Biochem. J. (1981) **199**, 155–161
Printed in Great Britain

A generalized theory of the transition time for sequential enzyme reactions

John S. EASTERBY
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

(*Received 7 April 1981/Accepted 12 June 1981*)

In a sequence of coupled enzyme reactions the steady-state production of product is preceded by a lag period or transition time during which the intermediates of the sequence are accumulating. Provided that a steady state is eventually reached, the magnitude of this lag may be calculated, even when the differential equations describing the process have no analytical solution. The calculation may be made for simple systems in which the enzymes obey Michaelis–Menten kinetics or for more complex pathways in which intermediates act as modifiers of the enzymes. The transition time associated with each intermediate in the sequence is given by the ratio of the appropriate steady-state intermediate concentration to the steady-state flux. The theory is also applicable to the transition between steady states produced by flux changes. Application of the theory to coupled enzyme assays allows a definition of the minimum requirements for successful operation of the assay. The theory can be extended to deal with sequences in which the enzyme concentration exceeds substrate concentration.

It has previously been shown that the practice of coupling several enzyme reactions, in order to generate a measureable product, results in a time-lag in the appearance of product (McClure, 1969; Hess & Wurster, 1970). Where the first enzyme of the sequence is severely rate-limiting and the reactions are irreversible, pseudo-first-order kinetic behaviour is observed and the time-lag or transition time for the sequence is the sum of the transition times for the individual enzymes (Easterby, 1973). Each of these transition times represents the part of the elapsed time required to generate the steady-state concentration of the particular intermediate that is substrate for the enzyme and is defined as the $K_m/V_{max.}$ ratio for the associated coupling enzyme. This prompted the use of the term time-limiting enzyme to describe the enzymes of the sequence with greatest $K_m/V_{max.}$ ratio (Easterby, 1973). These enzymes would largely determine the time required for a sequence of reactions to become established in the steady state. The equations derived to describe the transition time for a sequence of irreversible reactions were subsequently verified by other authors and the 'time-limiting' designation was adopted (Heinrich & Rapoport, 1975). More recently it has been shown that the equations can be used to optimize assay conditions and improve cost-effectiveness (Cleland, 1979). This may be particularly important in automated or clinical applications of coupling enzymes, where large numbers of assays are being routinely performed. A review of the application of the method has appeared (Rudolph *et al.*, 1979).

One limitation of the previous analysis was that it gave no indication of the restrictions on the validity of the assumption of first-order behaviour. Nor did it allow for the inclusion of enzymes in the sequence whose kinetics followed the normal Michaelis–Menten hyperbola. The application of Michaelis–Menten behaviour to a two-enzyme system has, however, been considered by Storer & Cornish-Bowden (1974).

The most important parameter describing a coupled enzyme system is the transition time, otherwise known as the transient or transit time. This quantity gives an indication of the speed with which a pathway enters a steady state and largely determines the accuracy with which steady-state rate measurements can be made. The present paper describes a general theory of the transition time that allows its calculation and description in terms of the kinetic constants of the system, even when the differential equations describing the progress curve are not readily amenable to analytical solution. It applies to all systems that enter a steady state (generally those in which the first enzyme reaction of the sequence is the slowest) and is equally valid when reactions are reversible or subject to feedback or feedforward modification of rate. It has previously been common practice to associate the transition time of a particular step in a reaction sequence with the enzyme catalysing the step. This can lead to a

0306-3275/81/100155-07$01.50/1

cumbersome definition of the overall transition time if reversible steps or steps subject to modification of rate by effector molecules are included. If the transition time is associated with an intermediate rather than the enzymes catalysing its conversion, a simple definition is obtained, and the individual transition times may be added to obtain that for the sequence.

The types of sequence covered by this analysis have been described elsewhere (Hess & Wurster, 1970; Easterby, 1973), but good examples would be the coupling of hexokinase to glucose 6-phosphate dehydrogenase to generate NADPH as the measureable product or the coupling of other kinases, through ADP, to pyruvate kinase and lactate dehydrogenase. In the latter example NAD^+ would be regarded as the end product of the sequence and the disappearance of NADH would be monitored.

