beta}\,\frac{1 + P/S + k_3/k_{-2}}{1 + P/S + Pk_3/Sqk_{-2}} \tag{26}$$

where β denotes the Brønsted coefficient of the second reaction step in Scheme 2 [47, 48]. Under the assumption $\beta = 0.5$

Table 1. *Optimal solutions* L_n *for the rate constants* k_i, k_{-i} *(i = 1, 2, 3) for the enzymatic reaction depicted in Scheme 2 as functions of the concentrations of substrate and product for* $q \geq 1$ *and* $k_m = k_r$ *as derived in [55, 56]*

The solutions are expressed in dimensionless units. Concentrations and rate constants are normalized as follows: $k_dS/k_r \rightarrow S$, $k_dP/k_r \rightarrow P$, $k_\alpha/k_d \rightarrow k_\alpha$, $k_\beta/k_m \rightarrow k_\beta$, $k_\gamma/k_r \rightarrow k_\gamma$, with $\alpha = 1, -3$, $\beta = 2, -2$ and $\gamma = -1, 3$. The thin lines separate solutions differing in the number (l) of rate constants assuming nonmaximal values. The broken line separates two subgroups of solutions with $l = 2$. For L_4, L_5 and L_6 normalized rate constants of only backward reactions are unequal to unity while for L_7, L_8 and L_9 nonmaximal values are obtained also for rate constants of forward reactions. The solutions are valid for $q \geq 1$. For $q < 1$ the optimal rate constants may be obtained from the solutions derived for $q > 1$ by the transformations $v \rightarrow -v$, $q \rightarrow 1/q$ and by interchanging the meaning of the symbols k_1 and k_{-3}, k_2 and k_{-2} and k_{-1} and k_3 as well as of S and P

Solution	k_1	k_{-1}	k_2	k_{-2}	k_3	k_{-3}
L_1	1	$\frac{1}{q}$	1	1	1	1
L_2	1	1	1	$\frac{1}{q}$	1	1
L_3	1	1	1	1	1	$\frac{1}{q}$
L_4	1	$\sqrt{\frac{P}{q}}$	1	1	1	$\sqrt{\frac{1}{Pq}}$
L_5	1	$\sqrt{\frac{S+P}{q(1+P)}}$	1	$\sqrt{\frac{1+P}{q(S+P)}}$	1	1
L_6	1	1	1	$\sqrt{\frac{2P}{q(1+S)}}$	1	$\sqrt{\frac{1+S}{2Pq}}$
L_7	$\sqrt{\frac{2q(1+P)}{S}}$	1	1	$\sqrt{\frac{2(1+P)}{Sq}}$	1	1
L_8	1	1	$\sqrt{\frac{2q(1+S)}{P}}$	1	1	$\sqrt{\frac{2(1+S)}{Pq}}$
L_9	1	$\sqrt{\frac{2(S+P)}{q}}$	1	1	$\sqrt{2q(S+P)}$	1
L_{10}	$k_1 = k_2 = k_3 = 1, k_{-1}^4 + k_{-1}^3 - \frac{Pk_{-1}}{q} - \frac{SP}{q} = 0, k_{-2} = \frac{P}{qk_{-1}^2}, k_{-3} = \frac{1}{qk_{-1}k_{-2}}$					

[35], it follows from Eqn (26) that for $Sq > P$ (i.e. $v > 0$) the internal equilibrium constant becomes greater than unity. For near-equilibrium enzymes ($Sq \approx P$) one obtains from Eqn (26) that $K_{int} \approx 1$ irrespective of the overall equilibrium constant q. This conclusion was criticized since β may vary between zero and values even greater than unity [34]. Furthermore, the concept of rate-equilibrium relations, which is the basis for the use of Brønsted coefficients, has severe limitations [48]. Some experimental data support the theoretical prediction $K_{int} \approx 1$ [49–53] but for many enzymes a value of K_{int} much higher than unity seems to be more realistic (e.g. [33, 34]).

Probably, the basic assumption of Eqn (20) stating that the second-order rate constants k_1 and k_{-3} are not subject to evolutionary changes cannot be proclaimed as a dogma. The values of k_1 and k_{-3} can be much higher than the diffusional limit if, for example, a direct transfer of metabolites of one enzyme to the next in a pathway takes place [34, 54]. Furthermore, there are no reasons why k_1 or k_{-3} should not be smaller than k_d if this is compatible with a high catalytic rate. Thus, the optimization analysis was generalized by replacing the equality conditions (Eqn 20) by the inequalites

$$k_1, k_{-3} \leq k_d \tag{27a}$$

[37, 55]. Furthermore, upper limits were taken into account also for the first-order rate constants, i.e.

$$k_{-1}, k_3 \leq k_r; k_2, k_{-2} \leq k_m. \tag{27b,c}$$

Under the conditions of inequalities (27a–c), the optimization principle $|v| = v_{max}$ yields ten solutions L_n listed in Table 1 for the case $q \geq 1$. These solutions depend on S and P and differ in the number (l) of rate constants attaining nonmaximal values in the optimal state. All solutions except for L_{10} can

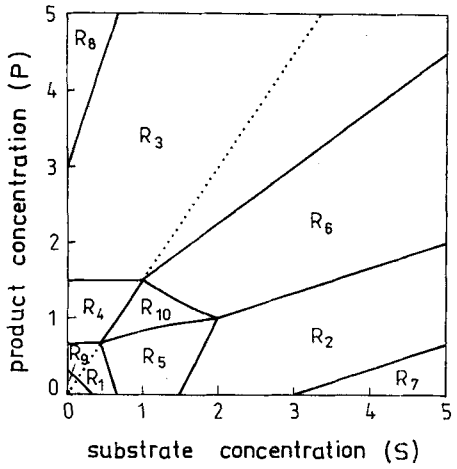

Fig. 3. *Subdivision of the* (S,P) *plane into subregions* R_n *corresponding to the various solutions* L_n *for optimal microscopic rate constants of the reversible three-step kinetic mechanism depicted in Scheme 2 for* q = *1.5 [55].* Concentrations *S* and *P* are normalized as explained in the legend to Table 1

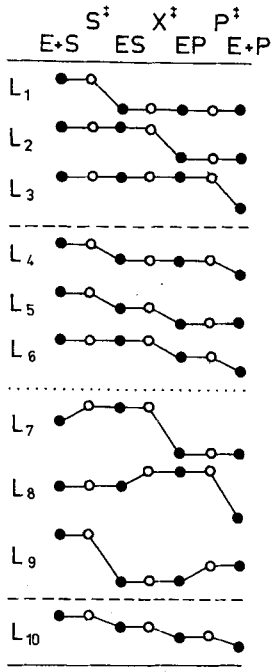

Fig. 4. *Schematic free energy profiles for solutions* L_n *(n = 1,...,10) listed in Table 1.* (●) The 'free states' E + S and E + P and the 'bound states' ES and EP; (○) the transition states $S^{\ddagger}$, $X^{\ddagger}$ and $P^{\ddagger}$. Horizontal lines between ● and ○ indicate that the rate constant of the corresponding process attains its maximal value

be given analytically. For the special case $S = P = 1$, an explicit solution of the fourth-order equation given in Table 1 for L_{10} can be obtained:

$$k_{-1} = k_{-2} = k_{-3} = \sqrt[3]{\frac{1}{q}}. \tag{28}$$

Conditions (27a–c) define the allowed (*S*,*P*) region R_n for each solution L_n (Fig. 3).

For example, solution L_{10} applies only if

$$S \leq \frac{2q}{P} - 1, S \geq \frac{P}{q}, S \leq P(P^2q + Pq - 1) \quad (29a-c)$$

where *S* and *P* are expressed in dimensionless units (cf. legend to Table 1).

The solutions L_n for the optimal rate constants may be illustrated also by the corresponding free energy profiles as shown in Fig. 4.

From Table 1 and Figs 3 and 4, the following properties of the optimal solutions may be derived.

a) At low substrate concentrations an optimal enzymatic activity is achieved by improvement of the enzyme-substrate binding ($k_1 = 1, k_{-1} < 1$: solutions $L_1, L_4, L_5, L_9, L_{10}$).

b) At high concentrations *S* or *P* the substrate and product are weakly bound to the enzyme ($k_1 < 1, k_{-1} = 1$: solution L_7, and $k_3 = 1, k_{-3} < 1$: solutions $L_3, L_4, L_6, L_8, L_{10}$, respectively).

c) k_2 is always maximal except for region R_8 where the reaction proceeds backwards.

d) In contrast to previous assumptions [17, 19, 35, 36], an optimal enzymatic activity is not compulsorily achieved by maximal values of the second-order rate constants. As to k_1 this is the case for solution L_7 and as to k_{-3} for solutions L_3, L_4, L_6, L_8 and L_{10}.

e) $K_{int} = 1$ is obtained for solutions L_1, L_3, L_4 and L_9. $K_{int} \approx 1$ is valid for all near-equilibrium enzymes ($Sq \approx P$). The latter result corresponds to conclusions derived in [35] (see discussion above).

Optimal values for the kinetic parameters V^+, V^-, K_m^+ and K_m^- can be obtained by introducing k_i and k_{-i} from Table 1 into Eqns (16) and (17). In this way these macroscopic parameters become functions of the external concentrations *S* and *P* as well as of the equilibrium constant *q*. Although these dependences differ in the various regions R_n [37, 55], a general conclusion is that in most subregions R_n the relations

$$\frac{V^+}{V^-} \geq 1; \frac{K_m^+}{K_m^-} \leq 1 \tag{30}$$

are fulfilled [56]. Exceptions are R_8 (where $V^+/V^- \leq 1$) and R_7 (where $K_m^+/K_m^- \geq 1$).

For the special case $S = P = 1$ (for the normalization of concentrations see legend to Table 1) where the optimal values of the rate constants are given by Eqn (28) one obtains

$$V^+ = \frac{q^{1/3}}{2q^{1/3} + 1}, V^- = \frac{1}{q^{1/3}(2 + q^{1/3})} \tag{31a,b}$$

$$K_m^+ = \frac{1 + q^{1/3} + q^{2/3}}{q^{1/3}(1 + 2q^{1/3})}, K_m^- = \frac{1 + q^{1/3} + q^{2/3}}{2 + q^{1/3}} \tag{32a,b}$$

(V^+ and V^- are expressed as multiples of $k_r E_0$, K_m^+ and K_m^- in multiples of k_r/k_d). From Eqn (32a,b) it is immediately derived that, for the special cases $q = 1$,

$$\frac{S}{K_m^+} = 1, \frac{P}{K_m^-} = 1 \tag{33a,b}$$

and $q \gg 1$

$$\frac{S}{K_m^+} \approx 2, \frac{P}{K_m^-} \approx q^{-1/3}. \tag{34a,b}$$

Eqns (33a) and (34a) support previous conclusions that there is a matching of the Michaelis constant K_m^+ to the substrate concentration *S* [16, 18]. Moreover, for equilibrium constants not very different from unity, the theory predicts also a matching of the Michaelis constant K_m^- to the product concen-

Table 2. *Elementary rate constants of three different β-lactamases*
The original values are taken from [58]. The normalized values are recalculated with the normalization constants $k_d = 10^8\ M^{-1}\ s^{-1}$ and $k_m = 10^4\ s^{-1}$

Constant	Value for β-lactamase		
	I	PCI	RTEM
k_1 ($\mu M^{-1}\ s^{-1}$)	41	22	123
k_{-1} (s^{-1})	2320	196	11800
k_2 (s^{-1})	4090	173	2800
k_3 (s^{-1})	3610	96	1500
k_1/k_d	0.41	0.22	1.23
k_{-1}/k_m	0.23	0.02	1.18
k_2/k_m	0.41	0.017	0.28
k_3/k_m	0.36	0.01	0.15

tration P [37, 55, 56] (cf. Eqns 33b and 34b). One should note, however, that Eqns (33) and (34) were derived for special values of S and P (see above). For extremely low or high substrate or product concentrations the matching of K_m^+ and K_m^- to S and P, respectively, is much less pronounced [37, 55, 56]. In particular, at low substrate concentrations the enzymatic rate becomes maximum if $K_m^+ > S$ while for high substrate concentrations optimization leads to $K_m^+ < S$ [37].

Comparison with experimental data. The comparison of theoretically predicted optimal values of rate constants with enzyme data has usually been based on the rather special measure for enzyme efficiency proposed in [17] for the three-step Haldane mechanism (cf. Eqn 23). In the light of this optimization criterion, the triose-phosphate isomerase and some β-lactamases have been classified as almost perfect catalysts [17, 57, 58]. Since triose-phosphate isomerase actually obeys a four-step reversible kinetic mechanism, this finding has to be considered with precaution.

Employing the more detailed mathematical treatment presented in [37], one is faced with the difficulty that the upper limits for the elementary rate constants which enter the theory as normalization factors are not precisely known and, moreover, may vary for different enzymes and for different types of ligands. Recently, experimental data for the acyl-enzyme mechanism of β-lactamases

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \qquad \text{(Scheme 3)}$$

have been presented [58]. Some of these data are listed in Table 2.

Scheme 3 is a special case of the Haldane mechanism shown in Scheme 2 with k_{-2} and k_{-3} being negligibly small ($q \gg 1$). Amongst the ten solutions of the flux maximization problem listed in Table 1, there is only one solution (L_6) for which with $q \gg 1$ the rate constants k_{-2} and k_{-3} tend to zero and the four other constants remain finite, so that Scheme 3 holds. As predicted by this solution L_6, the first-order rate constants for each β-lactamase listed in Table 2 are indeed of the same order of magnitude. For a better comparison with the theory, the rate constants shown in Table 2 are normalized by the upper limits $k_d = 10^8\ M^{-1}\ s^{-1}$ and $k_m = 10^4\ s^{-1}$ representing the largest values observed within the group of β-lactamases. The fact that the absolute values for the three enzymes differ appreciably means that either not every β-lactamase is catalytically perfect [59] or that the upper limits of the rate constants differ for the particular β-lactamases.

