

A Linear Steady-State Treatment of Enzymatic Chains

Critique of the Crossover Theorem and a General Procedure to Identify Interaction Sites with an Effector

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A theoretical analysis of the crossover theorem is presented based on a linear approximation. Cases are considered in which the simple crossover theorem may lead to erroneous conclusions. Among them are the following: more than one interaction site of an effector with the enzymatic chain; influx and efflux of metabolites regulated by outer metabolic processes; existence of inner effectors; conservation equations for metabolite concentrations; and changes of the state of complexes with the metabolites. It is shown that the action of an effector does not always produce a crossover at the affected enzyme. On the other hand, examples are given where "pseudo-crossovers" occur at unaffected enzymes. It is concluded that for real systems the identification of the interaction sites of an effector with an enzymatic chain cannot be achieved by the simple crossover theorem. Furthermore, even the identification of "rate controlling" or "regulatory important" enzymes by means of crossovers must be done with great caution.

A simple and general procedure for the identification of interaction sites of an outer effector with an enzymatic chain is proposed. It requires the determination of the flux through the chain, the concentrations of the substrates and products of the enzymatic step under consideration and the rate law by which an inner effector, if present, influences the reaction rate of this step.

The crossover theorem was first formulated by Chance *et al.* [1–4]. It deals with the influence of outer effectors on the levels of the metabolites in an enzymatic chain. In its simplest form, the "classical" crossover theorem can be stated in the following way: the variations of the concentrations of the metabolites before and beyond an enzyme which is influenced by an effector have different signs. It has been widely used to identify the interaction sites of effectors within the chain. Chance *et al.* [1–5] have applied the crossover theorem for the investigation of the changes in the steady-state oxidation-reduction levels of the components of the respiratory chain. With the help of this theorem they were able to identify the sites of phosphorylation. An inspection of the literature on metabolic regulation shows that subsequently the crossover theorem has been applied to a variety of systems including very complicated ones. It was tacitly assumed that in all these cases the crossover theorem is valid in its simple form (*e.g.* [6–8]).

Only few theoretical considerations of the crossover theorem have been published so far. Chance *et al.* [7] have studied the crossover behaviour of

the respiratory chain by means of analogue computers. In a more general manner, Holmes [9] considered several types of sequences of chemical reactions and proved the theorem for some simple cases. For the characterization of a crossover he used pairs of neighbouring metabolites where the signs (–, +) and (+, –) indicate the direction of the variation of the concentrations produced by the effector. For the condition that the effector increases the flux the pairs were called "forward" and "backward" crossovers, respectively, by Williamson [10].

The present paper deals with the limitations of the simple crossover theorem in its application to real systems. We shall consider five situations where the crossover theorem is not valid. It will be shown that the uncritical application of the crossover theorem may lead to serious misinterpretations. On the other hand, the procedure proposed in this paper for the identification of the interaction sites of an effector with an enzymatic chain will give correct results if the linear approximation holds.

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Analytical Expressions of Crossovers in Linear Enzymatic Chains

For simple linear enzymatic sequences the crossover theorem can be written by the formalism of the preceding paper [11]. It was shown that the following equations hold for the relative changes of the metabolite concentrations as functions of the relative variations of the effector concentration F

$$\begin{aligned} i < j: \quad d \ln S_i &= -C_j \frac{C_{1,i}}{1 - C_{1,i}} d \ln F \\ i \geq j: \quad d \ln S_i &= C_j X_j d \ln F \end{aligned} \quad (1)$$

where the symbols have the following meanings [11]: C_j , control strength of the affected enzyme E_j ; $C_{1,j}$, control strength of the enzyme sequence $E_1 - \dots - E_j$; X_j , effector strength of the effector F at the enzyme E_j ; S_i , metabolite concentrations.

Equation (1) immediately reveals that the concentrations of the metabolites before and beyond the enzyme which is influenced by the effector, change in opposite directions. In particular, we have for the substrate S_{j-1} and the product S_j of the enzyme E_j the variations

$$X_j > 0: \quad \Delta S_{j-1} < 0, \quad \Delta S_j > 0, \quad (2a)$$

$$X_j < 0: \quad \Delta S_{j-1} > 0, \quad \Delta S_j < 0 \quad (2b)$$

i.e. a crossover occurs between the metabolites S_{j-1} and S_j . In the first case, Eqn (2a), the changes are produced by an activator and in the second case, Eqn (2b), by an inhibitor of the enzyme E_j .

