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Kinetics of metabolic pathways

A system *in vitro* to study the control of flux

Néstor V. TORRES,* Fatima MATEO,* Enrique MELÉNDEZ-HEVIA*‡ and Henrik KACSER†
*Departamento de Bioquímica, Facultad de Biología, Universidad de La Laguna, 38000 Tenerife, Canary Islands, Spain, and †Department of Genetics, University of Edinburgh, Edinburgh EH9 3JN, Scotland, U.K.

A method for determining Control Coefficients is proposed for systems studied *in vitro* and applied to a model pathway. Rat liver extract, which converts glucose into glycerol 3-phosphate, was used with the addition to the incubation mixture of fructose-bisphosphate aldolase, triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase as 'auxiliary' enzymes, which leaves all the control on the first three enzymes. The flux of the metabolic pathway was recorded by assaying NADH decay. Flux Control Coefficients (C^J_E) of hexokinase, glucose-6-phosphate isomerase and phosphofructokinase were calculated by titration of the system with increasing quantities of extraneous enzymes. It is shown that the summation property is fulfilled. The applicability of this procedure to study the control in any metabolic pathway is discussed. Possible relevance of the method to conditions *in vivo* and its limitations are considered.

INTRODUCTION

Control of flux in metabolic pathways is shared among several, if not all, points of the system. The quantitative contribution of each element can be expressed by means of several coefficients of control, rigorously defined in their theory of control of Kacser & Burns (1973) and Heinrich & Rapoport (1974*a*). Earlier steps towards a theory are Higgins (1963, 1965), Kacser (1963), Kacser & Burns (1968), Burns (1969) and Savageau (1969, 1971*a*,*b*, 1972), and later developments are Kacser & Burns (1979, 1981), Heinrich & Rapoport (1974*b*,*c*, 1975, 1977, 1983), Rapoport *et al.* (1976), Kacser (1983), Fell & Sauro (1985) and others [see reviews by Groen *et al.* (1982*a*), Porteous (1983) and Westerhoff *et al.* (1984)]. Control Coefficients of a flux with respect to enzyme activity, C^J_E ('Sensitivity Coefficients' or 'Control Strengths' in the previous literature; see Burns *et al.*, 1985), are systemic coefficients of which there are as many involved in a given metabolic pathway as there are enzymes. The Flux Control Coefficient, $C^J_{E_i}$, of the metabolic pathway flux for an individual enzyme, E_i, within it, is defined as:

$$C^J_{E_i} = \frac{\delta J/J}{\delta e_i/e_i} \quad (1)$$

where J is the flux of the system and e_i is the activity of any enzyme E_i. Thus the Control Coefficient is defined as the fractional change of flux produced as a result of an infinitesimal fractional change in the activity of an individual enzyme. Sensitivity of the systems to changes in concentration or activity of different enzymes can be very different, and thus the particular values of these coefficients give information on the distribution of control in the metabolic pathways among all enzymes involved in it.

A number of studies have dealt with the relationships between the flux of a metabolic system *in vivo* and the concentrations or activities of particular enzymes with the use of genetic means to alter the enzyme parameters (see, e.g., Flint *et al.*, 1981; Kacser & Burns, 1981; Middleton & Kacser, 1983). With the use of a variety of theorems and methods arising from the Control Theory, mitochondrial respiration has been investigated in a part-system of isolated mitochondria. Values of the Flux Control Coefficients, at various rates of respiration, have been determined for the different steps in this stystem (see, e.g., Groen *et al.*, 1982*b*; Wanders *et al.*, 1984).

Control Theory is concerned with the study of metabolic pathways within the whole system in order to determine the regulatory effects of each enzyme on the total flux, i.e. it applies to situations *in vivo* (Kacser & Burns, 1979). Although a study of metabolic regulation must be made *in vivo* in order to draw physiological conclusions, part-systems or systems studied *in vitro* can nevertheless be used to explore certain aspects of control distribution. In the present paper such a method is described and is demonstrated by reference to a model system *in vitro*. The possible relevance of such results to the situation *in vivo* and the limitations of the method are indicated.