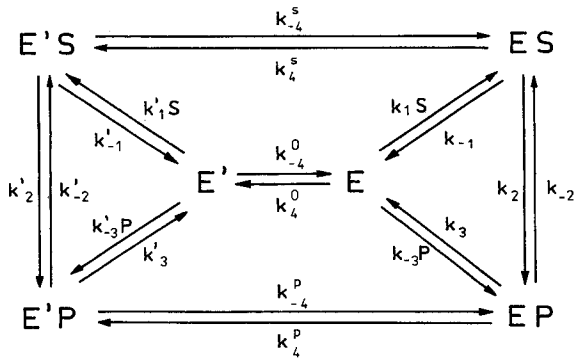

Fig. 5. *General model of kinetic cooperativity for a one-substrate/one-product monomeric enzyme occurring in two basic conformational states E and E'.* The elementary rate constants for the i-th reaction step (i = 1, 2, 3; −1, −2, −3) within the two conformational 'pools' are denoted by k_i and k'_i, the rate constants for the transconformational transitions are k_4^v and $k^v{}_{-4}$ (v = o, s, p) and depend on the ligandation v of the enzyme

Cooperative enzymes

Besides catalytic capacity, other enzyme-kinetic properties related to metabolic regulation were studied as targets of evolutionary optimization. One striking feature of most regulatory enzymes is their cooperative kinetic behaviour. Two alternative fundamental models were proposed for cooperativity of oligomeric enzymes: the all-or-none transition model [60] and the sequential induced-fit model [61]. But also monomeric enzymes with a single active site may display cooperativity provided that they can occur in distinct conformational ground states [62, 63].

In general, the steady-state rate equation for a cooperative enzyme can be written as a rational function

$$v = \frac{\sum_{i=1}^{n} p_i S^i}{1 + \sum_{i=1}^{n} p'_i S^i} \qquad (35)$$

where the coefficients p_i and p'_i are functions of the elementary rate constants for the association, dissociation, catalytic interconversion and conformational transition of the various enzyme complexes. The order n of the two polynomials is determined by the number of subunits or (and) the number of possible conformational ground states.

A straightforward mathematical inspection of the local minima, maxima and saddle points of expression Eqn (35) reveals for $n = 3$ the existence of 26 curve characteristics in the double-reciprocal representation $1/v$ vs $1/S$ [64]. For higher values of n this complicated kinetic behaviour is even more pronounced. On the other hand, such kinetic complexity has only been observed for very few enzymes. Regulatory enzymes of cellular metabolism usually exhibit a rather simple functional behaviour such as positive cooperativity, negative cooperativity or substrate inhibition. Therefore 'functional simplicity' might represent one goal of evolution of enzyme function [65]. Functional simplicity means that the parameters in Eqn (35) attain special values, so that the number of curve characteristics is lowered. This can be achieved either by establishing certain constraints among the elementary rate constants or by dropping some of them to zero values, i.e. by blocking some of the elementary reaction steps [65]. The problem of functional simplicity of cooperative enzymes was stud-

ied for two simple but illustrative models of kinetic cooperativity [65–68]: (a) the monomeric one-site enzyme existing in two conformational ground states and (b) the dimeric enzyme constituted by two interacting identical subunits.

Monomeric enzymes occurring in two conformational ground states. For a monomeric one-site enzyme existing in two conformational ground states the general model of kinetic cooperativity is shown in Fig. 5. A similar model was considered in [66] neglecting the reversible binding of product. There are 18 elementary rate constants, 12 of them (k_i and k'_i for $i = \pm 1, \pm 2, \pm 3$) belonging to the complete catalytic cycle in the pools $E_0 = E + ES + EP$ and $E'_0 = E' + E'S + E'P$ and six of them (k_4^v, k_{-4}^v, v = o,s,p) belonging to the conformational transitions between these pools.

Owing to the thermodynamic equilibrium relation

$$\frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}} = \frac{k'_1 k'_2 k'_3}{k'_{-1} k'_{-2} k'_{-3}} = q \tag{36}$$

and the principle of microscopic reversibility which relates the rate constants within a closed loop to each other,

$$k_4^o k'_1 k_{-4}^s k_{-1} = k_{-4}^o k_1 k_4^s k'_{-1} \tag{37a}$$

$$k_4^p k'_{-2} k_{-4}^s k_2 = k_{-4}^p k_{-2} k_4^s k'_2 \tag{37b}$$

there remain 14 independent parameters. Considering the kinetic scheme in Fig. 5 as that of two interacting Haldane enzymes (cf. Scheme 2) with total concentrations E_0 and E'_0, the steady-state rate equation is given by

$$v = (qS - P)\left(\frac{E_0}{N} + \frac{E'_0}{N'}\right) \tag{38}$$

where the denominators N and N' are given by Eqn (19b) written for the parameter sets $\{k_i\}$ and $\{k'_i\}$ respectively. The relations between the pools E_0 and E'_0 follow from the steady-state condition $dE_0/dt = dE'_0/dt = 0$, i.e.

$$E_0 + E'_0 = E_{tot} \tag{39}$$

and

$$\frac{k_4^o f_E + k_4^s f_{ES} + k_4^p f_{EP}}{k_{-4}^o f_{E'} + k_{-4}^s f_{E'S} + k_{-4}^p f_{E'P}} = \frac{E_0}{E'_0} \tag{40}$$

where the fractions f are defined by

$$f_E = (1/\tilde{N})[k_{-1}k_{-2} + k_{-1}k_3 + k_2 k_3] \tag{41a}$$

$$f_{ES} = (1/\tilde{N})[k_1 (k_{-2} + k_3) S + k_{-2} k_{-3} P] \tag{41b}$$

$$f_{EP} = (1/\tilde{N})[k_1 k_2 S + k_{-3} (k_{-1} + k_2) P]. \tag{41c}$$

The denominator $\tilde{N}$ in Eqn (41) is identical with that in Eqn (14).

Replacing E_0 and E'_0 according to Eqns (39) and (40) in Eqn (38), v can be rewritten in the form of Eqn (35), whereby $n = 2$. Thus, for the mechanism depicted in Fig. 5, kinetic cooperativity can be expected.

In the particular case where the rates of the conformational transitions do not depend upon how the enzyme is liganded,

$$q_c = \frac{k_4^o}{k_{-4}^o} = \frac{k_4^s}{k_{-4}^s} = \frac{k_4^p}{k_{-4}^p} \tag{42}$$

it follows from Eqns (39) and (40) that $E_0 = E_{tot}/(1 + q_c)$ and $E'_0 = E_{tot}\, q_c/(1 + q_c)$ so that the 2:2 rate equation degenerates to a sum of two Michaelis-Menten equations, which does not display kinetic cooperativity.

A detailed derivation and thermodynamic substantiation of Eqn (42) for functional simplicity of a two-state monomeric enzyme based on the concepts of structural kinetics [69] and of generalized microscopic reversibility [70] was outlined in [66].

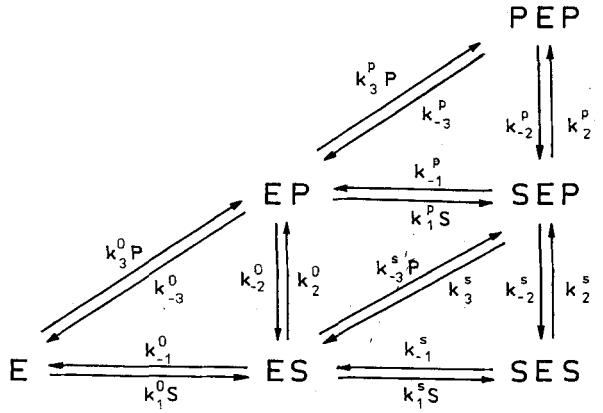

Fig. 6. *General model of kinetic cooperativity for a one-substrate/one-product dimeric enzyme constituted by two identical subunits.* The elementary rate constants k_i^v for the i-th reaction step ($i = \pm 1, \pm 2, \pm 3$) proceeding at one subunit depend on the occupation v (v = o, s, p) of the other subunit

One easily verifies that Eqn (42) and the two constraints of Eqns (37a) and (37b) are consistent with each other if and only if the relation

$$\frac{k_i}{k_{-i}} = \frac{k'_i}{k'_{-i}} \tag{43}$$

is met, which means that the differences $\Delta G_i^{\ddagger} - \Delta G_i'^{\ddagger}$ between the free energies of the various transition states in the catalytic cycle of the two conformers are equal to one another [66]. It was assumed that Eqn (43) holds for the two sets of optimal rate constants where the enzyme has reached catalytic perfection in both conformational states, thus arriving at the conclusion that catalytic perfection of the two conformations and cooperativity of the monomer cannot occur simultaneously [66]. In other words, cooperativity of the monomer can only be achieved at the expense of its catalytic perfection.

Dimeric enzymes constituted by identical subunits. The general scheme of elementary reactions for a dimeric enzyme comprising two identical interacting subunits is shown in Fig. 6. As in the case of the two-state monomer, the reduction of the general 4:4 steady-state rate equation corresponding to this scheme was qualitatively achieved [65]. The activation free energy $\Delta G_{a,i}$ of any elementary reaction step proceeding in a polymeric enzyme is split up into two main contributions:

$$\Delta G_{a,i} = \Delta G'_{a,i} + \Delta G_{a,i}^{(int)}, \tag{44}$$

an intrinsic component $\Delta G'_{a,i}$ being the activation energy for that step of the isolated subunit and an interaction component $\Delta G_{a,i}^{(int)}$. Thus, the rate constant can be written as $k_i = k'_i a_i$ where the interaction coefficient a_i is approximately unity for weak coupling of the subunits or much smaller or much greater than unity in case of tight coupling.

Functional simplicity of oligomeric enzymes rests on two alternative requirements [65, 67, 68].

a) The conformation of a given subunit is the same for all transition states $S^{\ddagger}$, $X^{\ddagger}$ and $P^{\ddagger}$ in the course of the reaction. The subunits are loosely coupled ($k_i^o = k_i^s = k_i^p$) and there is a catalytic balance, i.e. the forward and backward catalytic rates are approximately the same ($k_{+2}^v \approx k_{-2}^v$).

b) The conformation of a given subunit is the same for all transition states. The subunits are tightly coupled, which

means that a conformational change of one subunit is accompanied with one and the same conformational change of all other subunits. The forward catalytic rate constant is much higher than the backward catalytic rate constant. The second requirement implies a blocking of some reactions connected with 'mixed' conformational states.

In contrast to monomers there is no antagonism between catalytic perfection and functional simplicity of oligomeric enzymes [68]. Depending on the values of the interaction coefficients a_i, an increase in catalytic efficiency need not necessarily diminish the cooperativity of the enzyme.

A thorough mathematical justification of the above-mentioned qualitative findings is still lacking. In particular, it is still not known whether the emergence of cooperative enzymes is linked to flux rate optimization or rather to regulatory requirements as, for example, high sensitivity to external signals (cf. below).

Optimization of multienzyme systems

Due to the multitudinous interactions between metabolites and enzymes in metabolism (cf. results of metabolic control analysis [71 – 73]), additional features of enzyme function (e.g. allosteric regulation, allocation of enzyme amounts) occur, which can only be understood by taking into account the organization of enzymes into metabolic networks rather than by considering single enzymes [19, 29, 30, 36]. Nevertheless, the maximization of catalytic efficiency as studied for single enzymes remains relevant also in the context of enzymatic networks.

The temporal behaviour of the reactant concentrations of multienzyme systems is, on the basis of some simplifying assumptions (cf. [74]), usually described by systems of ordinary differential equations

$$\frac{\mathrm{d}S_i}{\mathrm{d}t} = \sum_{j=1}^{r} n_{ij} v_j, \; i = 1,\ldots,n \tag{45}$$

where n_{ij} denote the elements of the stoichiometric matrix.

The performance functions of metabolic systems investigated in the literature are mostly confined to steady states and depend on variables characterizing the metabolic state, e.g. on the concentrations (S_i) or the reaction rates (v_i). Moreover, optimization studies have usually been restricted to special stoichiometries such as unbranched enzymatic chains.