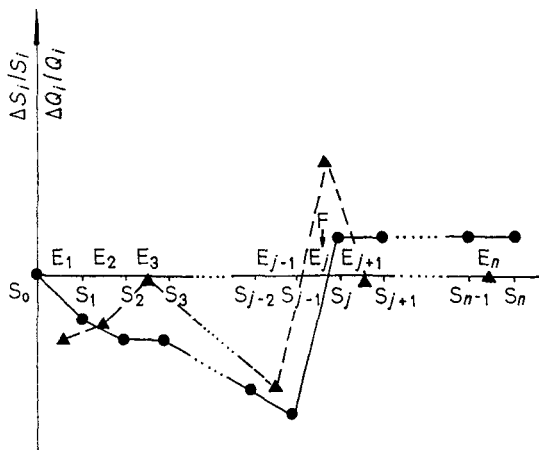


Fig. 1. Crossover plot of a hypothetical linear enzymatic system. Action of an activator on the enzyme E_j . $\Delta S_i/S_i$, relative variations of the metabolite concentrations (●—●); $\Delta Q_i/Q_i$, relative variations of the mass action ratios (▲—▲). The control strength of the enzyme E_j was assumed to be zero

For the study of the action of an effector on the enzymatic chain commonly a graphic representation of the percentage variations of each metabolite is used. For the action of an activator on a simple linear enzymatic chain such a "crossover plot" is shown schematically in Fig. 1. The characteristics of this plot may be derived from Eqn (1). It is seen that the metabolites following the enzyme E_j show all the same relative variations depending only on the effector strength X_j and the control strength, C_j . The variations of the metabolites preceding E_j depend in addition on the value of $C_{1,i}$. The greater $C_{1,i}$ the more pronounced is the decrease of these metabolites. Since $C_{1,i}$ increases along the chain, the decrease of the metabolites is greater in the neighbourhood of the interaction site.

If C_j of the affected enzyme E_j is zero its product S_j will be unchanged by an effector. If the conditions $C_j = 0$ (but $\tau_j \neq 0$, τ_j -characteristic time of E_j) and $C_{1,j-1} = 1$ hold, *i.e.* that an enzyme preceding E_j catalyses an irreversible step [11], the concentration of the substrate will be affected. The resulting variations, where only one of two neighbouring metabolites is changed, may be called "half crossovers".

The influence of an effector on the metabolite concentrations may be also described by the variations of the mass action ratios Q_i of the enzymatic reactions

$$\begin{aligned} i < j: \quad d \ln Q_i &= -\frac{C_i C_j}{(1 - C_{1,i})(1 - C_{1,i-1})} X_j d \ln F \\ i = j: \quad d \ln Q_i &= \frac{C_j}{(1 - C_{1,j-1})} X_j d \ln F \\ i > j: \quad d \ln Q_i &= 0. \end{aligned} \quad (3)$$

While the mass action ratios of the steps beyond the interaction site do not change, all other ratios show variations provided that the corresponding steps are catalyzed by enzymes with control strength greater than zero (as before, the exception $C_i = 0$, $C_j = 0$ ($\tau_i, \tau_j \neq 0$), $C_{1,i-1} = 1$ must be taken into account). For an activator the only positive variation of the mass action ratio Q_i occurs at the interaction point.

The analysis of this linear enzymatic chain reveals that the relative variations of the concentrations of metabolites as well as the relative variations of the mass action ratios can be used for an identification of the interaction site of an effector. In the first case, crossovers may be characterized by the symbols $(-, +)$ and $(+, -)$ which indicate activators and inhibitors, respectively. In the second case one can use the triplets $(-, +, 0)$ and $(+, -, 0)$ [see Eqn (3)]. The mass action ratios do not distinguish between complete and half crossovers.

LIMITATIONS OF THE CROSSOVER THEOREM

Outer Effectors with More than One Interaction Site in an Enzymatic Chain

If an effector has an influence not only on one but on several enzymes of a chain restrictions of the crossover theorem must be taken into account. For simplicity we shall deal only with two interaction sites of the outer effector. The general case of n interaction sites may be treated analogously.

It is assumed that an effector F influences the enzymes E_j and E_k with the effector strengths X_j and X_k , respectively. Then we may write for the relative variations in S_i assuming small variations ΔF in the effector concentration

$$\frac{\Delta S_i}{S_i} = (S_{i,j}X_j + S_{i,k}X_k) \frac{\Delta F}{F} \quad (4)$$

where $S_{i,j}$ and $S_{i,k}$ are elements of the control matrix defined in [11] [cf. Eqn (45) therein]. Thus, we have for the relative changes of the substrates and products of the affected enzymes E_j and E_k

$$\begin{aligned} \frac{\Delta S_{j-1}}{S_{j-1}} &= - \left(\frac{C_j C_{1,j-1}}{1 - C_{1,j-1}} X_j + \frac{C_k C_{1,j-1}}{1 - C_{1,j-1}} X_k \right) \frac{\Delta F}{F} \\ \frac{\Delta S_j}{S_j} &= \left(C_j X_j - \frac{C_k C_{1,j}}{1 - C_{1,j}} X_k \right) \frac{\Delta F}{F} \\ \frac{\Delta S_{k-1}}{S_{k-1}} &= \left(C_j X_j - \frac{C_k C_{1,k-1}}{1 - C_{1,k-1}} X_k \right) \frac{\Delta F}{F} \\ \frac{\Delta S_k}{S_k} &= (C_j X_j + C_k X_k) \frac{\Delta F}{F}. \end{aligned} \quad (5)$$

Depending on the location of the rate-controlling enzymes in the chain and on the ratio of the effector strengths X_j/X_k different crossover patterns are obtained.