METHODOLOGY

The flux carried by a pathway is normally a positive function of the activity of any of its enzymes. In many cases this relationship can be represented by a hyperbolic function (Kacser & Burns, 1979; see also Waley, 1964):

$$J = \frac{Q_1 e_i}{Q_2 + e_i} \quad (2)$$

where Q_1 and Q_2 are constants that are functions of the kinetic parameters of all the other (constant) enzymes [for examples of this see, e.g., Kacser & Burns (1981), Flint *et al.* (1981), Middleton & Kacser (1983) and Salter *et al.* (1986)]. The Control Coefficient defines the response of the flux, at the value it has, to a small (infinitesimal)

‡ To whom correspondence should be addressed.

change in the activity of the enzyme at the value it has. From its definition (eqn. 1) we can derive the expression for the coefficient by forming the derivative of function (2) and multiplying this by the scaling or normalizing factor e_i/J. This gives:

$$C^{J}_{Ei} = \frac{\partial J}{\partial e_i}\cdot\frac{e_i}{J} = \frac{Q_1 - J}{Q_1} \quad (3)$$

or

$$C^{J}_{E_i} = \frac{Q_2}{Q_2 + e_i} \quad (4)$$

We here use eqns. (2) and (3) to obtain a formulation that can be used experimentally. Inverting eqn. (2) gives:

$$\frac{1}{J} = \frac{Q_2}{Q_1}\cdot\frac{1}{e_i} + \frac{1}{Q_1} \quad (5)$$

a straight line whose intercept at $1/e_i = 0$ gives the value of Q_1. The data for such a line can be obtained if a series of enzyme activities are available and the fluxes measured.

Q_1 is a parameter of the system whose physical significance is the maximal flux of the pathway obtained when the activity of E_i tends to infinity, i.e. the flux of the system after addition of sufficient enzyme activity so that this enzyme in no way limits the flux.

$$Q_i = \lim_{e_i \to \infty} J = J_{\max.(e_i)}$$

so that eqn. (3) is:

$$C^{J}_{E_i} = \frac{J_{\max.(e_i)} - J}{J_{\max.(e_i)}} \quad (6)$$

For each enzyme in a pathway there will, in general, be a different $J_{\max.}$, and the determination of these values will allow the calculation of the control coefficients for each enzyme at a given J.

An important consequence of the theory is the establishment of the summation property that states that the sum of all Flux Control Coefficients of a metabolic pathway is equal to unity, i.e.:

$$\sum_{i=1}^{n} C^{J}_{E_i} = 1 \quad (7)$$

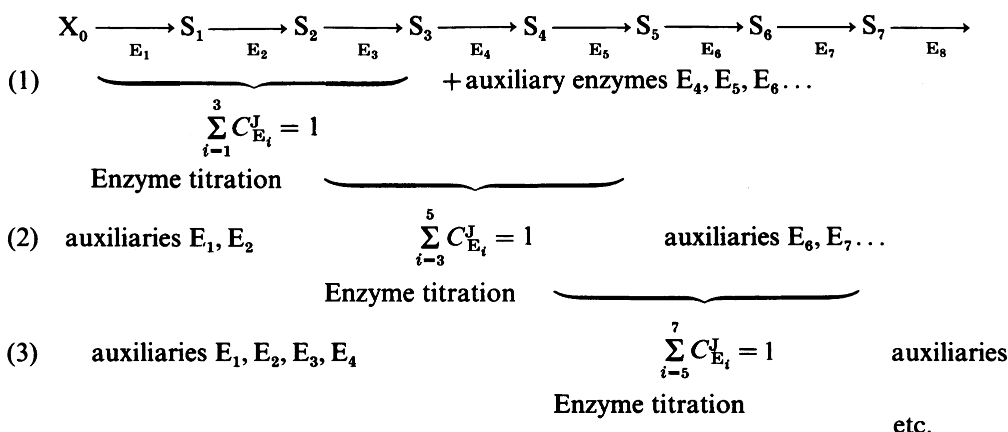
For a studied system *in vitro*, such as the extract of a particular tissue, the variation in each enzyme activity can be produced by adding more enzyme to the extract. One can thus 'titrate' the flux (measured in some suitable manner) by successive additions of enzyme. In this way, by using eqn. (5), the values of the different $J_{\max.}$ are obtainable. The source of such added enzyme is irrelevant, provided that account is taken of the difference in kinetic constants of the endogenous and added enzyme species. We must bear in mind that e_i values express total activity of the enzymes in the system, which are those of the extract plus those added in the titration procedure. This makes it necessary to assay the enzyme activity in the extract before titration. On the other hand the system can be titrated with an enzyme from any biological source, provided that e_i values, i.e. V/K_m, are plotted. Control Coefficient values can thus be calculated according to eqn. (3) by using only the value of J (the flux before enzyme addition in titration experiments) and Q_1 values, after verification of the hyperbolic relationship between flux and enzyme activity, processing data by means of eqn. (5).