Basic equations for unbranched reaction chains

$$\mathrm{S} \underset{v_1}{\rightleftarrows} \mathrm{S}_1 \underset{v_2}{\rightleftarrows} \mathrm{S}_2 \underset{v_3}{\rightleftarrows} \ldots\ldots \underset{v_n}{\rightleftarrows} \mathrm{S}_n \underset{v_{n+1}}{\rightleftarrows} \mathrm{P}. \qquad \text{(Scheme 4)}$$

For the unbranched enzymatic chain depicted in Scheme 4 which was studied in [22, 26, 71, 75 – 78] the governing system of Eqn (45) simplifies to

$$\frac{\mathrm{d}S_i}{dt} = v_i - v_{i+1}, \; i = 1,\ldots,n. \tag{46}$$

The concentrations of initial substrate and end product are usually assumed to be fixed ($S_0 = S = \text{const.}$, $S_{n+1} = P = \text{const.}$). In steady states, it holds $v_i = J \, (i = 1,\ldots,n+1)$. Using the reversible form of the Michaelis-Menten equation (Eqn 15) for the reaction rates v_i, the following implicit equation may be derived for the steady-state flux [30]

$$P = S \prod_{j=1}^{i} \frac{K_j^- (V_j^+ - J)}{K_j^+ (V_j^- + J)} - J \sum_{l=1}^{i} \frac{K_l^+}{V_l^+ - J} \prod_{j=l}^{i} \frac{K_j^- (V_j^+ - J)}{K_j^+ (V_j^- + J)} \tag{47}$$

which may be rearranged into a polynomial equation for J. An explicit expression for J can only be obtained for linear rate equations

$$v_i = k_i S_{i-1} - k_{-i} S_i = \left(k_i S_{i-1} - \frac{S_i}{q_i} \right) \tag{48}$$

with

$$k_i = \frac{V_i^+}{K_i^+}, k_{-i} = \frac{V_i^-}{K_i^-}, q_i = \frac{k_i}{k_{-i}} \tag{49}$$

which result from Eqn (15) under the condition that the concentrations of all intermediates are much lower than the corresponding Michaelis constants ($S_i \ll K_{i+1}^+, S_i \ll K_i^-$). Substituting Eqn (48) into Eqn (46) one obtains for the steady-state flux

$$J = \frac{S \prod_{j=1}^{n+1} q_j - P}{\sum_{j=1}^{n+1} \frac{1}{k_j} \prod_{m=j}^{n+1} q_j} \tag{50}$$

[72, 75, 76]. In the absence of enzyme-enzyme interactions, the maximal activities and, therefore, also the first-order rate constants are linearly dependent on the enzyme concentrations,

$$k_i = \varkappa_i E_i. \tag{51}$$

Using Eqn (51), Eqn (50) may be reformulated as follows:

$$J = \frac{B}{\sum_{j=1}^{n+1} \frac{1}{c_j E_j}} \tag{52}$$

with

$$c_j = \varkappa_j \prod_{m=j}^{n+1} \frac{1}{q_m}, B = S \prod_{j=1}^{n+1} q_j - P. \tag{53}$$

Using the linear rate laws (Eqn 48), one may derive also an explicit expression for the steady-state concentrations

$$S_k = S \prod_{j=1}^{k} q_j - J \sum_{l=1}^{k} \frac{1}{k_l} \prod_{j=l}^{k} q_j \tag{54}$$

[30, 71, 76] with J given by Eqn (50).

Maximization of steady-state fluxes

Similarly as for single enzymes (cf. above), the optimization principle

$$J = J_{\max} \tag{55}$$

was investigated for unbranched enzymatic chains [22, 29, 30, 75]. The fact that the total amount of enzymes is limited can be expressed by the condition

$$\sum_{j=1}^{n+1} E_j = E_t = \text{const.} \tag{56}$$

The extremum principle of Eqns (55) and (56) was first considered for a three-enzyme system [75]. A more general solution was derived in [22, 29, 30]. Using the method of Lagrange multipliers, one arrives, with the help of Eqn (51), at

$$\frac{\partial J}{\partial k_i} + \mu \frac{\partial}{\partial k_i} \sum_j \frac{k_j}{\varkappa_j} = 0. \tag{57}$$

Using Eqns (50 – 53), one derives from Eqn (57):

$$\frac{E_i}{E_j} = \sqrt{\frac{c_j}{c_i}} = \sqrt{\frac{\varkappa_j}{\varkappa_i} \prod_{k=i}^{j-1} q_k}. \tag{58}$$

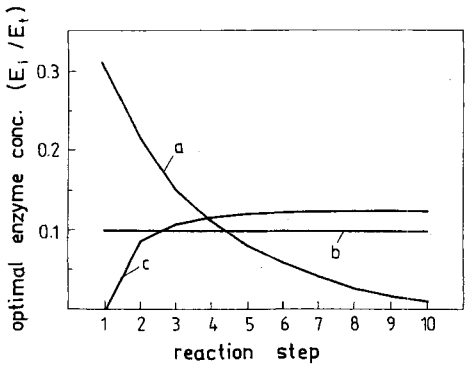

Fig. 7. *Enzyme concentrations in states of maximal steady-state flux for an unbranched chain of* r = *10 enzymatic reactions (cf. [29, 30]).* Curves a and b, without osmotic constraint: (a) $q_i = q = 2$; (b) $q_i = q = 1$. Note that these curves also represent the distribution of the flux control coefficients (cf. Eqn 80). Curve c, with osmotic constraints ($\Omega^\circ/S = 0.998$ and $S = P = 1$). In case (a) most reactions are slow (low time hierarchy) whereas in case (c) there is only one slow reaction (high time hierarchy); for further explanations see text (*Time hierarchy in biochemical networks*)

With Eqn (56), the solution of Eqn (57) may also be written in the form

$$E_i = \frac{E_t \sqrt{\frac{1}{\varkappa_i} \prod_{j=1}^{i-1} \frac{1}{q_j}}}{\sum_{k=1}^{n+1} \sqrt{\frac{1}{\varkappa_k} \prod_{j=1}^{k-1} \frac{1}{q_j}}}. \tag{59}$$

Eqns (58) and (59) predict an inverse relationship between the specific rate constants $\varkappa_i$ and the optimal enzyme concentrations E_i, i.e. in states of maximal steady-state activity, poor catalysts with low specific rate constants should be present in high concentrations. In the special case that all specific rate constants are equal ($\varkappa_i = \varkappa$) Eqns (58) and (59) yield for $q_i > 1$ a monotonic decrease of the enzyme concentrations from the beginning toward the end of the chain (cf. Fig. 7).

It is worthwile noting that the optimization problem of Eqns (55) and (56) is equivalent to the problem of minimizing the total enzyme concentration, $E_t = E_{\min}$, at fixed flux J.

Inserting Eqn (59) into Eqn (52), an expression for the maximal steady-state flux may be derived. For the special case $q_i = 1$ and $\varkappa_i = \varkappa$, where the theory predicts a uniform distribution of the optimal enzyme concentrations, $E_i = E_t/(n+1)$, one obtains with Eqns (50) and (51):

$$J_{\max} = \frac{(S - P)\varkappa E_t}{(n+1)^2}. \tag{60}$$

This formula is in line with the hypothesis [30] that, as a general feature, the optimal flux decreases with increasing chain length (cf. below).

There is a close relation between flux maximization and the maximization of total entropy production. The latter principle, i.e. the establishment of system states far from thermodynamic equilibrium, was suggested to play an important role in the evolution of biochemical systems (see pp. 442–445 of [79]).For the linear chain, the state where the total entropy production,

$$\sigma_{\text{tot}} = \sum_{i=1}^{n+1} \frac{v_i A_i}{T} = JR \ln\left(\prod_{i=1}^{n+1} q_i \frac{S}{P}\right) \tag{61}$$

becomes maximum coincides with the state of maximum flux [30].

Osmotic constraints. In the context of optimization properties of metabolic systems, considerations about the limited solvent capacity and the osmotic balance of living cells play an important role [13, 30, 52, 78, 80]; for a detailed analysis see section entitled *Implications of the solvent capacity and osmotic balance.* In particular, the concentrations of metabolites cannot exceed upper limits [13, 30, 80]. It is easy to see that this osmotic condition is not always fulfilled by Eqn (59). In the optimal state the intermediates accumulate progressively towards the end of the chain if all equilibrium constants are greater than unity (cf. Eqns 54 and 59). The osmotic condition can be included as an equality constraint [30]

$$\Omega = \sum_{i=1}^{n} S_i = \Omega^\circ = \text{const.} \tag{62}$$

where Ω denotes the total osmolarity of the intermediates of the unbranched pathway. Solutions of the optimization problem $J = J_{\max}$ subject to E = const. and Ω = const. can be obtained in closed form only for the limiting case $q_i \to \infty$. One obtains [30]

$$E_1 = \frac{E_t \Omega^\circ}{\Omega^\circ + n^2 S}; \; E_i = \frac{n E_t}{\Omega^\circ + n^2 S} \tag{63}$$

with $i \geq 2$. This equation shows that inclusion of the osmotic constraint (Eqn 62) makes the results more realistic since it impedes the occurrence of zero solutions for E_i. For finite values of the equilibrium constants, numerical solution of the optimization problem shows that the magnitude of Ω° remarkably affects the distribution of enzyme concentrations (cf. Fig. 7). This distribution may even increase along the chain for sufficiently small values of Ω°. Moreover, inclusion of the osmotic constraint (Eqn 62) implies the optimal enzyme distribution to depend not only on the equilibrium constants but also on the concentrations S and P [30].

Branched pathways. Defining optimal states in terms of flux maximization for branched pathways one encounters the problem that the number ϱ of independent steady state fluxes J_i

$$\varrho = r - \text{rank}(\mathbf{N}) \tag{64}$$

is greater than unity [27, 30]. In Eqn (64) r denotes the number of reactions and $\mathbf{N}$ the stoichiometric matrix.

It was proposed that ϱ is related to the number of different biological functions of a metabolic network [27]. One way of modelling flux maximization in such 'multifunctional' systems is by using the performance function [29, 30]

$$\Phi = \prod_i J_i. \tag{65}$$

Eqn (65) meets the requirement that the performance function becomes zero if one of the fluxes vanishes. Based on Eqn (65), optimal states of branched metabolic systems containing three linear segments were calculated under the side condition $E_t = E_{t,1} + E_{t,2} + E_{t,3}$ = const. [30]. The results indicate that in the case where one end product is produced from two alternative substrates most enzyme protein is allocated to that pathway for which the free energy difference of the substrate and the end product is greatest. For the alternative case that two end products are produced from one substrate that branch which is unfavourable, from a thermodynamic point of view, i.e. which exhibits the smaller free energy decrease, will gain a higher total enzyme amount than the other.

Consideration of detailed enzymatic mechanisms. The calculation of kinetic parameters maximizing the steady-state fluxes becomes rather complicated if nonlinear rate equations are used. This calculation can, however, be facilitated by taking into account relations of metabolic control analysis [71–73]. The variations δv_j and δJ of the reaction rate of a single enzyme and the steady-state flux can be related through

$$\frac{\delta J}{J} = \mathbf{C}_j^J \frac{\delta v_j}{v_j} \tag{66}$$

with $\mathbf{C}_j^J$ denoting the flux control coefficient. Under the conditions

$$\frac{\partial v_i}{\partial S_{i-1}} > 0, \frac{\partial v_i}{\partial S_i} < 0 \tag{67}$$

which are always fulfilled for the reversible form of the Michaelis-Menten equation (Eqn 15), all flux control coefficients of unbranched chains are positive so that

$$\operatorname{sign}\left(\frac{\partial J}{\partial k_{i,j}}\right) = \operatorname{sign}\left(\frac{\partial v_j}{\partial k_{i,j}}\right) \tag{68}$$

where $k_{i,j}$ ($i = \pm 1, \pm 2, \pm 3$ and $j = 1,\ldots, n+1$) denote the elementary rate constants. From Eqn (68) it follows that rate constants maximizing the steady-state rate $J(k_{i,1},\ldots, k_{i,n+1}; S, P)$ will also maximize the rates of the individual enzymes $v_j(k_{i,j}; S_{i-1}, S_i)$. In other words, an optimal state of an unbranched enzymatic reaction is characterized by values of the kinetic parameters listed in Table 1 with the only difference that the concentrations S_{i-1} and S_i will replace the concentrations S and P. An iteration procedure for determining all S_i values in optimal states was developed [37]. The optimal kinetic parameters for the various enzymes are generally obtained from different types of solutions L_n listed in Table 1 depending on the location of enzyme within the chain.

Influence of substrate specificity. It was proposed that the evolution of metabolic pathways involved the specialization of a smaller set of enzymes that had a much broader substrate specificity than the enzymes of present-day metabolism [22, 81–83]. Such an ambiguity may be regarded as necessary to make a metabolic system possible despite the limited gene content of primitive cells [82]. Probably, the translation process itself evolved from a less accurate mechanism [84]. The effect of substrate specificity on steady-state fluxes in multienzyme systems was analysed in [22]. The calculations were confined to unbranched enzymatic chains with reactions described by linear rate equations (Eqns 48 and 49). Although a chain of reversible reactions was considered, for the specific rate constants $\varkappa_j$ Eqn (6) derived for irreversible reactions was applied. In this formula the free energy for the formation of the transition state can be subdivided into the activation energy ΔG_a and the binding energy ΔG_b

$$\Delta G_2^{\ddagger} - \Delta G_o = \Delta G_a + \Delta G_b \tag{69}$$

(cf. Fig. 1). Under the assumption that the coupling constant r defined in Eqn (7) equals unity, which corresponds to the hypothesis that mutations affect the binding energies ΔG_b rather than the activation energies ΔG_a, one obtains, with Eqns (6) and (7):

$$\varkappa = \varkappa_o \exp\left(-\frac{\gamma}{RT}\right). \tag{70}$$

Mutations providing negative values of γ improve the complementarity between the enzyme and the substrate (cf. Eqn 7).

The mathematical treatment becomes rather simple if only one multifunctional enzyme exists, say E_i [22]. After appropriate renumbering of the reaction steps, the denominator in Eqn (52) for the steady-state flux may be split into two terms, one for the reactions catalyzed by E_i and another term Z for the remaining reactions:

$$J = \frac{B}{\frac{1}{E_i}\sum_k \frac{1}{c_k} + Z} \tag{71}$$

with B and c_k defined in Eqn (53). The analysis was based on the assumption that a mutation affecting enzyme E_i may increase its complementarity towards the substrate of reaction j only if, at the same time, it diminishes the substrate specificity towards all other substrates. This can be expressed through the relation $\gamma = \gamma_j = -\gamma_k < 0\,(k \neq j)$ [22]. Under consideration of Eqns (52) and (70), one easily derives from Eqn (71) that such a mutation of E_i increases the flux if

$$\exp\left(\frac{\gamma}{RT}\right) > \frac{\sum_{k \neq j} 1/c_k}{1/c_j}. \tag{72}$$

Since $\gamma < 0$, the condition of Eqn (72) can only be fulfilled if

$$\frac{1}{c_j} > \sum_{k \neq j} \frac{1}{c_k}. \tag{73}$$

Thus, an enhancement of the steady-state flux due to mutations of a multifunctional enzyme can only be achieved if the catalytic coefficients are increased of reactions which are much slower than all other reactions catalyzed by the same enzyme. Furthermore, Eqn (72) indicates that the absolute amount $|\gamma|$ of the change of the binding energy must be very small in order to be effective. There may be cases where Eqn (72) cannot be fulfilled for $\gamma < 0$. Under such circumstances, the enzyme is 'trapped' in its configuration and all mutations are of selective disadvantage [22].