We consider two cases shown in Table 1: (a) F is an activator of E_j and E_k ($X_j > 0$, $X_k > 0$); and (b) F is an activator of E_j and an inhibitor of E_k ($X_j > 0$, $X_k < 0$).

For case (a) the metabolites before the enzyme E_j decrease and those beyond E_k increase. With regard to the location of the crossover points there are three possibilities of metabolite variations (Table 1). In the first and third case crossovers occur only either at the enzyme E_j or at E_k . Crossovers can be found also simultaneously at both enzymes. A necessary condition is

$$\frac{C_{1,j}}{1 - C_{1,j}} < \frac{C_{1,k-1}}{1 - C_{1,k-1}}. \quad (6)$$

Of course, this condition can be met only if there are enzymes between E_j and E_k which have control strengths unequal to zero. Thereby, a third crossover is produced which is not, however, the result of an interaction of an effector with the enzyme at which it occurs. Therefore, we call it a "pseudo-crossover". In the case mentioned the pseudo-crossover occurs only at an enzyme with a τ -value greater than zero.

For case (b) where $X_j > 0$ and $X_k < 0$ there are only two possibilities of crossover patterns. Depending on the ratio of the control strengths as well as of the effector strengths the overall effect is an elevation or diminution of the flux ($\Delta S_k > 0$ and $\Delta S_k < 0$, respectively). A crossover occurs either at the enzyme E_j (the flux increases) or at the enzyme E_k (the flux decreases).

In order to obtain the crossover patterns for the cases $X_j < 0$, $X_k < 0$ and $X_j < 0$, $X_k > 0$ only the signs in Table 1 need to be interchanged.

Table 1. Crossover patterns of a linear enzymatic chain for an effector F acting simultaneously on the enzymes E_j and E_k . For case (a) F is an activator of E_j as well as of E_k ; for case (b) F is an activator of E_j and an inhibitor of E_k .

Case	$\frac{\Delta S_{j-1}}{S_{j-1}}$	$\frac{\Delta S_j}{S_j}$	$\frac{\Delta S_{k-1}}{S_{k-1}}$	$\frac{\Delta S_k}{S_k}$	Condition
(a) $X_j > 0$	-	+	+	+	$\frac{C_k C_{1,k-1}}{C_j (1 - C_{1,k-1})} < \frac{X_j}{X_k}$
	-	+	-	+	$\frac{C_k C_{1,j}}{C_j (1 - C_{1,j})} < \frac{X_j}{X_k} < \frac{C_k C_{1,k-1}}{C_j (1 - C_{1,k-1})}$
	-	-	-	+	$\frac{X_j}{X_k} < \frac{C_k C_{1,j}}{C_j (1 - C_{1,j})}$
(b) $X_j > 0$	-	+	+	+	$-\frac{C_k}{C_j} < \frac{X_j}{X_k}$
	$X_k < 0$	+	+	-	$\frac{X_j}{X_k} < -\frac{C_k}{C_j}$

We conclude that in all cases considered interaction sites may be overlooked or falsely postulated if one uses for their identification crossovers of the metabolite concentrations.

As mentioned before, interaction points of effectors may be also detected by an analysis of the mass action ratios of the metabolites. This method has the advantage that the effector of the enzyme E_j does not change the mass action ratios of the succeeding steps [see Eqns (3) and (5)]. Therefore, in the case of two interaction points, the second crossover in the chains is independent from the first in the kind and the extent of its variation. On the other hand, the second crossover will have no influence on the preceding one only if at least one irreversible step is located between the affected enzymes.

Influx and Efflux of Metabolites

Now we shall deal with a linear enzymatic system where one of its enzymes, say E_j , catalyzes one reaction with two substrates. Fig. 2 shows the three possibilities for influx and efflux of the metabolites P and Q which are product and substrate, respectively, of a metabolic pathway branching off. We call these substances "outer metabolites" since they are not solely determined by the enzyme system under investigation. It suffices to treat only

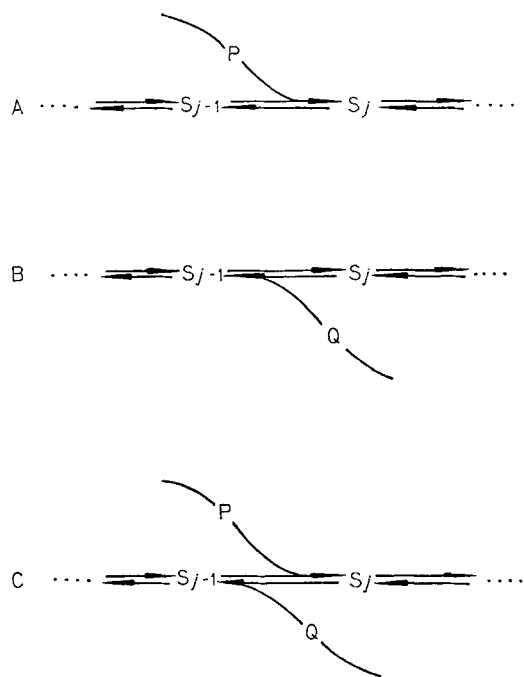


Fig. 2. Influx and efflux of metabolites. Three possibilities for the "outer metabolites" P and Q to take part in the reactions of a linear enzymatic system

the first case (influx of P) since the other cases are completely analogous.