A practical problem of this titration method arises from the summation property. If the number of enzymes is reasonably large some Control Coefficients will be small, and this has often been observed (Kacser & Burns, 1981). Titration of such enzyme steps is not likely to result in clearly observable increases in flux. If the pathway is 'shortened' by the addition of large enzyme activities distal to the part to be investigated, then Control Coefficients of steps with such 'auxiliary' enzymes become virtually zero, and the coefficients of the remaining steps are increased, since $\Sigma C^{J}_{E_i} = 1$. As is discussed below, the ratios of the Control Coefficients so determined will be the same as in the whole system.

The method is shown by Scheme 1.

THE MODEL SYSTEM

Our experimental system consists of a soluble extract from rat liver with glucose, ATP and NADH as substrates, which converts glucose into glycerol 3-phosphate by means of enzymes of the glycolytic



Scheme 1. Arrangement of the steps in the experimental approach to obtain all Control Coefficient values in a metabolic pathway

The ratios of Control Coefficients of the pathway enzymes are obtained by determining these coefficients in fragments of the pathway with the use of auxiliary enzymes.

pathway. Previous experiments on glycolysis *in vitro* (Meléndez-Hevia *et al.*, 1984) show an important flux towards glycerol 3-phosphate with rat liver extracts, hexokinase (EC 2.7.1.2) and phosphofructokinase (EC 2.7.1.11) being the enzymes mainly responsible for the control of the system. Thus glycerol 3-phosphate production by rat liver extracts is a useful system to be used as a model to study control distribution. Fructose-bisphosphate aldolase (EC 4.1.2.13), triose-phosphate isomerase (EC 5.3.1.1) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) were added in large concentrations as 'auxiliary' enzymes in all experiments so that their Control Coefficients were virtually zero. The other enzymes, hexokinase (EC 2.7.1.1), glucose-6-phosphate isomerase (EC 5.3.1.9) and phosphofructokinase (EC 2.7.1.11), were present at liver extract concentrations. Therefore they must account for the total control of this modified metabolic pathway and their Control Coefficients must add up to unity. Acceleration of the last three steps by adding their corresponding auxiliary enzymes also results in a simple linear pathway from glucose to glycerol 3-phosphate, thus avoiding drainage of glyceraldehyde 3-phosphate towards L-lactate or to the pentose phosphate cycle, which would involve negative Control Coefficients. Steady state was ensured by the following experimental conditions. (1) The flux produced with 5 mM-glucose does not result in significant changes of glucokinase saturation. (2) ATP concentration was buffered by phosphocreatine and creatine kinase (EC 2.7.3.2). (3) The system as described, however, has a long transition time, since fructose 2,6-bisphosphate, an effector for phosphofructokinase, is produced (Hers & Van Schaftingen, 1982). The long transition time was shortened by adding this effector to the incubation mixture in order to obtain a more rapid approach to the steady state. This steady state was verified by observing a constant flux and constant glucose 6-phosphate and fructose 6-phosphate concentrations during each experiment. Flux of the pathway was assayed by recording NADH decay in the glycerol-3-phosphate dehydrogenase reaction. The kinetics of the three enzymes were studied in the same conditions as those for the system flux experiments; their activities were assayed and their K_m values were determined and found to be as described in the literature under similar conditions (Pilkis, 1975; Castaño *et al.*, 1979) (results not shown). In applying this approach it is necessary to assay the commercial enzymes used in titration experiments under the same conditions as occur in the system, in order to obtain their kinetic parameters. The results of these activity assays were the same as those indicated by the manufacturer, and their K_m values were also the same as those described in the literature (Bergmeyer, 1974). Glycogen degradation during the assays was not significant, glucose being the only source of carbon. Enzyme activities were assayed after a number of experiments; there was no significant loss of activity, indicating that proteolysis was negligible.