Growth rate maximization. According to [22] the growth rate can be expressed as

$$G = \frac{1}{V}\frac{\mathrm{d}V}{\mathrm{d}t} \tag{74}$$

where V denotes the cell volume. The mathematical treatment is based on Scheme 4 and, in addition, takes into account that enzymes are not only the catalysts of the metabolic system but also their net product. Neglecting the synthesis of structural proteins and using the assumption that the cell volume is proportional to the protein content, the growth rate G may be expressed as follows

$$G = G(E_1,\ldots, E_{n+1}) = \frac{J}{\sum_{i=1}^{n+1} E_i}. \tag{75}$$

The effect of changes of enzyme concentrations on the growth rate is described by the following variational equation

$$\frac{\partial G}{\partial E_j} = \frac{1}{E_t}\frac{\partial J}{\partial E_j} - \frac{J}{E_t^2}. \tag{76}$$

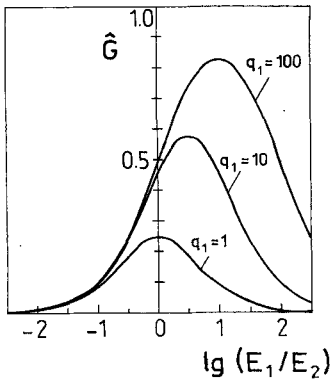

Fig. 8. *Growth rate constant for the model depicted in Scheme 4 as function of the ratio* E_1/E_2 *of enzyme concentrations for different values of the equilibrium constant* q_1 *with* $\varkappa_1 = \varkappa_2 = \varkappa$. $\hat{G}$ denotes the normalized growth rate constant $G/\varkappa S$. With Eqns (78) and (79) one obtains $\hat{G}_{opt} = q_1/(1+\sqrt{q_1})^2$

The optimal distribution of the enzyme concentrations is determined by $\partial G/\partial E_j = 0$, i.e. by

$$\frac{\partial J}{\partial E_j} = \frac{J}{E_t}. \tag{77}$$

Since the condition of Eqn (77) is mathematically equivalent to Eqn (57), the enzyme distribution maximizing the steady-state flux under the constraint of fixed total enzyme concentrations and that maximizing the exponential growth rate G are the same.

In [85, 86] the simple scheme

$$S \underset{v_1}{\rightleftarrows} X \underset{v_2}{\rightarrow} E_1 + E_2 \qquad \text{(Scheme 5)}$$

was considered. With $q_2 \rightarrow \infty$ and $n = 1$ in Eqns (50) and (51), one obtains for the growth rate constant

$$G = \frac{J}{E_1 + E_2} = \frac{Sq_1\varkappa_1\varkappa_2}{\varkappa_1\left(1+\frac{E_1}{E_2}\right) + \varkappa_2 q_1\left(1+\frac{E_2}{E_1}\right)}. \tag{78}$$

The function $G(E_1/E_2)$ is depicted in Fig. 8 for different values of q_1. The maximum value of G is obtained for

$$\frac{E_1}{E_2} = \sqrt{\frac{\varkappa_2}{\varkappa_1}q_1} \tag{79}$$

which is in accordance with Eqn (58).

Distribution of control coefficients. The flux control coefficients (Eqn 66) give a quantitative measure for the relative importance of a reaction for flux limitation in metabolic pathways [71 – 73]. Since they depend on the state of the system, kinetic parameters which optimize a certain performance function give rise to a special distribution of the control coefficients. For unbranched metabolic systems in states of maximal steady-state flux, one obtains from the variational Eqn (57), by using Eqn (66):

$$C_i^J = \frac{k_i/\varkappa_i}{\sum_{j=1}^{r} k_j/\varkappa_j} = \frac{E_i}{E_t} \tag{80}$$

with the optimal enzyme concentrations E_i given by Eqn (59) (cf. [29]). This result indicates that, in the optimal state, flux control coefficients are distributed in the same way as enzyme concentrations. That is to say, when the equilibrium constants are significantly greater than unity, flux control is mainly exerted by the reactions at the beginning of the chain (cf. Fig. 7).

Transient times

A necessary condition for the occurrence of a steady state is its stability. However, this property may not suffice for the maintenance of this state in the presence of permanent non-infinitesimal fluctuations [87]. Thus, besides stability, a rapid relaxation towards the steady state is of importance for the biological function of a metabolic pathway [12, 78, 88]. The speed of a relaxation process towards a stable steady state may be characterized by transient times.

An agreed definition for transient times only exists for isolated reactions obeying first-order kinetics [$\tau = 1/(k_+ + k_-)$]. However, biochemical pathways being multicomponent systems cannot be characterized by only one relaxation time. If small perturbations of a steady state are considered [$S_i(t) = S_i + \sigma_i(t)$] the solutions for $\sigma_i(t)$ may be expressed by the eigenvectors and the eigenvalues (λ_i) of the Jacobian matrix. If $\mathrm{Re}(\lambda_i) < 0$ an appropriate measure for the characterization of relaxation processes are the characteristic times

$$\tau_i = \frac{1}{|\mathrm{Re}(\lambda_i)|} \tag{81}$$

where $\mathrm{Re}(\lambda_i)$ denotes the real part of λ_i.

There are several approaches to define average relaxation times. However, all of these definitions are applicable only under very special conditions.

In [89, 90] a transient time for a linear reaction sequence (corresponding to Scheme 4) was defined:

$$\tau = \frac{1}{J}\sum_{i=1}^{n} S_i = \sum_{i=1}^{n} \tau_i. \tag{82}$$

If all reactions are irreversible and can be described by Michaelis-Menten equations one obtains [90]

$$\tau = \sum_{i=2}^{n+1} \frac{K_i^+}{(V_i^+ - J)}. \tag{83}$$

If $J \ll V_i^+$ the reactions may be described by first-order kinetic equations and Eqn (83) simplifies to

$$\tau = \sum_{i=2}^{n+1} \frac{K_i^+}{V_i^+} = \sum_{i=2}^{n+1} \frac{1}{k_i}. \tag{84}$$

Another definition of an average transient time is

$$\tau_i = \frac{\int_0^\infty t\,\sigma_i(t)\mathrm{d}t}{\int_0^\infty \sigma_i(t)\mathrm{d}t} \tag{85}$$

[91]. This definition is identical with that of a 'mean response time' in [92]. If Eqn (85) is, with $i = n$, applied to an unbranched chain of irreversible reactions one again obtains Eqn (84) if the perturbation pertains to the first intermediate S_1. From Eqn (82) or (85) one may also derive explicit expressions for the transient times of reversible reaction chains

[90, 91]. Furthermore, transient times of relaxation processes after perturbation of the concentration of any metabolites S_α can be calculated. With Eqn (85) one obtains

$$\tau_n^{(\alpha)} = \sum_{j=\alpha+1}^{n} \frac{1}{k_j} \left(\frac{1 + \sum_{i=2}^{j-1} \prod_{l=2}^{i} q_l}{\prod_{l=2}^{j-1} q_l} \right) \tag{86}$$

[78, 91] where it is assumed that the first reaction is irreversible, i.e. $q_1 \to \infty$. The quantity $\tau_n^{(\alpha)}$ may be regarded as the propagation time of a perturbation of the α-th intermediate to the end of the chain.

In [93], the optimization principle

$$\tau = \tau_{\min} \tag{87a}$$

subject to

$$\chi = \sum_{i=2}^{n+1} M_i E_i = \chi^\circ = \text{const.} \tag{87b}$$

(M_i: molecular mass of the i-th enzyme) was studied.

The cost function (Eqn 87b) proposed also in [26] takes into account that the number of ATP molecules necessary for the synthesis of a protein is proportional to the number of peptide bonds. It was assumed that the turnover numbers k_{cat} of the enzymes are all equal, so that $V_i^+/V_j^+ = E_i/E_j$ [26]. Furthermore, the simple expression of Eqn (84) for the transient time was used. For determining the optimal parameter distribution one has to solve the variational equation

$$\frac{\partial}{\partial V_i} \left\{ \sum_{j=2}^{n+1} \frac{K_j^+}{V_j^+} + \mu \left[\sum_{j=2}^{n+1} \left(\frac{1}{k_{\text{cat}}} M_j V_j^+ - \chi^\circ \right) \right] \right\} = 0. \tag{88}$$

From that it follows

$$\frac{V_i^+}{V_j^+} = \sqrt{\frac{K_i^+ M_j}{K_j^+ M_i}}. \tag{89}$$

Taking into account the accessory condition $\chi = \chi^\circ$, one obtains from Eqns (88) and (89):

$$V_i^+ = \frac{k_{\text{cat}} \chi^\circ \sqrt{K_i^+/M_i}}{\sum_{j=2}^{n+1} \sqrt{K_j^+ M_j}} \tag{90}$$

Besides the optimization principle (Eqn 87), the principle $\chi = \chi_{\min}$ with $\tau = \tau^\circ = \text{const.}$ was also considered [26]. The solution of the latter problem again leads to Eqn (89) while instead of Eqn (90) the following formula is obtained:

$$V_i^+ = \frac{1}{\tau^\circ} \sqrt{\frac{K_i^+}{M_i}} \sum_{j=2}^{n+1} \sqrt{K_j^+ M_j}. \tag{91}$$

This formula was first derived in [93] where the optimization of coupled enzyme assays was considered.

States of minimal transient times of unbranched reaction chains were calculated in [78] for the more general case of reversible reactions. In particular, the optimality principle

$$\tau_n^{(\alpha)} = \tau_{n(\min)}^{(\alpha)} \tag{92}$$

(cf. Eqn 86) was considered under two different types of constraints: (a) $J = J^\circ = \text{const.}$ and (b) $J = J^\circ = \text{const.}$, $\Omega = \Omega^\circ = \text{const.}$ Since it was assumed that $q_1 \to \infty$, the steady-state flux reads $J = k_1 S$ instead of Eqn (50). In case (a), one obtains the solution $k_1 = S/J^\circ$, $k_i \to \infty$ for $i > \alpha$ and arbitrary rate constants for the reactions with $1 < j \leq \alpha$. In case (b), the solution is characterized by two slow and $n-1$ very fast reactions. Also in this case, the first reaction is slow since k_1 is fixed by the condition $J = J^\circ$. The location of the other slow reaction depends on the value of α [78].

An alternative approach to comprehend relaxation processes quantitatively is the consideration of the longest characteristic time. For the reaction sequence depicted in Scheme 4 the Jacobian **M** with the elements m_{ij} has a tridiagonal form [78]

$$m_{ij} = -(k_{-i} + k_{i+1})\delta_{ij} + k_{-j}\delta_{i,j+1} + k_{-j}\delta_{i+1,j} \tag{93}$$

for $i,j = 1,\ldots, n$, with δ_{ij} being the Kronecker symbol. There are no analytical expressions for the eigenvalues of Eqn (93) but it is known that they are real and negative (cf. [94]). After a sufficiently long time, only the eigenvector belonging to the eigenvalue with the smallest absolute value (denoted, say, by λ_n) determines the relaxation process. The requirement that the steady state must be attained as fast as possible after any perturbation can be formulated as follows

$$|\lambda_n| = |\lambda_n|_{\max}. \tag{94}$$

Obviously, this is equivalent to the minimization of the largest characteristic time $\tau_n = 1/|\lambda_n|$. Principle (94), subject to $J = J^\circ = \text{const.}$ and $\Omega = \Omega^\circ = \text{const.}$, leads in the case of linear reaction sequences to optimal states with a distinct separation of time constants where two adjacent reactions are slow and the others are very fast [78, 95].

Sensitivity to external signals

Definition of sensitivity measures. The sensitivity of a biochemical system is defined as the change of a given state function Y (e.g. activity of a particular enzyme, steady-state flux etc.) caused by a variation of a given environmental signal Z (e.g. external substrate, hormone concentration etc.). If infinitesimal changes are considered, this can be expressed by the unscaled response function

$$R = \frac{\mathrm{d}Y}{\mathrm{d}Z} \tag{95}$$

introduced in [96] as 'control strength'.

Besides Eqn (95), the scaled (dimensionless) 'response coefficient' [71, 97]

$$\hat{R} = \frac{\mathrm{d}\ln Y}{\mathrm{d}\ln Z} = \frac{Z}{Y} R \tag{96}$$

has been used as sensitivity measure by many authors, mainly in the field of metabolic control analysis. This coefficient can be calculated by summing up the products of the control coefficients with respect to those enzymes which are affected by the signal Z and the corresponding elasticity coefficients with respect to Z.

In contrast to the quantities defined by Eqns (95) and (96), the 'amplification factor'

$$A_z = \frac{\Delta Y/Y}{\Delta Z/Z} = \frac{(Y_\mathrm{f} - Y_\mathrm{i})/Y_\mathrm{i}}{(Z_\mathrm{f} - Z_\mathrm{i})/Z_\mathrm{i}} \tag{97}$$

[98] takes into account a finite change of the signal and of the response from an initial level (i) to a final level (f).