Regarding P as a given parameter we can use for the calculation of the metabolite concentrations the formulae, previously derived in [11] [cf. Eqn (10) therein]. By a suitable transformation the two-substrate reaction of the enzyme E_j may be expressed in the following manner by means of its equilibrium constant and characteristic time:

$$q_j \rightarrow q_j P, \tau_j \rightarrow \tau_j(P) = \frac{1}{k_j P + k_{-j}}. \quad (7)$$

The kinetic parameters of all other enzymes remain unchanged. Then we obtain for the concentrations of the metabolites S_{j-1} and S_j

$$S_{j-1} = \frac{S_0}{N} \prod_{m=1}^{j-1} q_m (1 + \gamma [\tau_j(P) (1 + q_j P) \prod_{m=j+1}^n q_m + \sum_{k=j+1}^n \tau_k (1 + q_k) \prod_{m=k+1}^n q_m]) \quad (8)$$

and

$$S_j = \frac{S_0}{N} \prod_{m=1}^j q_m P (1 + \gamma [\sum_{k=j+1}^n \tau_k (1 + q_k) \prod_{m=k+1}^n q_m]) \quad (9)$$

where

$$N = 1 + \gamma [\sum_{k=1}^{j-1} \tau_k (1 + q_k) \prod_{m=k+1}^n q_m P + \tau_j(P) (1 + q_j P) \prod_{m=j+1}^n q_m + \sum_{k=j+1}^n \tau_k (1 + q_k) \prod_{m=k+1}^n q_m] \quad (10)$$

and, S_0 is the fixed substrate of the enzyme E_1 and γ the first order rate constant of the irreversible off-transport of the last metabolite S_n . It follows from the Eqns (8), (9) and (10) that

$$\frac{\partial S_{j-1}}{\partial P} < 0, \quad \frac{\partial S_j}{\partial P} > 0. \quad (11)$$

Variation of P, e.g. by action of an effector on other metabolic pathways, produces therefore a crossover at the enzyme E_j . According to our notation it is a pseudo-crossover. As Eqns (8), (9) and (10) reveal such "crossovers" may even happen at steps which are catalyzed by equilibrium enzymes, which have a very low τ -value.

For special conditions half crossovers may occur: $\frac{\partial S_{j-1}}{\partial P} = 0$ and $-\frac{\partial S_j}{\partial P} > 0$ if $\tau_i = 0$ ($i = 1, \dots, j-1$) and $\frac{\partial S_{j-1}}{\partial P} < 0$ and $\frac{\partial S_j}{\partial P} = 0$ if at least one of the enzymes preceding the step j catalyzes an irreversible step. Such half crossovers can also be produced at equilibrium steps ($\tau_j = 0$). This demonstrates that equilibria may differ in their behaviour

in the steady state as compared with isolated conditions where in any case one would expect changes of both S_{j-1} and S_j .

As before, the mass action ratios are more useful for the identification of real crossovers. If one uses the ratio $Q_j = \frac{S_j}{S_{j-1}P}$ no change is expected at variations of P if E_j is an equilibrium enzyme.

When in the junction (Fig.3A) P is increased the control strengths of the enzymes preceding E_j are increased while those of the subsequent enzymes are decreased.

The Action of Inner Effectors

So far we have dealt with a simple structure of the enzymatic chain which could be treated analytically on the assumption of linearity between the enzyme velocity v_i and the concentrations of the metabolites S_{i-1} and S_i . Generally, a real system is complicated by the fact that some metabolites are inner effectors, *i.e.* they act on enzymes of the chain.

For the investigation of the limitations of the crossover theorem we shall deal analytically only with the case of small effector strengths of the inner effectors and treat the effect in a first-order approximation.

The systems which are investigated are shown in Fig.3. In case A we have a forward activation or inhibition, in case B a feedback activation or inhibition. Three different positions of the enzyme E_j influenced by the effector F are possible which are