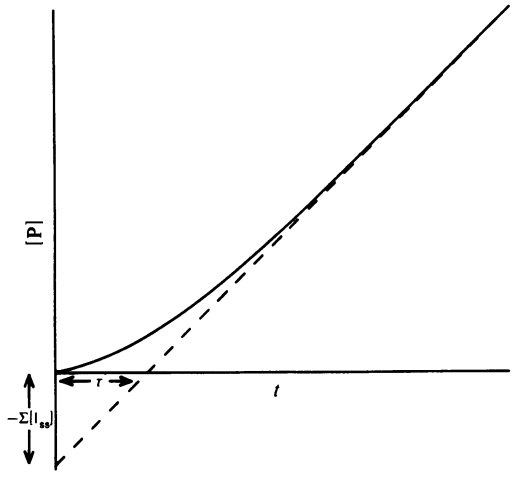

Fig. 1. *Relationship between product concentration and time in a generalized reaction sequence*
The abcissa and ordinate intercepts define the transition time and the steady-state concentration of intermediates respectively.

Results and discussion

Consider a reaction sequence consisting of the conversion of a buffered substrate S to an end product P through a series of intermediates I:

$$S \xrightarrow{v_0} I_1 \rightleftharpoons I_2 \rightleftharpoons \cdots \rightleftharpoons I_j \rightleftharpoons \cdots \rightleftharpoons I_n \longrightarrow P$$

where v_0 represents the rate of the initial flux-generating enzyme. At any time from the initiation of the reactions, mass conservation requires that:

$$v_0 \cdot t = [P] + \sum_{j=1}^{n} [I_j] \quad (1)$$

or

$$[P] = v_0 \cdot t - \sum_{j=1}^{n} [I_j] \quad (2)$$

$$[P] = v_0\left(t - \sum_{j=1}^{n} [I_j]/v_0\right) \quad (3)$$

In the steady-state one may write:

$$[P] = v_0\left(t - \sum_{j=1}^{n} [I_j]_{ss}/v_0\right) \quad (4)$$

This line represents the steady-state asymptote to the progress curve (Fig. 1). The line by definition intersects the time axis at τ, the transition time for the sequence. Thus:

$$\tau = \sum_{j=1}^{n} [I_j]_{ss}/v_0 = \sum_{j=1}^{n} \tau_j \quad (5)$$

where

$$\tau_j = [I_j]_{ss}/v_0 \quad (6)$$

Thus the overall transition time is the sum of the individual transition times for intermediates j and each is given by the ratio of the steady-state concentration to the steady-state flux. In general the τ_j will contain kinetic coefficients relating to more than one enzyme of the sequence. $[I_j]_{ss}$ may be readily calculated, when the rate laws of the individual enzymes are known, by writing $d[I_j]/dt = 0$. No solution of differential equations is necessary. It should be noted that the $[I_j]_{ss}$ terms include the concentrations of enzyme-bound intermediates where these are significant.

Examples of applications of the theory

1. *Enzymes obeying Michaelis–Menten kinetics.* The first obvious application of the theory is to coupled enzyme reactions in which the enzymes obey Michaelis–Menten kinetics. No theory of transition time for such a system has previously been available. The reactions considered are as follows:

$$S \xrightarrow{v_0} I_1 \xrightarrow{\frac{V_1[I_1]}{K_1+[I_1]}} I_2 \xrightarrow{\frac{V_2[I_2]}{K_2+[I_2]}} \cdots\cdots I_j \xrightarrow{\frac{V_j[I_j]}{K_j+[I_j]}} \cdots\cdots I_n \xrightarrow{\frac{V_n[I_n]}{K_n+[I_n]}} P$$

In the steady state:

$$d[I_j]/dt = 0 = v_0 - V_j[I_j]_{ss}/(K_j + [I_j]_{ss}) \quad (7)$$

Hence:

$$[I_j]_{ss} = v_0 K_j/(V_j - v_0) \quad (8)$$

and:

$$\tau_j = K_k/(V_j - v_0) \quad (9)$$

$$\tau = \sum_{j=1}^{n} K_j/(V_j - v_0) = \sum_{j=1}^{n} \tau_j \quad (10)$$

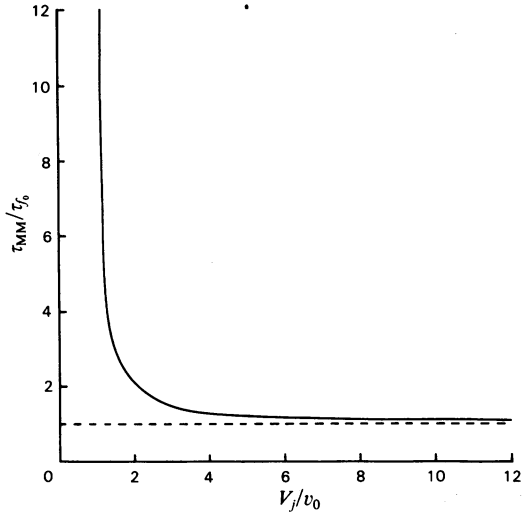

Fig. 2. *Relationship between transition times for Michaelis–Menten and pseudo-first-order enzymes*
The Figure demonstrates that the transition time of an enzyme obeying Michaelis–Menten kinetics rapidly converges to the first-order value as the amount of coupling enzyme is increased. For V_j/v_0 ratios greater than 2 (i.e. for intermediate concentrations less than the K_m of the coupling enzyme) little further improvement towards pseudo-first-order behaviour is obtained.

If $V_j \gg v_0$ the reactions tend to become pseudo-first-order and τ_j becomes K_j/V_j (i.e. the reciprocal of the pseudo-first-order rate coefficient). This is the description of τ_j previously obtained by making the first-order assumption (Easterby, 1973). The τ value obtained for a sequence of enzymes obeying Michaelis–Menten kinetics is greater than for a corresponding sequence of first-order reactions and is the same as that derived for a relaxation kinetic analysis of the system (Varfolomeev, 1977). The ratio of transition times obtained for pseudo-first-order and Michaelis–Menten analyses is given by $(1-v_0/V_j)$. The ratio of the steady-state rate to V_j therefore determines the error in τ arising from the first-order assumption (Fig. 2).

2. *Reversible first-order reactions.* The next logical development from the simple, linear, irreversible reaction scheme previously analysed is to provide sufficient coupling enzyme to allow the first-order assumption to be made but to consider reversible reactions within the sequence:

$$S \xrightarrow{v_0} I_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} I_2 \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \cdots\cdots I_j \underset{k_{-j}}{\overset{k_{+j}}{\rightleftharpoons}} \cdots\cdots I_n \xrightarrow{k_{+n}} P$$

The final step must be irreversible or a steady state is not obtained. In the steady state, detailed balancing procedures may be employed as the net flux at each step will be v_0. Hence:

$$k_{+j}[I_j]_{ss} - k_{-j}[I_{j+1}]_{ss} = v_0 \quad (11)$$

and

$$k_{+n}[I_n]_{ss} = v_0 \quad (12)$$

or:

$$[I_n]_{ss} = v_0/k_{+n} \quad (13)$$

and:

$$\tau_n = 1/k_{+n} \quad (14)$$

Similarly:

$$\tau_{n-1} = 1/k_{n-1} + \rho_{n-1}/k_{+n} \quad (15)$$

$$\tau_{n-2} = 1/k_{n-2} + \rho_{n-2}/k_{n-1} + \rho_{n-2}\cdot\rho_{n-1}/k_{+n} \quad (16)$$

$$\tau_j = 1/k_{+j} + \rho_j/k_{j+1} + \rho_j\cdot\rho_{j+1}/k_{+j+2} + \cdots + \rho_j\rho_{j+1}\rho_{j+2}\rho_{j+3}\cdots\rho_{n-1}/k_{+n} \quad (17)$$

or:

$$\tau_j = 1/k_{+j} + \sum_{x=j+1}^{n} 1/k_{+x} \prod_{y=j}^{x-1} \rho_y \quad (18)$$

where ρ_j is the equilibrium constant for the reverse reaction k_{-j}/k_{+j}. Hence each 'irreversible' τ_j value, $1/k_{+j}$, is perturbed by all subsequent steps up to the next irreversible step ($\rho_y = 0$), and is not a property of a single enzyme. If all steps are made irreversible the τ_j values reduce to $1/k_{+j}$, the reciprocal of the first-order rate coefficient, as described previously (Easterby, 1973). Heinrich & Rapoport (1975) have analysed this system but have defined τ_j values in terms of the kinetic parameters of individual enzymes. Consequently their τ_j values are not additive in the manner described in the present paper when the sequence as a whole is considered. However, when all steps are made irreversible their equations reduce to the same description. For the present treatment it should be emphasized that the transition time for the complete sequence is given by:

$$\tau = \sum_{j=1}^{n} \tau_j \quad (19)$$

3. *Systems including rate modification by effectors.* In many pathways intermediates act as feedback inhibitors of earlier steps. This is shown for a simple two-intermediate sequence as follows:

$$S \xrightarrow{v_0} I_1 \xrightarrow{\overset{i}{\downarrow}} I_2 \xrightarrow{\frac{V_2[I_2]}{K_2+[I_2]}} P$$

Case a: I_2 is a non-competitive inhibitor with respect to I_1. If both enzymes obey Michaelis–Menten kinetics and I_2 is a feedback inhibitor of the first coupling enzyme, one may write in the steady state:

$$v_0 = V_1[I_1]_{ss}/(1+[I_2]_{ss}/K_i)(K_1+[I_1]_{ss}) = V_2[I_2]_{ss}/(K_2+[I_2]_{ss}) \quad (20)$$

where K_i represents the inhibition constant of the enzyme. This leads to:

$$\tau_1 = \frac{K_1}{\left(\frac{V_1}{(1+[I_2]_{ss}/K_i)} - v_0\right)} \quad (21)$$

$$\tau_2 = K_2/(V_2 - v_0) \quad (22)$$

and:

$$[I_2]_{ss} = v_0 K_2/(V_2 - v_0) \quad (23)$$

Thus the effect of I_2 on the transition time of I_1 is simply to increase it by lowering V_1, as expected.

Case *b*: I_2 is a competitive inhibitor with respect to I_1. If inhibition by I_2 is competitive to I_1 the transition time becomes:

$$\tau_1 = \frac{K_1(1+[I_2]_{ss}/K_i)}{(V_1 - v_0)} \quad (24)$$

The descriptions of τ_2 and $[I_2]_{ss}$ are the trivial ones given above. From these equations it can be seen that the effects of inhibitors and activators on the transition time can be predicted quite easily. They merely alter the kinetic coefficients in the same way as in single-enzyme systems. Feedforward can be treated in a directly analogous manner.

It should be emphasized that this approach is not appropriate if an intermediate is an inhibitor of the first enzyme of the sequence. Then a full solution of the differential equation describing the conservation condition with variable v_0 is required. In general the τ value calculated will be too great. Where an end product is the inhibitor, it is possible that the pathway will have closely approached the steady state before sufficient inhibitor has accumulated to curtail the reactions. In the case of coupled assays it will usually be possible to abolish inhibitory effects by selection of high substrate or coupling enzyme concentrations.

However, feedback inhibition clearly has a role as a rapid switching mechanism. The initial activity of the rate-determining enzyme can be higher than required in the steady state and therefore facilitate a rapid build up of intermediates.

4. *Transition between steady states.* If a sequence of reactions in a steady state is perturbed by alteration of flux, a transition between steady states occurs. If the initial flux is given by $(v_0)_a$ and this is altered to $(v_0)_b$, one may write:

$$(v_0)_b \cdot t = \Delta[P] + \sum_{j=1}^{n} \Delta[I_j]_{ss} \quad (25)$$

where t is the time elapsed from the transition point or perturbation. Thus:

$$\Delta[P] = (v_0)_b \cdot \left(t - \sum_{j=1}^{n} \Delta[I_j]_{ss}/(v_0)_b\right) \quad (26)$$

This is analogous to eqn. (1) and may be written:

$$\Delta[P] = (v_0)_b \cdot (t-\tau) \quad (27)$$

where:

$$\tau = \sum_{j=1}^{n} \Delta[I_j]_{ss}/(v_0)_b \quad (28)$$

τ is also given by:

$$\tau = \Delta \sum_{j=1}^{n} [I_j]_{ss}/(v_0)_b = 1/(v_0)_b \cdot \left(\sum_{j=1}^{n} [I_j]_{ss_b} - \sum_{j=1}^{n} [I_j]_{ss_a}\right) \quad (29)$$

$$\tau = \tau_b - (v_0)_a/(v_0)_b \cdot \tau_a \quad (30)$$

The transition time is thus decreased by an amount dependent on the previous steady-state concentration of intermediate and therefore on the transition time for the attainment of the first steady state. The graphical representation of this result is shown in Fig. 3. At the first transition point the flux