Sensitivity of single enzymes. For most enzymes the dependence of the steady-state rate v upon the concentration S of a

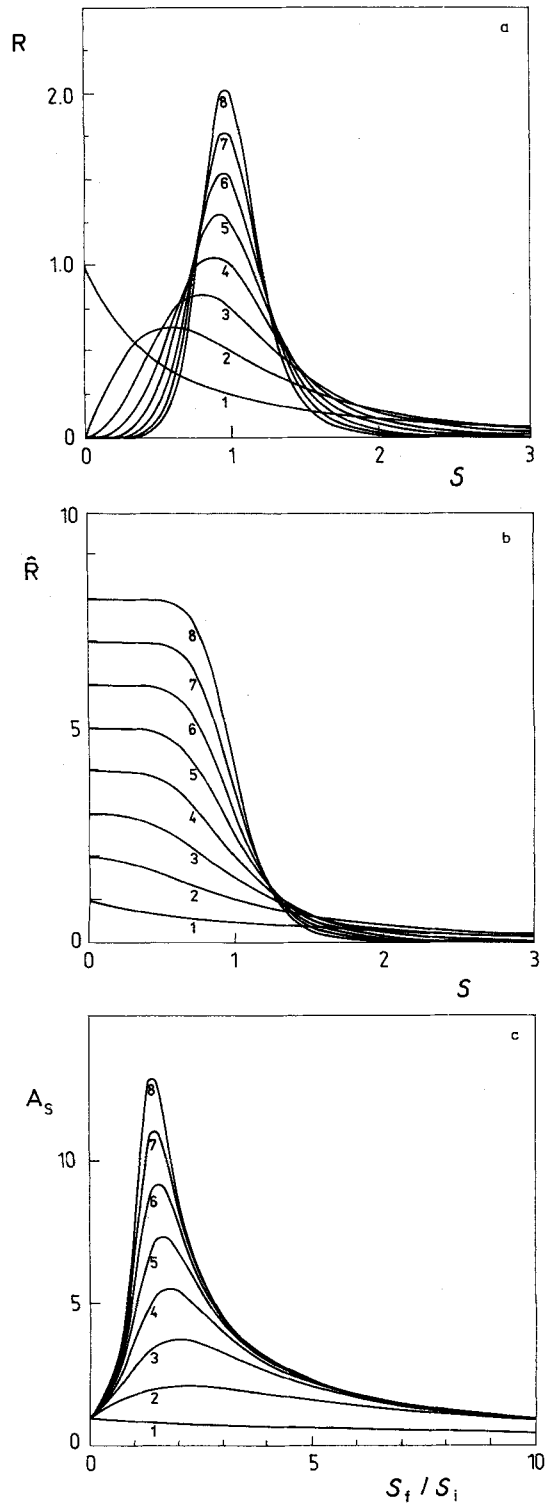

Fig. 9. *Dependence of the sensitivity measures (Eqns 95–97) on the concentrations* S *(a, b) and the ratio* S_f/S_i *(c) of the signal for an enzyme obeying the Hill equation.* Parameters: $S_{0.5} = 1$, $n_H = 1,\ldots,8$, $S_i = S_{0.5}(1/9)^{1/n_H}$, i.e. $Y_i = v_i/V_m = 0.1$

ligand (keeping the concentration of all the other ligands constant) can be described by the Hill equation

$$v = \frac{V_{max}}{1 + \left(\frac{S_{0.5}}{S}\right)^{n_H}} \tag{98}$$

where the half-saturation constant $S_{0.5}$ and the coefficient n_H are phenomenological parameters.

Defining the state function to be varied as relative flux rate, i.e. $Y = v/V_{max}$, the sensitivity measures (Eqns 95–97) read

$$R = \frac{n(S_{0.5}/S)^{n+1}}{S_{0.5}\left(1 + (S_{0.5}/S)^n\right)^2} \tag{99}$$

$$\hat{R} = \frac{n}{1 + (S/S_{0.5})^n} \tag{100}$$

$$A_s = \frac{(S_f/S_i)^n - 1}{(S_f/S_i - 1)[1 + (S_f/S_{0.5})^n]}. \tag{101}$$

As shown in Fig. 9a–c, the three sensitivity measures differently depend on the concentration of the signal. For given kinetic parameters $S_{0.5}$ and $n_H > 1$, the measures of Eqns (95) and (97) attain highest sensitivity at non-zero finite values S and S_f/S_i, respectively, whereas the response coefficient (Eqn 100) is monotonically decreasing with a small slope within the region $0 < S < S_{0.5}$ and maximum sensitivity at $S = 0$. The maximum of the response function (Eqn 95) is attained at $S = S_{0.5}[(n_H-1)/(n_H+1)]^{1/n_H}$. For the optimal value of the ratio S_f/S_i yielding the maximum amplification factor (Eqn 101), no analytical expression can be derived but numerical values can be found [98].

The response functions (Eqns 95–97) have in common that they monotonically ascend with increasing values of n_H. In other words, the higher the sigmoidicity of the enzyme, the higher its sensitivity. Formally, the maximum response for given values of the signal would be aquired at $n_H \to \infty$ where the rate curve degenerates to a step function. Obviously the degree of sigmoidicity may not exceed an upper critical value due to constraints pertaining to the number of interacting subunits and free energies of conformational changes in oligomers. Nervertheless, the parallel increase of sensitivity and sigmoidicity might be one reason for the observation that most regulatory key enzymes exhibit cooperativity.

Maximization of the unscaled sensitivity (Eqn 95) yields $S_{0.5} = S$, i.e. the predicted half-saturation constant should be comparable with the given substrate concentration. Maximization of the scaled response function (Eqn 96) and of the amplification factor (Eqn 97) yields $S_{0.5} \to \infty$, which implies that $S_{0.5}$ should increase as much as possible.

It is noteworthy that the result of the optimization problem in question strongly depends on the choice of the sensitivity measure.

Ultrasensitivity of covalent modification systems. A kinetic threshold behaviour, i.e. rate curves exhibiting super-linear parts as those of allosteric enzymes, may also originate from the dynamics of interconvertible enzyme systems [99, 100]. This was demonstrated for the monocyclic system of covalent modification shown in Scheme 6 and also for more complicated schemes taking into account the organization of interconvertible enzymes in cascades [101] and the various modes of action of effectors [100, 101]

$$W \underset{E_2(V_2,K_2)}{\overset{E_1(V_1,K_1)}{\rightleftharpoons}} W^*. \tag{Scheme 6}$$

For the simple two-component system in Scheme 6 where a protein W is reversibly modified by two Michaelian converter enzymes E_1 and E_2 the expressions for the steady-state fraction of modified protein W* has been derived for the first-order region of the converter enzymes [99] and for saturation

kinetics in [100]. In the latter case, one obtains an implicit expression for the concentration of the modified protein W*

$$\frac{V_1}{V_2} = \frac{W^*(W_o - W^* + K_1)}{(W_o - W^*)(W^* + K_2)} \tag{102}$$

with W_o being the total amount of protein. A plot of W^* as function of the ratio V_1/V_2 reveals a sigmoidal curve with the inflection point at $V_1/V_2 = 1$ [100, 102]. For large values of K_1 and K_2 (first-order kinetics of the converter enzymes) the slope of the curve is rather gentle whereas in the zero-order region (K_1, $K_2 \ll W_o$) the transition from W to W* proceeds very abruptly. Therefore, the name 'zero-order ultrasensitivity' was given to this phenomenon. For vanishingly small values of K_1 and K_2 the transition curve becomes a step function. The sensitivity of the above system has been analysed using the amplification factor (Eqn 97) [98]. The amplification factor plotted as a function of $(V_{1f}/V_{2f})/(V_{1i}/V_{2i})$ passes through a maximum. Thus, if the initial activities V_{1i} and V_{2i} of the converter enzymes are different from zero, then the highest amplification possible is obtained at finite and non-vanishing final values V_{1f} and V_{2f}.

The transition of the maximum activities of the two converter enzymes from their initial values to the final ones is usually achieved by effectors which may (a) activate the converter enzyme responsible for the transition into the active form, (b) inhibit the complementary converter enzyme, or (c) exert dual control by combination of mechanisms (a) and (b). The most efficient way of control is the latter [101, 103] which was termed 'multi-step ultrasensitivity' to stress the fact that amplification originates from multiple control exerted by one effector.

Implications of the solvent capacity and osmotic balance

Since most molecules in the cell, metabolites as well as proteins, contain polar groups or are even charged, they fix cellular water by hydration. Having in view the huge number of different substances in the cell, it was argued that the total solute concentration should be small enough in order to make diffusion processes possible and to impede osmotic swelling and disruption of cells [13, 78, 80 (ch. 10)].

A simple way of formalizing the evolutionary constraint caused by the limited solvent capacity is by using the solvate theory of Poynting and Callendar (outlined on p. 801 of [104]). In this theory, one assumes that each molecule of a solute S_i is permanently attached to v_i molecules of solvent. In order that the solvent capacity is not depleted, the following inequality must be fulfilled:

$$\sum_i v_i S_i \leq S_w \tag{103}$$

with S_w denoting the total concentration of water.

For all cells which have no cell wall, an additional constraint for concentrations results from the fact that these cells must be in osmotic equilibrium with the extracellular medium. This condition can be put as [1, 2, 105]

$$\pi_{in} = RT\sum_i g_i S_i = \pi_{ex}, \tag{104}$$

where π_{in} and π_{ex} denote the intracellular and extracellular osmotic pressure, respectively, and g_i is the osmolarity coefficient of solute S_i.

A possible way to simplify mathematical calculations is to put all g_i equal to unity (which is exact only for ideal solutions).

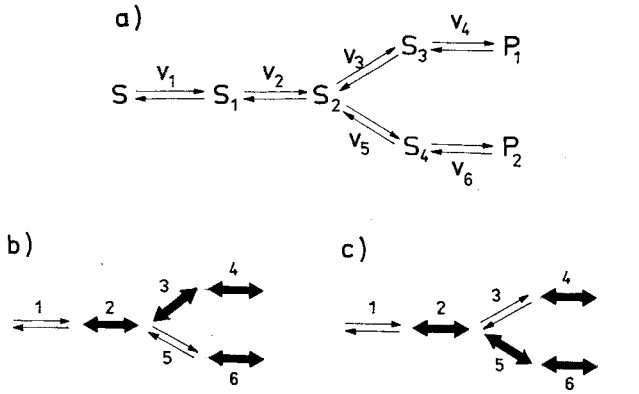

Fig. 10. *Solution to the problem of minimizing total osmolarity for a branched pathway (a)*. The result depends on whether $P_1(q_3q_4) > P_2/(q_5q_6)$ (b) or $P_1(q_3q_4) < P_2/(q_5q_6)$ (c). The thick arrows correspond to quasi-equilibrium reactions

Considering a given metabolic pathway, we can then write Eqn (104) as

$$\pi_{in} = RT\sum_{i=1}^{n} S_i + \pi'_{in} = \pi_{ex} \tag{105}$$

where the sum is taken over all metabolites involved in the pathway under consideration and π'_{in} denotes the contribution of all other intracellular compounds to the osmotic pressure [1, 30, 105]. Assuming π'_{in} to be constant, one obtains the constraint (Eqn 62) used above in the maximization of the steady-state flux and the minimization of transient times.

Several papers have dealt with the hypothesis that evolutionary pressure was directed towards minimization of the steady-state concentrations of all the metabolites which serve only as reaction intermediates [13, 52, 54 (ch.4), 78, 80 (ch.10), 106]. This leads to the minimization principle

$$\Omega = \sum_{i=1}^{n} g_i S_i = \Omega_{min} \tag{106}$$

where the sum does not include 'biologically important' substances whose levels must not be smaller than certain lower limits (e.g. storage metabolites). There is an interrelation between the principle of Eqn (106) and the minimization of transient times considered in the preceding section (cf. Eqn 82).

The extremum problem (Eqn 106) under the subsidiary condition of fixed steady-state fluxes was solved for various reaction systems [78, 106, 107].

For unbranched reaction chains as represented in Scheme 4 the inequalities

$$S_{i-1} \geq S_i/q_i, i = 1,\ldots,n+1 \tag{107}$$

have to be fulfilled in order that the steady-state flux, J, is positive (the case of negative fluxes can be treated similarly by renumbering of the metabolites). Equality in Eqn (107) applies if, and only if, reaction i is in quasi-equilibrium. From that it can readily be concluded that all S_i are simultaneously minimized if all reactions but the first are near equilibrium. In this state, also total osmolarity as given in Eqn (106) is minimized because this quantity is a monotonically increasing function of all concentrations S_i. This result is in agreement with the frequently observed feature that the first step of a pathway is a non-equilibrium reaction [90, 108, 109].

For the branched pathway depicted in Fig. 10a there are two different solutions depending on whether $P_1/(q_3q_4)$ is

greater or less than $P_2/(q_5q_6)$ [106]. In the former case, reactions 2, 3, 4, and 6 attain quasi-equilibrium in the optimal state (shown in Fig. 10b), while in the latter case this applies to reactions 2, 4, 5, and 6 (Fig. 10c).

The above theoretical treatment was also applied to systems of more complex stoichiometry including a model of glycolysis, the pentose monophosphate shunt and the glutathione system in human erythrocytes [106, 107]. The general conclusion is that minimization of total osmolarity always leads to a distinct decomposition of the network into near-equilibrium and non-equilibrium reactions.

The optimality principle (Eqn 106) was also applied to models including the enzymes as osmotically active substances by taking into account the elementary reactions. One obtains the result that all enzyme concentrations $E_{i,0}$ should be as small as possible whereas the rate constants should become as large as possible [107]. This is in qualitative accordance with properties of biochemical networks in that enzyme concentrations *in vivo* are generally very low in comparison with metabolite concentrations [110].