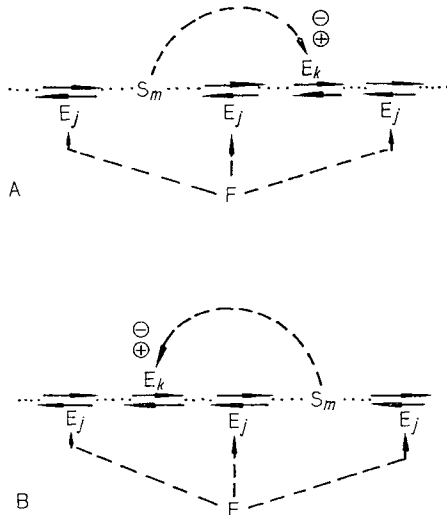


Fig.3. Schematic representation of the action of inner and outer effectors S_m and F , respectively. (A) Forward activation or inhibition of E_k by S_m ; (B) feedback activation or inhibition of E_k by S_m . Three different positions are possible for the enzyme E_j which is influenced by the outer effector F

indicated in the figure. If the influence of the metabolite S_m on the enzyme E_k is very small, we obtain for the relative variations of the metabolites produced by the effector F the approximate expressions

$$d \ln S_i = \left(\frac{\partial \ln S_i}{\partial \ln v_j} + \frac{\partial \ln S_i}{\partial \ln v_k} \frac{\partial \ln v_k}{\partial \ln S_m} \frac{\partial \ln S_m}{\partial \ln v_j} \right) \cdot \frac{\partial \ln v_j}{\partial \ln F} d \ln F = S_{i,j} X_j d \ln F + S_{i,k} S_{m,j} X_k X_j d \ln F. \tag{12}$$

$S_{i,j}$, $S_{i,k}$ and $S_{m,j}$ are elements of the control matrices, corresponding to a system without inner coupling, X_k the effector strength of the inner effector S_m acting on E_k , and X_j the effector strength of the outer effector F acting on E_j . Equation (12) can be regarded as a Taylor expansion of the relative changes of the metabolite concentrations $d \ln S_i$ with respect to X_k , quadratic and higher order terms being neglected. The first term in Eqn (12) takes into account that the outer effector F changes in the usual way *via* E_j the concentration of S_i and S_m . These changes of S_m produce *via* E_k additional variations of S_i given in the second term of the equation. If the items have opposite signs the action of the outer effector F

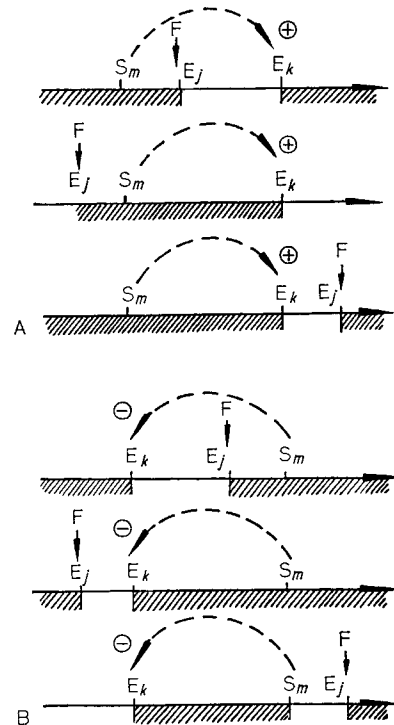


Fig.4. Regions of an enzymatic chain with inner coupling which are stable relative to the motion of an outer effector; regions of stability are hatched. (A) Forward activation of E_k by the inner effector S_m ; (B) feedback inhibition of E_k by the inner effector S_m

is damped compared with a system without coupling by inner effectors. Thus we have in the chain sections of relative stability and relative instability of metabolite concentrations with respect to changes of outer effectors (Fig.4).

It can be concluded that in most cases the effector F produces a crossover at the enzyme E_j between the metabolites S_{j-1} and S_j . In general, one of these metabolites is located in a region of stability and the other in a region of instability (Fig.4). Therefore, if the inner coupling is strong enough half crossovers may be produced. If the effectors F and S_m influence the same enzyme ($j = k$) the regions of stability and instability meet at this step and the variations of all metabolites are damped. In such a special case the action of an effector may fail to produce a crossover.

As Eqn (12) reveals crossovers occur only at the enzyme E_j which is influenced by the outer effector. This means that no additional pseudo-crossovers are produced, not even at the enzyme which is influenced by the inner effector. These statements are valid for the linear approximation, (Eqn (12)). Further work must be done in order to test their validity in the general nonlinear case

The Existence of Conservation Equations for Metabolite Concentrations

Let us consider an enzymatic chain which consists of a main pathway (metabolites S_i) and of a closed by-pass (metabolites A_j) (Fig.5). Both ways are coupled by the metabolites A_r and A_1 , the former participating with S_{j-1} and S_j in a reaction catalyzed by enzyme E_j and the latter (A_1) correspondingly with S_{m-1} and S_m in a step catalyzed by enzyme E_m . In [11] a set of differential equations was derived which describes the time-dependence of the metabolite concentrations of a linear enzymatic chain. By analogy, a system of differential equations for the description of the more complicated system, represented in Fig.5 can be easily obtained. A

simple analysis of the system shows that the following equation holds

$$\frac{d}{dt} \left(\sum_{k=j}^{m-1} S_k + \sum_{k=1}^r A_k \right) = 0. \quad (13)$$

By integration of Eqn (13) we obtain immediately a conservation relation

$$\sum_{k=j}^{m-1} S_k + \sum_{k=1}^r A_k = T. \quad (14)$$

It means that the sum T of the metabolite concentrations A_i and S_i between the enzymes E_i and E_m is independent of time. The increase of some metabolites of the cycle must be compensated by a decrease of others. Therefore, if the system is influenced by an effector acting on an enzyme which does not belong to the cycle, at least at one of its enzymes a pseudo-crossover must be produced.