MATERIALS AND METHODS

Chemicals

ATP, NADH, phosphocreatine, fructose 6-phosphate, fructose 2,6-bisphosphate, glucose 6-phosphate, chicken egg-white trypsin inhibitor (type II), Hepes, streptomycin sulphate, penicillin, hexokinase, glucose-6-phosphate isomerase, phosphofructokinase, fructose-bisphosphate aldolase, triose-phosphate isomerase, glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and creatine kinase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucose monohydrate and other reagents were obtained from E. Merck, Darmstadt, Germany.

Animals and diets

Female Wistar albino rats (180–220 g), fed on a standard laboratory diet (65% carbohydrate, 11% fat, 24% protein) *ad libitum*, were used in all experiments. Animals were housed at 20–22 °C with light from 07:00 to 19:00 h. They were killed between 90 and 100 days of age.

Preparation of rat liver extracts

Livers were obtained under ether anaesthesia between 09:00 and 10:00 h, cooled, chopped and homogenized at 1 g/3 ml in 50 mM-Hepes/10 mM-sodium phosphate buffer, pH 7.4, containing 100 mM-KCl, 10 mM-$MgCl_2$ and 1 mg of trypsin inhibitor/ml by using a Potter–Elvehjem homogenizer with Teflon pestle (about 1000 rev./min during 1 min) in an ice-cold bath. The homogenates were clarified twice by centrifugation at 27000 *g* in a Sorvall RC-5B centrifuge with a Sorvall RC-34 rotor at 3–4 °C for 20 and 10 min respectively. The resulting supernatants were used immediately for kinetic experiments, enzyme assays and protein measurements.

Enzyme and protein assays

Hexokinase (glucokinase) assays were carried out as described by Pilkis (1975), with 1 mM-ATP. Glucose-6-phosphate isomerase was assayed as described by Noltmann (1966). The phosphofructokinase assay was designed in our laboratory and in accordance with published methods (Castaño *et al.*, 1979), with 1 mM-ATP. All enzyme activities are expressed in units (μmol of product produced/min). Velocity of the reaction was continuously monitored by recording change in absorbance at 340 nm with a Hitachi 100-60 spectrophotometer (Hitachi, Tokyo, Japan). Temperature was stabilized at 35 °C with a thermocirculator (Churchill Instruments Co., Perivale, Middx., U.K.). Fructose-bisphosphate aldolase, triose-phosphate isomerase, glycerol-3-phosphate dehydrogenase and the commercial enzymes used in titration experiments (hexokinase, glucose-6-phosphate isomerase and phosphofructokinase) were assayed as indicated in Bergmeyer (1974) and their K_m values determined in the same conditions as that of the experimental system. Protein concentrations were assayed in a Hepes-free sample by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co.) as standard.