Metabolic channelling

There is now ample evidence for the existence of channelling mechanisms based on functional heterologous enzyme–enzyme interactions [52, 54, 111–116]. The assembly of enzymes can be transient (reversible, dynamic) or permanent (irreversible, static). Aggregations of globular proteins in the cytosol and mitochondria [52, 113, 116] and multienzyme complexes (metabolons) such as fatty-acid synthase [112], respectively, are well-known examples of these two types.

In static complexes the intermediates are directly transferred between enzyme sites, as illustrated in the following Scheme 7

$$\begin{aligned} S_1 + E_1E_2 \ldots E_m &\rightarrow \overset{S_1}{\underset{|}{E_1}}E_2 \ldots E_m \rightarrow E_1\overset{S_2}{\underset{|}{E_2}}\ldots E_m \rightarrow \ldots \\ &\rightarrow E_1E_2 \ldots \overset{S_m}{\underset{|}{E_m}} \rightarrow E_1E_2 \ldots E_m + P. \end{aligned}$$

(Scheme 7)

The polypeptide chains E_i may be linked covalently (multifunctional proteins) or non-covalently (multienzyme aggregates).

The possible functioning of dynamic complexes can be visualized as in Scheme 8 which is similar to that in [52]. It takes into consideration the fact that the reactions catalyzed by the associable enzymes are, in general, sequential in a particular metabolic pathway.

$$\begin{aligned} &S_1 + E_1 + E_2 + \ldots E_m \rightarrow E_1S_1 + E_2 + \ldots + E_m \\ &\rightarrow E_1S_2E_2 + E_3 + \ldots + E_m \rightarrow E_1 + E_2S_2 + \ldots + E_m \\ &\rightarrow E_1 + E_2S_3E_3 + \ldots + E_m \rightarrow \ldots \rightarrow E_1 + E_2 + \ldots E_m + P. \end{aligned}$$

(Scheme 8)

Enzymes operating in the 'hand-over' manner depicted in Scheme 8 have been called 'diazymes' [116].

In a broader sense, the term metabolic channeling is also used when multienzyme complexes or enzyme arrays attached to membranes or the cytoskeleton constitute a microenvironment which reduces diffusion lengths (microcompartmentation) [54 (ch.7), 117]. It is likely that most metabolic channels are leaky in that intermediates are allowed to escape into the aqueous medium [52, 54 (ch.4), 118].

The following selective advantages of metabolic channeling have been discussed in the literature. Most of these aspects have been derived from experimental findings and qualitative arguments. Mathematical simulations in this relatively new field are still at the very beginning [115, 116, 118–121].

a) Stabilization and preservation of intermediates. It is likely that by sequestration onto enzyme complexes, unstable intermediates (for example, 1,3-bisphosphoglycerate and phospho*enol*pyruvate) are stabilized against covalent reaction with water or other aqueous components [52, 111]. Furthermore, toxic metabolites are segregated [54 (ch.4)]. For intermediates which might become lost by diffusion, channelling can obviously provide a means of limiting this loss [111].

b) Flux enhancement. It was argued that channelling mechanisms enhance the flux through biochemical pathways by reducing the transit time, i.e. the time required for a metabolite to reach the next enzyme [52, 54 (ch.4), 113]. The main advantage is likely to be the fact that the time for solvation and desolvation of the substrate, product and active-site cleft of the enzyme is saved in each channelled reaction [52]. While flux enhancement is plausible for direct transfer mechanisms in multienzyme complexes, it is questionable for the diazyme mechanism since diffusion of enzyme-substrate complexes (E_iS_i in Scheme 8) should be much slower than diffusion of free intermediates. Another objection against the potential flux advantage was formulated on the basis of the concept of the 'flux-generating step' according to which the flux is always determined by the input to the pathway [118]. However, metabolic control analysis has shown [122] that this is only true if the input reaction is completely irreversible.

c) Stereospecific transfer. Direct transfer can preserve metabolites in specific steric conformations which are favourable for rapid binding to the subsequent enzyme [52].

d) Shift of equilibria. By direct transfer mechanisms, the equilibrium constants of individual reactions can be changed considerably, since the free energy change for biochemical reactions is determined to a large extent by the free energy of solvation. It has been found experimentally that the equilibrium constants for reactions of enzyme-bound substrates are mostly close to unity [52], which is in accordance with the theoretical prediction that an internal equilibrium constant of about unity is advantageous for efficient catalysis [17] (for critical discussion see section above on *Reversible three-step kinetic mechanism*).

e) Spatial organization. An obvious functional consequence of microcompartmentation, as well as of multienzyme complexes, is the orientation of reactions in space [52, 114, 118]. 'Metabolons' can couple metabolic reactions with the transport across membranes [114]. Theoretical considerations about the possible occurrence of autowaves in multienzyme complexes [123] are not yet solidified by experimental findings.

f) Maintenance of low intermediate concentrations. It was sometimes argued that a major benefit of channelling is the preservation of the limited solvent capacity of the cell by sequestration of metabolites into the active-site clefts of enzymes [52, 54 (ch.4), 118]. Based on an exemplifying reaction system similar to Scheme 9, it has recently been shown that this hypothesis is incorrect as long as the unchannelled pathway (branch F) is not completely blocked [121].

$$\begin{aligned} &\overset{\mathbf{F}}{\overbrace{\phantom{S \overset{v_0}{\rightarrow} S_1 \overset{v_1}{\rightleftarrows} S_2 \overset{v_2}{\rightleftarrows} \ldots \overset{v_{n-2}}{\rightleftarrows} S_{n-1} \overset{v_{n-1}}{\rightleftarrows} S_n \overset{v_n}{\rightleftarrows} P}}} \\ &S \overset{v_0}{\rightarrow} \underset{\underbrace{\phantom{S_1 \overset{v_1}{\rightleftarrows} S_2 \overset{v_2}{\rightleftarrows} \ldots \overset{v_{n-2}}{\rightleftarrows} S_{n-1} \overset{v_{n-1}}{\rightleftarrows} S_n}}_{v_c \qquad \mathbf{C}}}{S_1} \overset{v_1}{\rightleftarrows} S_2 \overset{v_2}{\rightleftarrows} \ldots \overset{v_{n-2}}{\rightleftarrows} S_{n-1} \overset{v_{n-1}}{\rightleftarrows} S_n \overset{v_n}{\rightleftarrows} P \end{aligned}$$

(Scheme 9)

g) Reduction of transient times. A further advantage of channelling, in particular of multienzyme complexes, seems to be the reduction of transient times [54, 113, 115, 118, 120]. This potential advantage is closely related to the preceding one since the transient time is a monotonically increasing function of steady-state concentrations (cf. Eqn 82). So this hypothesis should be scrutinized in the light of the above reasoning about equilibration of intermediates with the aqueous medium. Applying the definition of the transient time (Eqn 82) to reaction Scheme 9 one obtains

$$\tau = \frac{\Sigma S_i + S_c}{v_0}. \tag{108}$$

where S_c is the total concentration of the channel intermediates. Thus, τ can only be less than in the case without channelling if the sum of free intermediate concentrations is likewise diminished.

h) Coordinate regulation. Metabolic channelling offers multitudinous facilities for coordinate metabolic regulation [52, 54, 113, 115, 118, 123]. For example, ligandation at one enzyme site can affect the conformationally dependent catalysis at a complementary enzyme site. Such mechanisms can prevent an inhibition of an enzyme causing accumulation of substrates formed in preceding reactions. Furthermore, alternative aggregation of enzymes located at branching points is probably of special importance for an efficient switching from one path to another [54 (ch.4], 113].

Owing to the high mass percentage of proteins in living cells and the multitude of attractive forces between proteins, enzyme–enzyme interactions are certainly inevitable *in vivo*. So it may be supposed that from a multitude of unspecific interactions occurring for physico-chemical reasons, various specific interactions between enzymes catalyzing sequential reactions have amplified during evolution.

Thermodynamic principles

General conclusions concerning possible evolutionary optimization strategies were obtained by applying the formalism of nonequilibrium thermodynamics [79, 124] to complex biochemical systems and studying their efficiency in terms of efficiency functions which are linked to the overall input/output behaviour [28, 125–132]. One striking benefit of the thermodynamic approach is that no detailed knowledge of the underlying kinetic mechanisms is necessary.

One of the fundamental principles dictated by the second law of thermodynamics can be formulated as

$$\sigma = \sum_{i=1}^{m} J_i X_i \geq 0 \tag{109}$$

which requires the entropy production σ of an arbitrary system comprising m macroscopic processes to be nonnegative with equality only applying to equilibrium [79, 124]. In Eqn (109), J_i and X_i denote the generalized net flow and force, respectively, of the i-th process.

In the linear range of nonequilibrium thermodynamics it holds

$$J_i = \sum_{i=1}^{m} L_{ij} X_j \tag{110}$$

with time-independent phenomenological coefficients L_{ij} which obey the Onsager symmetry relations (cf. [79])

$$L_{ij} = L_{ji}. \tag{111}$$

The range of applicability of the linear regime (Eqn 110) of nonequilibrium thermodynamics to biochemical reaction systems has been under heavy controversial debate [127].

Based on nonequilibrium thermodynamics a quantitative description of linear and nonlinear energy converters was elaborated [125] and extended to study, for example, the efficiency of oxidative phosphorylation [28] and of microbial growth [128].

For the phenomenological description of mitochondrial energy transduction, a system of three linear flux/force relations was proposed [28]

$$J_1 = L_{11} X_1 + L_{12} X_2 \tag{112a}$$

$$J_2 = L_{21} X_1 + L_{22} X_2 \tag{112b}$$

$$J_3 = L_{31} X_1 \tag{112c}$$

It encompasses the net flows of ATP production (J_1), oxygen consumption (J_2) and ATP utilization (J_3) and two forces X_1 and X_2, the phosphorylation potential and the redox potential, respectively. The overall coupling between the energy-yielding reaction (oxidation of reducing equivalents in the respiratory chain) and ATP synthesis catalysed by the F_o F_1-ATPase is encapsulated in the phenomenological cross-coupling coefficient $L_{12} = L_{21}$.

The two cardinal terms entering the thermodynamic theory of linear energy converters are the 'degree of coupling'

$$q = L_{12}/\sqrt{L_{11} L_{22}} \tag{113}$$

characterizing the strength of coupling between the driving and the driven processes and the efficiency

$$\eta = -\frac{\text{output power}}{\text{input power}} = -\frac{J_1 X_1}{J_2 X_2}. \tag{114}$$

Definitions of the thermodynamic efficiency other than Eqn (114) and their benefits and drawbacks were considered in [126, 129]. Analysing the general properties of the efficiency (Eqn 114) for linear and nonlinear energy converters, it was suggested [130] that evolutionary pressure had selected linear energy converters since the maximum of the efficiency usually occurs in the linear flux/force region.

Introducing the normalized force ratio $x = X_1\sqrt{L_{11}}/X_2\sqrt{L_{22}}$ the efficiency (Eqn 114) can be written as

$$\eta = -\frac{x + q}{q + (1/x)}. \tag{115}$$

The optimal force ratio at which the efficiency (Eqn 115) attains its maximum for a given degree of coupling q is given by

$$x_{opt} = -\frac{q}{1 + \sqrt{1 - q^2}}. \tag{116}$$

The corresponding value of the optimal efficiency is

$$\eta_{opt} = x_{opt}^2 = \frac{q^2}{(1 + \sqrt{1 - q^2})^2} \tag{117}$$

Thus, with respect to maximization of thermodynamic efficiency, perfectly tight coupling, i.e. $q = 1$, would be the most favourable situation for mitochondrial energy transduction. In this situation, however, the net rate of ATP synthesis is zero. To make this point more transparent, Eqn (112a) can be rewritten as

$$J_1 = (q + x)\sqrt{L_{11} + L_{22}} L_{22} X_2. \tag{118}$$

Table 3. *Optimal strategy of the 'combinatorial game' proposed in [133] for the explanation of the stoichiometry of the nonoxidative phase of the pentose phosphate pathway (F-type)*
For the rules of the 'game', see section on *optimal stoichiometries*. TK = transketolase; TA = transaldolase; Ald = fructose-1,6-bisphosphate aldolase

Step	Boxes						Step
	1	2	3	4	5	6	
	5	5	5	5	5	5	
1A		TK					
	3	7	5	5	5	5	
2A		TA					
	6	4	5	5	5	5	
3A			TK				
	6	6	3	5	5	5	
					TK		1B
	6	6	3	5	7	3	
					TA		2B
	6	6	3	5	4	6	
				TK			3B
	6	6	3	3	6	6	
4			Ald				4
	6	6	0	6	6	6	

At optimal force ratio x_{opt} given by Eqn (116) the net rate of ATP synthesis reads

$$J_1 = \frac{q\sqrt{1-q^2}}{1+\sqrt{1-q^2}}\sqrt{L_{11}+L_{22}}\,L_{22}\,X_2. \qquad (119)$$

Expression (119) becomes zero as q tends to unity, i.e. the state of maximal efficiency is accompanied with vanishing flux rates. On the other hand, J_1 given by Eqn (119) becomes maximum at a degree of coupling $q = 0.786$. Hence, the rate of ATP synthesis and the thermodynamic efficiency cannot be maximized simultaneously with respect to q. In [28, 132] this conflict has been resolved by determining optimal values for the parameters x and q in such a manner that a function $F(x,q)$ is maximized under the constraint that the thermodynamic efficiency possesses a local maximum with respect to the parameter x, i.e.

$$F(x,q) + \mu\frac{\partial\eta(x,q)}{\partial x} = F_{max}. \qquad (120)$$

Choosing the function F as (a) the net rate of ATP synthesis (J_1), (b) the output power of oxidative phosphorylation (J_1X_1), (c) the net rate of ATP synthesis weighted by the energy costs ($J_1\eta$), or (d) the output power weighted by the energy costs ($J_1X_1\eta$), optimal degress of coupling $q = 0.786$, $q = 0.91$, $q = 0.953$, and $q = 0.972$, respectively, were obtained. The two latter values are in good agreement with the degree of coupling reported for liver mitochondria in starved and fed rats, respectively (cf. [132]).