Since it is tedious to solve in an analytical form the general system, represented in Fig.5, we discuss only two simple examples from which all essential conclusions can be drawn (Fig.6). The conservation equations for these systems become

$$A + S_2 + S_3 = T \quad (15)$$

and

$$A_1 + A_2 + S_2 + S_3 = T. \quad (16)$$

Assuming S_0 to be constant, steady-state values for the metabolite concentrations can be obtained. For the system in Eqn (15) we get

$$S_1 = \frac{S_0 \tau_3 \left(T - \frac{S_0 \tau_3}{\tau_1} \right)}{q_2 \tau_1 \left(1 + \frac{S_0 \tau_5}{q_4 \tau_1} \right)}, \quad S_2 = \frac{S_0 \tau_3}{\tau_1},$$

$$S_3 = \frac{T - \frac{S_0 \tau_3}{\tau_1}}{1 + \frac{q_4 \tau_1}{S_0 \tau_5}}, \quad S_4 = \frac{S_0 \tau_5}{\tau_1}, \quad A = \frac{T - \frac{S_0 \tau_3}{\tau_1}}{1 + \frac{S_0 \tau_5}{\tau_1}} \quad (17)$$

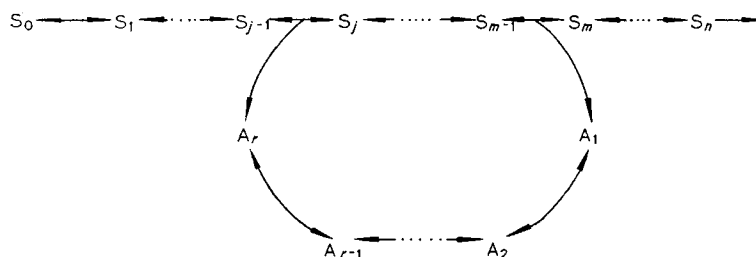


Fig.5. Scheme of a cycled metabolic pathway. The variations of the metabolite concentrations are restricted by the conservation equation $\sum_{k=j}^{m-1} S_k + \sum_{k=1}^r A_k = T$

and for the system in Eqn (16)

$$\begin{aligned}
 S_1 &= \frac{S_0 \tau_3}{\tau_1 q_2 \left[T - \frac{S_0 \tau_3}{\tau_1} - \frac{S_0 \tau_6}{\tau_1} - \left(\frac{S_0}{\tau_1} \right)^2 \frac{\tau_5 \tau_6}{q_4} \right]}, \\
 S_2 &= \frac{S_0 \tau_3}{\tau_1}, \\
 S_3 &= \left(\frac{S_0}{\tau_1} \right)^2 \frac{\tau_5 \tau_6}{q_4}, \\
 S_4 &= \frac{S_0 \tau_5}{\tau_1}, \\
 A_1 &= \frac{S_0 \tau_6}{\tau_1}, \\
 A_2 &= T - \frac{S_0 \tau_3}{\tau_1} - \frac{S_0 \tau_6}{\tau_1} - \left(\frac{S_0}{\tau_1} \right)^2 \frac{\tau_5 \tau_6}{q_4}.
 \end{aligned} \quad (18)$$

Now we consider the effect of an activator of the first enzyme E_1 on the metabolite concentrations. It is easily seen that the following inequalities hold for the system in Eqn (15)

$$\frac{\partial S_1}{\partial \tau_1}, \frac{\partial S_2}{\partial \tau_1}, \frac{\partial S_4}{\partial \tau_1} < 0, \quad \frac{\partial A}{\partial \tau_1} > 0. \quad (19)$$

Different signs can be obtained for the corresponding derivative of S_3 dependent on the kinetic constants of the enzymes and the values of S_0 and T

$$\frac{\partial S_3}{\partial \tau_1} \geq 0 \quad \text{if} \quad \tau_3 \geq \tau_1 \frac{T}{S_0 \left(2 + \frac{S_0 \tau_5}{q_4 \tau_1} \right)}. \quad (20)$$

Comparing the inequalities of Eqns (19) and (20) we conclude that the action of the activator on the enzyme E_1 produces a pseudo-crossover between the metabolites S_2 and S_3 if the reaction of the enzyme E_3 is slow. The characteristic time of this enzyme must be such that the upper inequality of the relation of Eqn (20) is fulfilled. If the reaction is fast all metabolites S_i increase and only A decreases. If the changes are related to the external metabolite A there appear to exist pseudo-crossovers between A and S_2 as well as between S_3 and A .