Titration experiments

All titration experiments were carried out in the cuvette of a Hitachi 100-60 spectrophotometer. Fluxes were measured by continuous recording of NADH decay at 340 nm and 35 °C. The total volume of the incubation mixture was 2 ml, containing 100 μl of diluted liver extract. Final concentrations are 1.5 mg of protein/ml, 5 mM-glucose, 1 mM-ATP, 0.28 mM-NADH, 2.5 mM-phosphocreatine, 15 μM-fructose 2,6-biphosphate, 5 units of creatine kinase/ml, 1 unit of fructose-bisphosphate aldolase/ml, 5 units of triose-phosphate isomerase/ml and 3 units of glycerol-3-phosphate dehydrogenase, the

auxiliary enzymes being added to the mixture in the same buffer described above. Reactions were started by addition of liver extract to the mixture with substrates and auxiliary enzymes. Flux was measured, after a transition time (2–3 min), in the absorbance range from 0.8 to 0.3 A, where NADH decay was always linear. In titration assays diluted enzymes were added to the mixture at convenient concentrations according to the experiment. Total assay time was about 10–15 min. Flux values were normalized with respect to the value of basal flux (without any titrating enzyme added, only the auxiliary enzymes being present). This basal flux was taken as 1 in each series of experiments. The sums of enzyme activities (basal and added) were divided by their respective K_m values to obtain the corresponding e_i parameter values. Reciprocal values of flux and e_i were inserted in eqn. (5) to obtain the normalized values of Q_1. By using eqn. (3), Control Coefficients, C^J_E, were calculated by:

$$C^J_{GK} = \frac{Q_{1(GK)} - 1}{Q_{1(GK)}};\; C^J_{GPI} = \frac{Q_{1(GPI)} - 1}{Q_{1(GPI)}};\; C^J_{PFK} = \frac{Q_{1(PFK)} - 1}{Q_{1(PFK)}} \quad (8)$$

where GK, GPI and PFK represent hexokinase (glucokinase), glucose-6-phosphate isomerase and phosphofructokinase respectively.

RESULTS AND DISCUSSION

The results of titration of the system with hexokinase, glucose-6-phosphate isomerase and phosphofructokinase are shown in Fig. 1. Reciprocal values of these data are plotted in Fig. 2, giving good linearity (coefficients of regression about 0.99), which confirms the hyperbolic relationship described in eqn. (5). Intercepts with the ordinate give Q_1 values, with which Control Coefficients were calculated as shown in Table 1. It can be seen that summation of all these coefficients is nearly unity (1.01). Thus these results confirm the application of several aspects of the theory: first, eqn. (5), where a hyperbolic relationship between flux and enzyme activity is described; secondly, the summation property for all Flux Control Coefficients, i.e. $\Sigma C^J_{E_i} = 1$; furthermore, it is also shown that Flux Control Coefficients of the auxiliary enzymes are equal to zero, which is, in fact, a condition for any auxiliary enzyme. For this system *in vitro*, hexokinase and phosphofructokinase account for the total control of this part of the system, the system not being sensitive to small changes in glucose-6-phosphate isomerase activity (or, of course, any auxiliary enzyme). From the data of Table 1 it can be seen that the endogenous activities of the last three enzymes were quite high, suggesting that their normal Flux Control Coefficients were small to start with. Further work is required to explore this aspect.

The hyperbolic relationship of flux plotted versus enzyme activity is the basic requirement of the method. This behaviour is theoretically expected if the enzymes of the pathway operate at low saturation. For such a case algebraic solutions can be obtained (Kacser & Burns, 1973, 1981) that give expressions for each enzyme titration as given in eqn. (5). Hyperbolic relationships are also obtained for any degree of saturation of one or any of the enzymes provided that the degree of saturation does not change significantly during the titration