Applying the concept of 'mosaic nonequilibrium thermodynamics' [131] to the energetics of bacterial growth [128] a similar treatment led to the conclusion that the relatively low efficiency of the ATP production observed in certain growth regimes of bacteria can be rationalized with optimization of these systems towards maximum growth rate.

Optimal stoichiometries

In the section entitled *Maximization of steady-state fluxes*, it was shown that, at limiting concentrations of enzymes, the optimal flux through an unbranched chain of reactions will decrease with increasing number of intermediate products (cf. Eqn 60). One may ask whether the reduction of the number of reaction steps in the transformation of an initial substrate S into an end product P may be considered as a general principle of evolutionary optimization. This problem has been analyzed in more detail for the nonoxidative phase of the pentose phosphate pathway [133]. The question was raised whether Nature manages the conversion of pentoses into hexoses in a minimum number of reaction steps. Several constraints were considered in the mathematical analysis of this problem including: (a) only moieties with two or three carbons can be transferred from any sugar to another by the enzymes transketolase and transaldolase and (b) all intermediates must contain at least three carbon atoms.

For the solution of this problem a 'game of combinatorial optimization' obeying the following rules was proposed [133]. (a) At the beginning six boxes are filled with five balls each; (b) each step involves the transfer of only two or three balls from one box into another; (c) any box which is not empty cannot contain less than three balls; (d) the goal is to fill five boxes with six balls each by a minimum number of steps. The optimal strategy for this game is shown in Table 3. Identifying steps 1A, 1B, 3A, 3B with the reactions of transketolase, step 2A and 2B with the reaction of transaldolase and step 4 with fructose-1,6-bisphosphate aldolase, it is seen that the solution given in Table 3 is exactly the same as the sequence of reactions taking place in the nonoxidative phase of the pentose phosphate pathway (so-called 'F-type' of this pathway [134, 135]). This analysis was extended [136, 137] to the 'L-type' of the pentose phosphate pathway [138, 139] as well as to the nonreductive phase of the Calvin cycle of photosynthesis.

The theoretical investigation of optimal stoichiometries of metabolic pathways is still at the very beginning. Probably, the problem may be tackled in the future by application of mathematical methods developed in theoretical chemistry for predicting the conceivable existence of chemical objects for a given collection of atoms as well as for generating reaction pathways by computers (cf. [140]).

Time hierarchy in biochemical networks

Biochemical reaction systems are generally characterized by time hierarchy, i.e. the wide-range separation of time scales inherent to dynamic behaviour [74, 80, 122]. This feature results from the enormous differences in magnitude of kinetic parameter values of the particular enzymes.

There is as yet no agreed definition of a quantitative measure of time hierarchy. A straightforward definition is [141]

$$H_\tau = \frac{\langle \Delta\tau_i \rangle}{\langle \tau_i \rangle} = \frac{r}{r-1} \frac{\sum_i (\tau_s - \tau_i)}{\sum_i \tau_i} \tag{121}$$

where τ_i denotes the relaxation time of the i-th reaction ($i = ,\ldots,r$) and

$$\tau_s = \max_i \tau_i. \tag{122}$$

It is readily verified that H_τ is bounded by

$$H_\tau \leq r \tag{123}$$

and attains its maximum value if one reaction is slow and all remaining reactions are as fast as possible.

It is of interest to inquire to what extent the phenomenon of time-scale separation can be accounted for by optimization principles. According to the results given in the section on *Optimization of multienzyme systems*, maximization of steady-state fluxes without osmotic constraint leads to a considerably lower time hierarchy than with inclusion of this constraint (cf. Fig. 7). Moreover, minimization of transient times (Eqns 87, 92 and 94) and the minimization of total osmolarity (Eqn 106) also imply time-scale separation. Consider, for example, the minimization of total osmolarity in an unbranched pathway (cf. section on *Implications of the solvent capacity and osmotic balance*). In the optimal state the measure of time hierarchy H_τ equals its maximum value r, since the characteristic times of all reactions but the first one tend to zero.

Temporal hierarchy implies hierarchy of control or, in other words, strongly non-uniform distribution of control coefficients. Specifically, a high value of H_τ predicted by several optimization principles implies that the majority of reactions have low control coefficients.

It is well known that feedback inhibition in metabolic pathways may lead to unstable steady states and oscillatory behaviour. Investigations on the suppression of potential instabilities in model systems have shown that this can be achieved by reducing the number of reaction steps or by separating the magnitudes of time constants [80 (ch.11), 109, 122, 141, 142]. This can be visualized with the aid of the reaction chain depicted in Scheme 4 with $n = 3$. Using the rate laws

$$v_1 = \frac{v}{1 + p S_3^m}. \tag{124}$$

$$v_i = k_i S_i, i = 2,3,4 \tag{125}$$

one obtains by means of the Routh-Hurwitz criterion that the steady state is stable whenever

$$m < C\left(2 + \frac{k_2}{k_3} + \frac{k_3}{k_2} + \frac{k_3}{k_4} + \frac{k_4}{k_3} + \frac{k_2}{k_4} + \frac{k_4}{k_2}\right) \tag{126}$$

with C = const. [122, 142]. Inequality (126) shows in a straightforward way that time hierarchy has a stabilizing effect since the stability range of m increases as the rate constants k_i become different from each other.

The evolutionary 'non-equalization' of kinetic constants in metabolic pathways [80] is somehow equivalent to a reduction of the number of independent dynamic variables and to a simplification of the kinetic behaviour [74, 92, 122, 141]. This result might be related to the observation that most biochemical systems exhibit a relatively simple behaviour in spite of their high structural complexity.

Final remarks

In this paper mathematical approaches to study the evolution and structural design of biochemical reaction systems on the basis of optimization principles have been outlined. The adequacy of these approaches depends essentially on the formulation of appropriate objective functions used to evaluate the efficiency (fitness, selective value, etc.) of a given system. The objective functions are usually derived from heuristic arguments, and their validity can only be judged by comparing theoretically predicted optimum system parameters with experimental observations.

For single enzymes, the steady-state rate is mostly regarded as an efficiency measure; however, other properties such as, for example, kinetic simplicity or sensitivity against external effectors were shown also to be of great physiological significance. It seems to be very unlikely that the structural design and dynamic behaviour of contemporary enzymes can be rationalized on the basis of only one objective function. This is true all the more for networks of reactions (metabolic systems) owing to the multitude of their biological functions and the interactions between them.

Accordingly, various objective functions have been proposed in the literature. Mostly, only one of them was favoured specifically, but there are also approaches based on a simultaneous inclusion of several objective functions [30, 78, 88, 106, 107]. One plausible method is to combine the various supposed evolutionary goals into one unified objective function. For example, flux maximization in branched pathways can be performed by constructing the objective function as a product of the independent fluxes [30]. Another way is to phrase some of the objectives as side constraints, having in view that these goals need be approached only to a certain extent rather than to be attained exactly [85]. A third method is to involve several objectives by multiple criteria optimization which is often used in engineering sciences (for mathematical theory see [143], for application to biochemical systems see [78, 107]). The basic idea of this approach is that the various objectives have to trade-off with one another so that a so-called compromise set of solutions is obtained. If all objective functions attain their maximum at the same parameter configuration (i.e. are not antagonistic to each other), the compromise set degenerates to a point; the simultaneous minimization of the total osmolarity and of the transient time $\tau_n^{(1)}$ may serve as an example of this case (cf. section on *Transient times*). For other combinations of antagonistic objective functions the compromise set may be of higher dimension (as, for example, when maximizing the thermodynamic efficiency and the flux of ATP utilization of linear energy converters as considered in *Thermodynamic principles*). This could be one plausible explanation for the large variation of biochemical data even within one species. Finally, it may happen that the compromise set is composed of several disconnected subsets within the parameter space [78]. Occurrence of such solutions could account for the fact that not every intermediary state between some frequently observed configurations is realized in animate nature.

In some cases, the optimality principle can be formulated equivalently by exchanging the roles of objective function and side constraint. So the minimization of the transient time at constant total enzyme concentration [93] leads to the same result as the minimization of the total enzyme concentration at constant transient time [26]. Regardless of the mathematical equivalence, the proper choice of the objective function should be in line with the biological function of the system in question.

Apart from some studies on glycolysis [30], mitochondrial energy transduction [28], and a few other pathways, most work on optimal properties of metabolism has been restricted to simple model reaction schemes. In the future, the analysis should be extended to such metabolic systems for which there already exists a large body of experimental data and model studies. For the treatment of complex systems, methodological concepts used for the optimization of bioreactors [144] and for optimal nutritional and pharmaceutical therapies [145] could be helpful. With respect to the optimization of single enzymes, the next important step could be the involvement of bimolecular reactions, which occur much more frequently than monomolecular ones. Data bases on enzymes and metabolic pathways [146, 147] should be used for the validation of theoretical results on a broader experimental scale.

REFERENCES

1. Werner, A. & Heinrich, R. (1985) *Biomed. Biochim. Acta 44*, 185–212.
2. Lew, V. L. & Bookchin R. M. (1986) *J. Membr. Biol. 92*, 57–74.
3. Pettersson, G. & Ryde-Pettersson, U. (1987) *Eur. J. Biochem. 175*, 661–672.
4. Schuster, R., Holzhütter, H.-G. & Jacobasch, G. (1988) *BioSystems 22*, 19–36.
5. Wright, B. E. & Reimers, J. M. (1988) *J. Biol. Chem. 263*, 14906–14912.
6. Steinmeyer, D. E. & Shuler, M. L. (1989) *Chem. Eng. Sci. 44*, 2017–2030.
7. Joshi, A. & Palsson, B. O. (1989) *J. Theor. Biol. 141*, 515–545.
8. Joshi, A. & Palsson, B. O. (1990) *J. Theor. Biol. 142*, 41–85.
9. Bartel, Th. & Holzhütter, H. G. (1990) *Biochim. Biophys. Acta 1035*, 331–339.
10. Kuchel, P. W., Berthon, H. A., Bubb, W. A., Bulliman, B. T. & Collins, J. G. (1990) *Biomed. Biochim. Acta 49*, 757–770.
11. Lowry, O. H. & Passoneau, J. V. (1964) *J. Biol. Chem. 239*, 31–42.
12. Rosen, R. (1967) *Optimality Principles in Biology*, Butterworths, London.
13. Atkinson, D. E. (1969) *Curr. Topics Cell. Reg. 1*, 29–43.
14. Fersht, A. R. (1974) *Proc. R. Soc. Lond. B 187*, 397–407.
15. Cleland, W. W. (1975) *Acc. Chem. Res. 8*, 145–151.
16. Crowley, P. H. (1975) *J. Theor. Biol. 50*, 461–475.
17. Albery, W. J. & Knowles, J. R. (1976) *Biochemistry 15*, 5631–5640.
18. Cornish-Bowden, A. (1976) *J. Mol. Biol. 101*, 1–9.
19. Brocklehurst, K. (1977) *Biochem. J. 163*, 111–116.
20. Hall, J. B. (1971) *J. Theor. Biol. 30*, 429–454.
21. Horvath, R. S. (1974) *J. Theor. Biol. 47*, 361–371.
22. Kacser, H. & Beeby, R. (1984) *J. Mol. Evol. 20*, 38–51.
23. Belfiore, F. (1980) *Enzyme regulation and metabolic diseases*, S. Karger, Basel, New York.
24. Reich, J. G. (1983) *Biomed. Biochim. Acta 42*, 839–848.
25. Rosen, R. (1986) *J. Math. Anal. Appl. 119*, 203–222.
26. Kuchel, P. W. (1985) in *Organized multienzyme systems* (Welch, G. R, ed.), pp. 303–380, Academic Press, New York.
27. Heinrich, R. (1990) in *Control of metabolic processes* (Cornish-Bowden, A. & Cárdenas, M. L., eds), pp. 329–342, Plenum Press, New York, London.
28. Stucki, J. W. (1980) *Eur. J. Biochem. 109*, 269–283.
29. Heinrich, R. & Holzhütter, H. G. (1985) *Biomed. Biochim. Acta 44*, 959–969.
30. Heinrich, R., Holzhütter, H. G. & Schuster, S. (1987) *Bull. Math. Biol. 49*, 539–595.
31. Ledermann, W. & Vajda, S.(eds) (1982) *Handbook of applicable mathematics* ch. 15, John Wiley, Chichester.
32. Chin, J. (1983) *J. Am. Chem. Soc. 105*, 6502–6503.
33. Stackhouse, J., Nambiar, K. P., Burbaum, J. J., Stauffer, D. M. & Benner, S. A. (1985) *J. Am. Chem. Soc. 107*, 2757–2763.
34. Ellington, A. D. & Benner, S. A. (1987) *J. Theor. Biol. 127*, 491–506.
35. Burbaum, J. J., Raines, R. T., Albery, W. J. & Knowles, J. R. (1989) *Biochemistry 28*, 9293–9305.
36. Pettersson, G. (1989) *Eur. J. Biochem. 184*, 561–566.
37. Heinrich, R. & Hoffmann, E. (1991) *J. Theor. Biol. 151*, 249–283.
38. Briggs, G. E. & Haldane, J. B. S. (1925) *Biochem. J. 19*, 338–339.
39. Damjanovich, S. & Somogyi, B. (1973) *J. Theor. Biol. 41*, 567.
40. Somogyi, B. & Damjanovich, S. (1975) *J. Theor. Biol. 48*, 393–401.
41. Somogyi, B., Karasz, F. E., Tron, L. & Condman, P. R. (1978) *J. Theor. Biol. 74*, 209–216.
42. Welch, G. R., Somogyi, B., Matko, J. & Papp, S. (1983) *J. theor. Biol. 100*, 211–238.
43. Welch, G. R., Somogyi, B. & Damjanovich, S. (1982) *Prog. Biophys. Mol. Biol. 39*, 109–146.
44. Haldane, J. B. S. (1930) *The enzymes*, Reprint (1965): MIT Press, Cambridge, MS.
45. Wong, T. J. (1975) *Kinetics of enzyme mechanisms*. Academic Press, London, New York, San Francisco.
46. Zhou, G. Q. & Zhong, W. Z. (1982) *Eur. J. Biochem. 128*, 383–387.
47. Markus, R. A. (1968) *J. Phys. Chem. 72*, 891–899.
48. Murdoch, J. R. (1972) *J. Am. Chem. Soc. 94*, 4410–4418.
49. Nageswara Rao, B. D., Kayne, F. J. Jr & Cohn, M. (1979) *J. Biol. Chem. 254*, 2689–2696.
50. Wilkinson, K. D. & Rose, I. A. (1979) *J. Biol. Chem. 254*, 2125–2131.
51. Nageswara Rao, B. D. & Cohn, M. (1981) *J. Biol. Chem. 256*, 1716–1721.
52. Srivastava, D. K. & Bernhard, S. A. (1986) *Curr. Top. Cell. Regul. 28*, 1–68.
53. Burbaum, J. J. & Knowles, J. R. (1989) *Biochemistry 28*, 9306–9317.
54. Friedrich, P. (1984) *Supramolecular enzyme organization*, Pergamon/Akademiai Kiado, Oxford/Budapest.
55. Heinrich, R., Hoffmann, E. & Holzhütter, H. G. (1990) *Biomed. Biochim. Acta 49*, 891–902.
56. Heinrich, R., Hoffmann, E. & Holzhütter, H. G. (1991) in *Proceedings of the 4th Workshop BioThermoKinetics, Noordwijkerhout 1990* (Westerhoff, H. V. ed.) Intercept, Andover, UK, in the press.
57. Albery, W. J. & Knowles, J. R. (1976) *Biochemistry 15*, 5627–5631.
58. Christensen, H., Martin, M. T. & Waley, G. (1990) *Biochem. J. 266*, 853–861.
59. Brocklehurst, K. & Topham, C. M. (1990) *Biochem. J. 270*, 561–564.
60. Monod, J., Wyman, J. & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.
61. Koshland, D. E., Nemethy, G. & Filmer, P. (1966) *Biochemistry 5*, 365–385.
62. Rabin, B. R. (1967) *Biochem. J. 102*, 226–236.
63. Ainslie, G. R., Shill, J. P. & Neet, K. E. (1972) *J. Biol. Chem. 247*, 7088–7096.
64. Bardsley, N. G. (1977) *J. Theor. Biol. 65*, 281–316.
65. Ricard, J. & Noat, G. (1982) *J. Theor. Biol. 96*, 347–365.
66. Ricard, J. (1978) *Biochem. J. 175*, 779–791.
67. Ricard, J. & Noat, G. (1984) *J. Theor. Biol. 111*, 737–753.
68. Ricard, J. & Noat, G. (1986) *J. Theor. Biol. 123*, 431–451.
69. Ricard, J., Mouttet, C. & Nari, J. (1974) *Eur. J. Biochem. 41*, 479–497.
70. Whitehead, E. P. (1976) *Biochem. J. 159*, 449–456.