For the system in Eqn (16) we get the inequalities

$$\frac{\partial S_1}{\partial \tau_1}, \frac{\partial S_2}{\partial \tau_1}, \frac{\partial S_3}{\partial \tau_1}, \frac{\partial S_4}{\partial \tau_1}, \frac{\partial A_1}{\partial \tau_1} < 0, \quad \frac{\partial A_2}{\partial \tau_1} > 0. \quad (21)$$

It can be concluded that an effector of E_1 produces in each case a pseudo-crossover at the enzyme E_6 between the metabolites A_1 and A_2 .

These examples show that for systems having a cyclic structure the crossover theorem is not applicable. Due to the existence of conservation terms for the metabolite concentrations pseudo-crossovers occur. This statement is valid whether or not a linear approximation is used. The metabolite variations of systems with several cycles may be restricted by more than one conservation term.

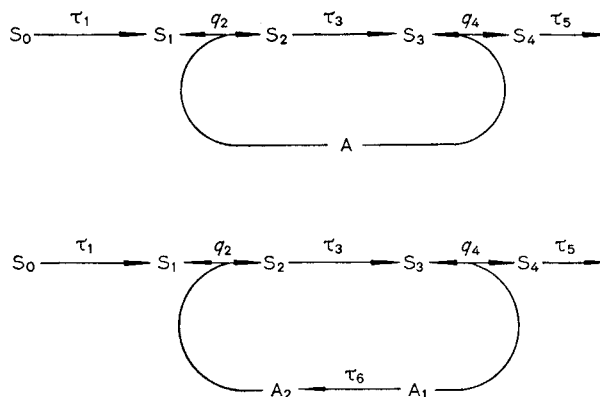


Fig. 6. Schemes of two cyclic metabolic pathways. It is assumed that the two-substrate reactions catalyzed by the enzymes E_2 and E_4 are very fast and that the slow enzymes E_1 , E_3 , E_5 and E_6 catalyze irreversible reactions

Of course, for such systems it is difficult to predict the crossover behaviour. However, it seems reasonable to assume that pseudo-crossovers can be found predominantly at slow or irreversible enzymes.

Change of the Complexation State of the Metabolites

In this section we consider again a linear enzymatic system where several metabolites react with the enzymes as metal complexes, for example as Mg-complexes. Depending on the metal ion concentration and on the association constants of the complexes there are varying concentrations of complexed and uncomplexed species of the metabolites in the system. Only the active forms of the metabolites must be considered for the regulation of any enzyme, if the inactive species are not effectors of any enzyme. With respect to the crossover theorem we are interested in the variations of the total concentrations of the metabolites S_i^t due to changes of the free metal ion concentration. These may be produced by changes either of the total metal concentrations or by variations of metabolites with high binding capacities. For the sake of simplicity we confine ourselves to 1:1 complexes between the metal and the metabolites and assume that for the metabolites S_j the uncomplexed species S_j^f and for the metabolites S_k ($k \neq j$) the complexed forms $S_k M$ are active. Then we have for all metabolites S_i the relationships

$$\frac{S_i M}{S_i^f \cdot M^f} = K_i \quad (22)$$

where K_i are the association constants of the complexes, M^f the concentration of the free metal ions. At given concentration of the free metal ions

the total concentrations S_j^t and S_k^t can be easily obtained as functions of their free and complexed forms, respectively

$$S_j^t = (1 + K_j M^t) S_j^f \quad (23)$$

$$S_k^t = \frac{(1 + K_k M^t) S_k M}{K_k M^t} \quad (24)$$

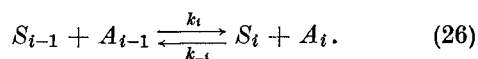
In the steady state the levels of S_j^f and $S_k M$ are determined by the rate and equilibrium constants of the enzymatic steps. Thus, they are constant for varying concentrations of the free metal ions. Therefore one obtains

$$\begin{aligned} \frac{\partial \ln S_j^t}{\partial \ln M^t} &= \frac{K_j M^t}{1 + K_j M^t} \\ \frac{\partial \ln S_k^t}{\partial \ln M^t} &= -\frac{1}{1 + K_k M^t} \end{aligned} \quad (25)$$

It is apparent that pseudo-crossovers produced by variation of the free metal ion concentration can only be expected at steps where the active forms of substrate and product are different, *i.e.* one reacts as a complex and the other as a free species. Neighbouring metabolites which react both either in the complexed or in the uncomplexed form are changed in the same direction but, depending on their association constants, to different extents. A change in the state of complexes could even cause differences in the relative variations of metabolites connected by an equilibrium enzyme. This may explain the "fine" structure of crossover plots for the influence of effectors which act not only at enzymes but change also the concentration of the free metal ions.

General Procedure for the Identification of Interaction Sites for Linear Systems

The following derivation is independent of the number of substrates and products which participate in the reactions. For the sake of simplicity, however, we regard a bimolecular reaction



For the flux through the chain the following equation holds if one uses a linear approximation

$$v = k_i S_{i-1} A_{i-1} - k_{-i} S_i A_i \quad (27)$$

This equation may be rearranged to

$$\frac{S_{i-1} A_{i-1}}{v} \left(1 - \frac{Q_i}{q_i}\right) = \frac{1}{k_i} = \frac{\tau_i (1 + q_i)}{q_i} \quad (28)$$

where Q_i is the mass action ratio of the reaction given in Eqn (26).