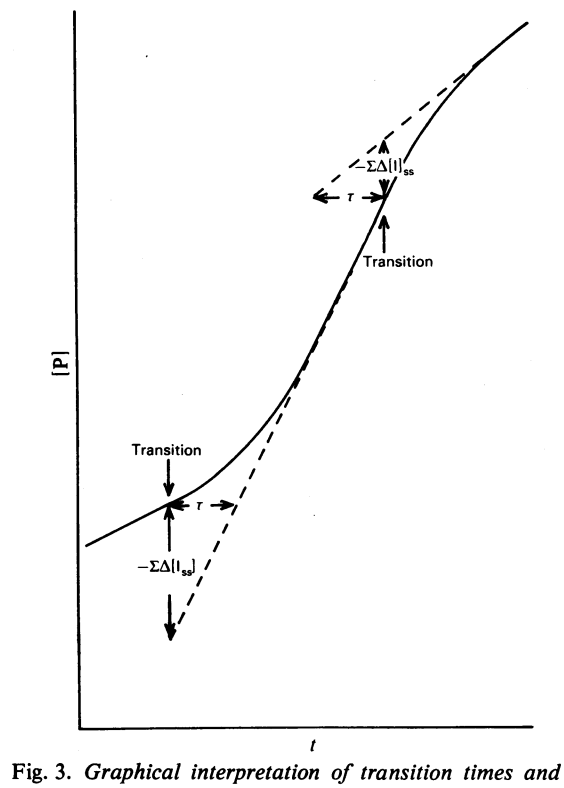

Fig. 3. *Graphical interpretation of transition times and steady-state concentrations during transitions between steady states*

through the pathway is increased and at the second it is decreased. This results respectively in an increase and a decrease in the steady-state concentrations of the intermediates. A similar approach to the relaxation kinetics of the process described in the present paper identified a relaxation time equivalent to τ_b (Varfolomeev, 1977). The present approach, however, lifts the restriction of small displacements from the steady state imposed by relaxation kinetics and looks at the transition in product rather than intermediates. This results in the more complex description of τ for the process given in eqn. (30).

5. *Limitations on the use of coupled enzyme assays.* It has previously been shown that when enzymes obey pseudo-first-order kinetic behaviour a time equivalent to approximately 5 times the transition time must elapse before the rate of product formation matches that of the initial rate-limiting enzyme to within 1% (Easterby, 1973). In general, enzymes do not follow such simple kinetics, and it is valuable to estimate how much coupling enzyme is required and what time must elapse before a steady state is approached in systems obeying Michaelis–Menten kinetics. This may be most readily done by considering a complete solution to the equations describing the following scheme:

$$S \xrightarrow{v_0} I_1 \xrightarrow{\frac{V_1[I_1]}{K_1+[I_1]}} P$$

in which a single coupling enzyme obeys Michaelis–Menten kinetics. A previous solution to this problem has been published (Storer & Cornish-Bowden, 1974), but in the present paper the equations are developed in terms of the transition-time concept. One may write:

$$d[I_1]/dt = v_0 - V_1[I_1]/(K_1+[I_1]) \qquad (31)$$

A solution to this equation is:

$$\frac{(V_1/v_0)}{(V_1/v_0-1)} \cdot \ln\frac{(V_1/v_0)(1-f)}{(V_1/v_0-f)} + f/(V_1/v_0-f) = -t_f/\tau \qquad (32)$$

where:

$$\tau = K_1/(V_1-v_0) \qquad (33)$$

as described in section 1 for enzymes obeying Michaelis–Menten kinetics, and f represents the fraction of the steady-state rate attained at time t_f. Substitution of 0.99 for f provides the curves of Fig. 4. The ratio $t_{0.99}/\tau$ is shown both for the true transition time obtained for Michaelis–Menten kinetics, τ_{MM}, and in terms of the more commonly assumed pseudo-first-order value τ_{f_0}. It can be seen that, as the amount of coupling enzyme is increased relative to the amount of enzyme being measured, the time required to approach steady-state conditions decreases rapidly. This effect is most noticeable up to a V_1/v_0 ratio of 2 (at which ratio the steady-state concentration of the intermediate is equal to the enzyme K_m). For ratios greater than about 6, further decrease in time is only achieved at the expense of vastly increased use of coupling enzyme. Considering this in conjunction with Fig. 2, one may conclude that providing sufficient coupling enzyme is present to give a V_1/v_0 ratio of 6, pseudo-first-order behaviour may be assumed and the time required to approach the steady-state is less than 6 times τ_{f_0}. Thus, in choosing conditions for a coupled assay involving an enzyme that follows Michaelis–Menten kinetics, the following relationships should be used to select conditions:

$$V_1 > 6v_0 \qquad (34)$$

and:

$$t_{0.99} < 6K_1/V_1 \qquad (35)$$

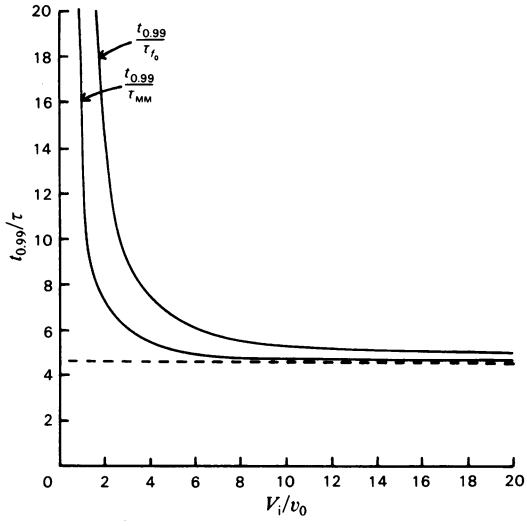

Fig. 4. *Determination of the time required to approach a steady state*

The time required to reach 99% of the steady-state reaction rate is shown as a function of transition time assuming either Michaelis–Menten or pseudo-first-order kinetics to prevail. τ_{MM} is defined by eqn. (33) and τ_{f0} is merely the ratio K_1/V_1. The time decreases rapidly as the amount of coupling enzyme is increased, but for V_1/v_0 ratios greater than 6 very little further improvement in the rate of attainment of the steady state can be obtained.

6. *Transition times in the presence of high enzyme concentrations.* Implicit in the previous analysis is the assumption that the concentrations of enzyme-bound intermediates are insignificant compared with free solution concentrations. In some applications, particularly *in vivo*, this may not be true. However, a simple correction to the transition time may then be made. As only steady-state concentrations need to be known in order to calculate τ, no detailed consideration has to be given to the pre-steady-state phase in the formation of enzyme–intermediate complexes.

The total intermediate concentration consists of two parts:

$$[I]_{ss} = [I_{free}]_{ss} + [EI]_{ss}$$

$[I_{free}]_{ss}$, the free solution concentration, can be calculated as above by using the appropriate rate equation, and $[EI_{ss}]$ is determined from a knowledge of the steady-state flux and enzyme maximum velocity.

It has been shown (Chaplin, 1981; Robson, 1981) that, when the enzyme concentration is significant compared with substrate concentration, a simple description of the total substrate concentration may be given:

$$[S_T] = \frac{v_0}{V_{max.} - v_0} \cdot K_m + \frac{v_0}{V_{max.}} \cdot [E_T] \qquad (36)$$

Where $[E]_T$ and $[S_T]$ represent the total concentrations of enzyme and substrate respectively. The first term of the equation represents the free concentration of substrate and is identical with the description obtained from Michaelis–Menten kinetics. The second term is the concentration of enzyme-bound intermediate. In the present context one may write for the total concentration of intermediate:

$$[I_T] = \frac{v_0}{V_{max.} - v_0} \cdot K_m + \frac{v_0}{V_{max.}} \cdot [E_T] \qquad (37)$$

and hence the transition time:

$$\tau = \tau_{free} + \tau_{bound} = \frac{K_m}{V_{max.} - v_0} + \frac{[E_T]}{V_{max.}} \qquad (38)$$