71. Kacser, H. & Burns, J. A. (1973) *Symp. Soc. Exp. Biol. 27*, 65–104.
72. Heinrich, R. & Rapoport, T. A. (1974) *Eur. J. Biochem. 42*, 89–95.
73. Cornish-Bowden, A. & Cárdenas, M. L. (eds) (1990) *Control of metabolic processes*, Plenum Press, New York, London.
74. Reich, J. G. & Selkov, E. E. (1981) *Energy metabolism of the cell*, Academic Press, London.
75. Waley, S. G. (1964) *Biochem. J. 91*, 514–517.
76. Heinrich, R. & Rapoport, T. A. (1973) *Acta Biol. Med. Germ. 31*, 479–494.
77. Kacser, H. & Burns, J. A. (1981) *Genetics 97*, 639–666.
78. Schuster, S. & Heinrich, R. (1987) *J. Theor. Biol. 129*, 189–209.
79. Nicolis, G. & Prigogine, I. (1977) *Self-organization in non-equilibrium systems*, John Wiley, New York.
80. Savageau, M. (1976) *Biochemical systems analysis*, Addison-Wesley, Reading, MS.
81. Waley, S. G. (1969) *Comp. Biochem. Physiol. 30*, 1–11.
82. Ycas, M. (1974) *J. Theor. Biol. 44*, 145–160.
83. Jensen, R. A. (1976) *Annu. Rev. Microbiol. 30*, 409–425.
84. Woese, C. R. & Rose, I. A. (1979) *Proc. Natl Acad. Sci. USA 54*, 1546–1550.
85. Reich, J. G. (1985) *Proc. 16th FEBS Congr.*, Part C, pp. 431–435, VNU Press, Utrecht.
86. Reich, J. G. (1985) *Biomed. Biochim. Acta 44*, 845–852.
87. Horsthemke, W. & Lefever, R. (1984) *Noise induced transitions*, Springer, Berlin.
88. Majewskij, R. A. & Domach, M. M. (1985) *BioSystems 18*, 15–22.
89. Easterby, J. S. (1973) *Biochim. Biophys. Acta 293*, 552–558.
90. Easterby, J. S. (1981) *Biochem. J. 199*, 155–161.
91. Heinrich, R. & Rapoport, T. A. (1975) *BioSystems 7*, 130–136.
92. Palsson, B. O., Palsson, H. & Lightfoot, E. N. (1985) *J. Theor. Biol. 113*, 231–259.
93. Cleland, W. W. (1979) *Anal. Biochem. 99*, 142–145.
94. Hearon, J. Z. (1953) *Bull. Math. Biophys. 15*, 121–141.
95. Schuster, S. (1989) in *Dynamical networks* (Ebeling, W. & Peschel, M., eds), pp. 173–183, Akademie-Verlag, Berlin.
96. Higgins, J. J. (1965) in *Control of energy metabolism* (Chance, B., Estabrook, R. K. & Williamson, R. J., eds), pp. 13–46, Academic Press, New York.
97. Hofmeyr, J. H. S., Kacser, H. & van der Merwe, K. J. (1986) *Eur. J. Biochem. 155*, 631–641.
98. Goldbeter, A. & Koshland, D. E. Jr (1982) *Q. Rev. Biophys. 15*, 555–591.
99. Stadtman, E. R. & Chock, P. B. (1977) *Proc. Natl Acad. Sci. USA 74*, 2761–2765.
100. Goldbeter, A. & Koshland, D. E. Jr (1981) *Proc. Natl Acad. Sci. USA 78*, 6840–6844.
101. Goldbeter, A. & Koshland, D. E. Jr (1984) *J. Biol. Chem. 259*, 14441–14447.
102. Goldbeter, A. & Koshland, D. E. Jr (1990) in *Control of metabolic processes* (Cornish-Bowden, A. & Cárdenas, M. L., eds) pp. 173–182, Plenum Press, New York, London.
103. Cárdenas, M. L & Cornish-Bowden A. (1989) *Biochem. J. 257*, 339–345.
104. Moelwyn-Hughes, E. A. (1964) *Physical chemistry*, Pergamon, Oxford.
105. Brumen, M., Glaser, R. & Svetina, S. (1979) *Bioelectrochem. Bioenerget. 6*, 227–241.
106. Schuster, S. & Heinrich, R. (1991) *J. Math. Biol., 29*, 425–442.
107. Schuster, S., Schuster, R. & Heinrich, R. (1991) *J. Math. Biol., 29*, 443–455.
108. Crabtree, B. & Newsholme, E. A. (1987) *Trends Biochem Sci. 12*, 4–12.
109. Dibrov, B. F., Zhabotinsky, A. M., Kholodenko, B. N. (1982) *J. Math. Biol. 15*, 51–64.
110. Albe, K. R., Butler, M. H. & Wright, B. E. (1990) *J. Theor. Biol. 143*, 163–196.
111. Stebbing, N. (1980) in *Cell compartmentation and metabolic channeling*. (Nover, L., Lynen, F. & Mothes, K.; eds) pp. 93–106. Fischer/Elsevier, Jena/Amsterdam.
112. Wakil, S. J., Stoops, J. K. & Joshi, V. C. (1983) *Annu. Rev. Biochem. 52*, 537–579.
113. Ovádi, J. (1988) *Trends Biochem. Sci. 13*, 486–490.
114. Watford, M. (1989) *Trends Biochem. Sci. 14*, 313–314.
115. Keleti, T. (1990) in *Control of metabolic processes* (Cornish-Bowden, A. & Cárdenas, M. L., eds) pp. 259–270, Plenum Press, New York, London.
116. Dillon, P. F. & Clark, J. F. (1990) *J. Theor. Biol. 143*, 275–284.
117. Welch, G. R., Keleti, T. & Vértessy, B. (1988) *J. Theor. Biol. 130*, 407–422.
118. Easterby, J. S. (1990) in *Control of metabolic processes* (Cornish-Bowden, A. & Cárdenas, M. L., eds) pp. 281–290, Plenum Press, New York, London.
119. Westerhoff, H. V. & Kamp, F. (1986) in *The organization of cell metabolism* (Welch, G. R. & Clegg, J. S., eds) pp. 339–356, Plenum Press, New York, London.
120. Ovádi, J., Tompa, P., Vértessy, B., Orosz, F., Keleti, T. & Welch, G. R. (1989) *Biochem. J. 257*, 187–190.
121. Cornish-Bowden, A. (1991) *Eur. J. Biochem. 195*, 103–108.
122. Heinrich, R., Rapoport, S. M. & Rapoport, T. A. (1977) *Prog. Biophys. Mol. Biol. 32*, 1–82.
123. Kurganov, B. I. (1986) *J. Theor. Biol. 119*, 445–455.
124. Katchalsky, A. & Curran, P. F. (1965) *Nonequilibrium thermodynamics in biophysics*, Harvard University Press, Cambridge, MS.
125. Kedem, O. & Caplan, S. R. (1965) *Trans. Faraday Soc. 61*, 1897–1911.
126. Stucki, J. W. (1978) in *Energy conservation in biological membranes* (Schaefer, G. & Klingenberg, M., eds) pp. 264–287, Springer-Verlag, Berlin, Heidelberg, New York.
127. Wilson, D. F. & Westerhoff, H. V. (1982) *Trends Biochem. Sci. 7*, 275–279.
128. Westerhoff, H. V., Lolkema, J. S., Otto, R. & Hellingwerf, K. (1982) *Biochim. Biophys. Acta 683*, 181–220.
129. Roels, J. A. (1983) *Energetics and kinetics in biotechnology*, Elsevier Biomedical Press, Amsterdam.
130. Stucki, J. W., Compiani, M. & Caplan, S. R. (1983) *Biophys. Chemistry 18*, 101–109.
131. Westerhoff, H. V. & van Dam, K. (1988) *Thermodynamics and control of biological free-energy transduction*, Elsevier, Amsterdam.
132. Stucki, J. W. (1988) in *From chemical to biological organization*, (Markus, M., Muller, S. C., Nicolis, G., eds) pp. 216–224, Springer, Berlin.
133. Meléndez-Hevia, E. & Isidoro, A. (1985) *J. Theor. Biol. 117*, 251–263.
134. Horecker, B. L., Gibbs, M., Klenow, H. & Smyrniotis, P. Z. (1954) *J. Biol. Chem. 207*, 393–403.
135. Wood, H. G. & Katz, J. (1958) *J. Biol. Chem. 233*, 1279–1282.
136. Meléndez-Hevia, E. & Torres, N. V. (1988) *J. Theor. Biol. 132*, 97–111.
137. Meléndez-Hevia, E. (1990) *Biomed. Biochim. Acta 49*, 903–916.
138. Williams, J. F., Clark, M. G. & Blackmore, P. F. (1978) *Biochem. J. 176*, 241–256.
139. Williams, J. F. & Blackmore, P. F. (1978) *Biochem. J. 176*, 257–282.
140. Bauer, J., Fontain, E. & Ugi, I. (1988) *Anal. Chim. Acta 210*, 123–134.
141. Heinrich, R. & Sonntag, I. (1982) *BioSystems 15*, 301–316.
142. Savageau, M. (1975) *J. Mol. Evol. 5*, 199–222.
143. Sawaragi, Y., Nakayama, H. & Tanino, T. (1985) *Theory of multiobjective optimization*, Academic Press, Orlando.
144. Dourado, A., Calvet, J. L., Sevely, Y. & Goma, G. (1987) *Biotechn. Bioeng. 29*, 195–203.
145. Giladi, R. & Sideman, S. (1989) *Math. Biosci. 97*, 179–197.
146. Selkov, E.E., Goryanin, I. I., Kaimachnikov, N.P., Shevelev, E. L. & Yanus, I. A. (1989) *Stud. Biophys. 129*, 155–164.
147. Barcza, S., Kelly, L. A. & Lenz, C. D. (1990) *J. Chem. Inf. Comp. Sci. 30*, 243–251.
148. Barrow, G. M. (1979) *Physical chemistry*, McGraw Hill, New York.