On the right side of Eqn (28) now stands a constant. If this step is influenced by an effector the characteristic time τ_i will be changed. Inversely,

if one wishes to identify the interaction sites of an effector it is only necessary to insert in Eqn (28) the experimental values before and after addition of the effector. All the enzymatic steps for which the expression in Eqn (28) is changed are interaction sites of the effector.

The use of Eqn (28) circumvents most of the difficulties mentioned for the application of the crossover theorem for the identification of interaction sites. Firstly, it is independent of the number of interaction sites of the effector with the enzymatic chain. Secondly, since all metabolites that are substrates or products of the reaction enter the Eqn (28) the influx and efflux of metabolites is taken into account. Thirdly, the existence of conservation equations for metabolite concentrations is no limitation for the application of Eqn (28). Fourthly, if one uses the concentrations of the active species instead of the total concentrations of the metabolites, changes in the state of complexes of the metabolites do not affect the use of Eqn (28). Lastly, if there exists a coupling by inner effectors it is still possible to apply Eqn (28) provided the rate law by which the rate constant on the right side of the Eqn (28) is influenced is known. For instance, for the simple cases of competitive or non-competitive inhibitions of the reaction i by the metabolite S_j

$$v = \frac{q_i S_{i-1} A_{i-1} - S_i A_i}{\tau_i \left(1 + \frac{S_j}{K_j}\right) (1 + q_i)} \quad (29)$$

(K_j is the inhibition constant of S_j) one derives under linear approximation

$$\frac{S_{j-1} A_{j-1} \left(1 - \frac{Q_i}{q_i}\right)}{v \left(1 + \frac{S_j}{K_j}\right)} = \frac{\tau_i (1 + q_i)}{q_i} \quad (30)$$

In this case, one has to determine also the concentration of the metabolite S_j and the inhibition constant K_j for the identification of an interaction of an outer effector at the step i .

The use of Eqn (28) for the identification of interaction sites is limited to the case of a linear dependence of the enzymatic velocity on the substrate concentrations. This is true for low substrate concentrations. However, for Michaelis-type enzymes less restricted conditions give the same equation (Rohde, Heinrich and Rapoport, unpublished results).

Two special cases of Eqn (28) should be discussed. If the enzyme E_i catalyses an irreversible step the following simplified expression obtains:

$$\frac{S_{i-1} A_{i-1}}{v} = \frac{1}{k_i} \quad (31)$$

If the enzyme E_i is an equilibrium enzyme ($Q_i \approx q_i$) the left hand side of Eqn (28) becomes zero and the evaluation of a change in the rate constant is impossible.

It should be mentioned that an equation similar to Eqn (28) was previously derived by Hess and Brand [12]. So far it was not applied, however, to the identification of interaction sites of an effector.

DISCUSSION

The crossover theorem has been used in the literature to analyse the action of effectors on complicated enzymatic systems. The present paper demonstrated that in many cases the analysis of simple crossover plots fails to identify the interaction sites. Even a system as simple as the glycolysis can show several pseudo-crossovers. For instance, there exist several points where outer metabolites take part in the reaction (*e.g.* glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase), there exists a conservation quantity for the oxidized metabolites under anaerobic conditions [13] and there are several reactions where the metal complexes of the metabolites play a significant role. Williamson studied the metabolic control of perfused rat heart, in particular the inhibition of the glycolysis by acetate and pyruvate [6,7]. Applying the simple crossover theorem he concluded that both the phosphofructokinase and glyceraldehyde-phosphate dehydrogenase are inhibited. The latter interaction site does not seem justified from the obtained crossover plot.

It is clear that in even more complicated enzymatic systems the conclusions drawn from the crossover plot are rather doubtful. Several authors stated that crossovers indicate the rate-limiting steps of an enzymatic chain. For instance, Wilhelm *et al.* [8] concluded by means of crossover plots that the rate control in the glycolysis of Ehrlich-ascites-tumor cells is shifted from the phosphofructokinase at low pH-values to the glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase at higher pH-values. As will be shown in a succeeding paper [13], this conclusion is not justified from the patterns of the crossover plots.

The application of the crossover theorem to time-dependent processes [7] lacks any theoretical basis.

Simple methods for the analysis of time-dependent processes have not been worked out so far.

The procedure proposed in this paper for the identification of interaction sites of an effector with the chain does not require much more information than a simple crossover plot. In addition to the behaviour of the metabolites of the chain there must be known: the concentrations of outer metabolites, the flux, the equilibrium constants of the various enzymatic steps and possible inner effectors and their rate laws. In most cases simplifications can be probably made (*cf.* [13]). Although the proposed procedure may be limited to some extent to a linear dependence of the reaction rates on the substrate concentrations it is much to be preferred to the use of the crossover theorem.

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