where the subscripts to the transition times refer to the status of the intermediate. τ_{free} has the definition previously obtained by applying Michaelis–Menten kinetics, but the total transition time is increased by the term $[E_T]/V_{max.}$. This is the reciprocal of the turnover number of the coupling enzyme and is independent of both steady-state flux and enzyme concentration. If the enzyme concentration is increased, $V_{max.}$ also increases and eqn. (38) reaches the limiting form:

$$\tau = \frac{K_m}{V_{max.}} + \frac{[E_T]}{V_{max.}} \qquad (39)$$

The first term now describes the pseudo-first-order behaviour of the enzyme. It can be seen that τ_{bound} is only significant if $[E_T]$ is comparable with K_m. Moreover, there is a lower limit for the transition time as the enzyme concentration is increased:

$$\tau_{min.} = [E_T]/V_{max.} = (\text{turnover number})^{-1} \qquad (40)$$

The transition time at a particular step in the sequence may generally be viewed as the sum of the times required for a molecule to pass through the intermediate pool and through the enzyme. It should be emphasized that the present equations only apply when a flux-generating (rate-limiting) initial step is present and the system enters a steady state. If this is not the case, then a more complex treatment of the pathway will be necessary (Küchel & Roberts, 1974). This applies when the transition in the first enzyme needs to be considered.

The practical significance of the transition time of enzyme-bound intermediate is readily demonstrated by considering the commonly used coupling of pyruvate kinase to lactate dehydrogenase. By using data on the best commercially available preparations of lactate dehydrogenase one may calculate that τ_{bound} for pyruvate is 3 ms and only becomes comparable with τ_{free} at coupling enzyme concentrations of between 5 and 35 mg/ml, depending on the source of enzyme. Similarly, when hexokinase is coupled to glucose 6-phosphate dehydrogenase, τ_{bound} is 1.6 ms and becomes significant at enzyme concentrations of about 30 mg/ml. When other kinases are coupled to pyruvate kinase and lactate dehydrogenase τ_{bound} for ADP is 5 ms and the critical pyruvate kinase concentration is about 18 mg/ml. It follows that τ_{bound} can generally be ignored in comparison with τ_{free} in coupled systems and probably also *in vivo*.

7. *General deductions from the theory of transition times.* The main deductions from the present approach to transition times are as follows.

(*a*) The transition times of a sequence are associated with intermediates, not enzymes.

(*b*) In feedback-free systems the transition times are also associated with individual enzymes. Feedback in this context encompasses feedforward and reversibility of reaction. Back reactions represent the most direct form of negative feedback.

(*c*) Transition times are always additive with individual transition times being given by the ratio of steady-state intermediate concentration to steady-state flux (eqns. 5 and 6).

(*d*) In a sequence containing reversible steps, a transition time is dependent on all subsequent steps up to the first irreversible step.

(*e*) Where high enzyme concentrations are encountered, simple modifications to the transition time can be made. These are only significant when the transition time is comparable with the reciprocal of the turnover number of the enzyme. This condition corresponds to enzyme concentrations that are comparable with K_m.

References

Chaplin, M. F. (1981) *Trends Biochem. Sci.* **6**, no. 1, VI

Cleland, W. W. (1979) *Anal. Biochem.* **99**, 142–145

Easterby, J. S. (1973) *Biochim. Biophys. Acta* **293**, 552–558

Heinrich, R. & Rapoport, T. A. (1975) *BioSystems* **7**, 130–136

Hess, B. & Wurster, B. (1970) *FEBS Lett.* **9**, 73–77

Küchel, P. W. & Roberts, D. V. (1974) *Biochim. Biophys. Acta* **364**, 181–192

McClure, W. R. (1969) *Biochemistry* **8**, 2782–2786

Robson, B. (1981) *Trends Biochem. Sci.* **6**, no. 5, XII–XIV

Rudolph, F. B., Baugher, B. W. & Beissner, R. S. (1979) *Methods Enzymol.* **63A**, 22–42

Storer, A. C. & Cornish-Bowden, A. (1974) *Biochem. J.* **141**, 205–209

Varfolomeev, S. D. (1977) *Mol. Biol.* (*Engl. Transl.*) **11**, 612–620