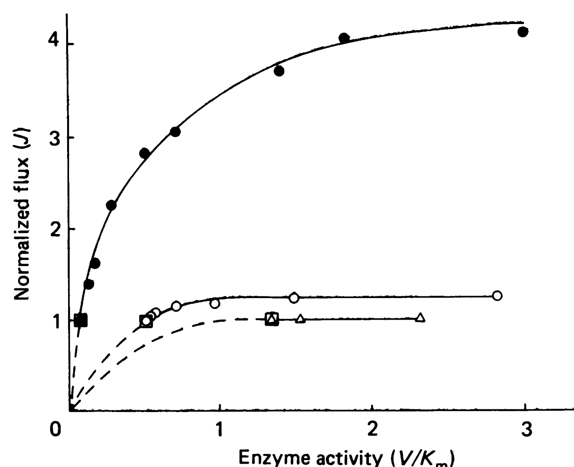


Fig. 1. Titration of the metabolic system with enzymes

The metabolic system was titrated with hexokinase (●), glucose-6-phosphate isomerase (△) and phosphofructokinase (○). Flux of the metabolic pathway without added enzymes is expressed as 1 on the ordinate axis, and the point marked □ in each curve corresponds to the flux with the enzyme present in the liver extract only. Addition of hexokinase or phosphofructokinase to the system enhances the flux, showing substantial values of the Flux Control Coefficients. Addition of glucose-6-phosphate isomerase does not modify the flux, demonstrating that its Flux Control Coefficient is virtually zero. Further addition of any auxiliary enzyme (results not shown) does not modify the flux either. Broken lines, arbitrarily traced, show zones of the curves without experimentally obtained data, corresponding to enzyme activities lower than those present in the extract. Values of hexokinase activity are multiplied by 10 in the graph for graphical convenience.

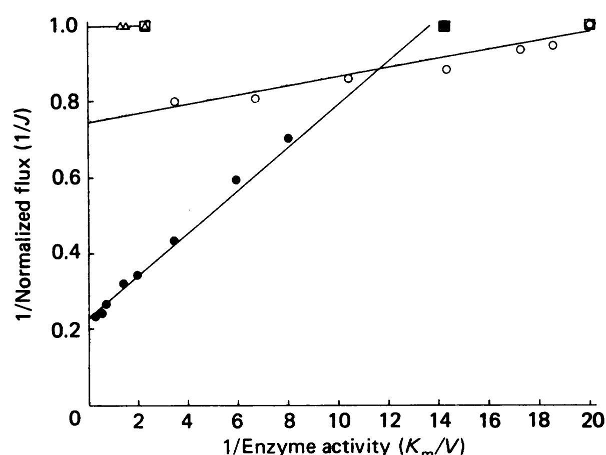


Fig. 2. Double-reciprocal plot of data shown in Fig. 1

Values of Q_1 and Q_2 shown in Table 1 were obtained from the intercepts at the ordinate and slopes respectively. Flux Control Coefficients were calculated by eqn. (3) (see Table 1). In this graph reciprocal values of phosphofructokinase activity have been multiplied by 10. ●, Hexokinase; △, glucose-6-phosphate isomerase; ○, phosphofructokinase.

Table 1. Parameters of the experimental system and Control Coefficients (C^J_E)

Enzyme activity is expressed as units (μmol of reaction product produced/min)/mg of protein. The metabolic system *in vitro* was carried out in the cuvette of the spectrophotometer to obtain values of flux. Q_1 and Q_2 parameter values, according to eqn. (5), were obtained from results shown in Fig. 2. Q_1 values were normalized, as described in the Materials and methods section, by setting the value of the flux equal to 1 in the system without added enzymes for titration (basal value of flux = 0.02 ± 2.5 nmol of glycerol 3-phosphate/min per mg of protein). Control Coefficients (C^J_E) were calculated from data of normalized Q_1 as described in eqn. (3). Note that, for enzymes with $C^J_E = 0$, $Q_1 = 1$. As expected from theory, summation of all Flux Control Coefficients of the system is near unity. Results are given as means ± S.D. for three experiments.

Enzyme	Activity (units/mg)	Q_1	Q_2	C^J_E
Hexokinase	0.07 ± 0.002	4.36 ± 0.13	0.025 ± 0.0007	0.77 ± 0.025
Glucose-6-phosphate isomerase	0.27 ± 0.008	1.00 ± 0.06	—	0.00 ± 0.005
Phosphofructokinase	0.05 ± 0.015	1.33 ± 0.05	0.15 ± 0.0004	0.24 ± 0.01
Fructose-biphosphate aldolase*	1.33 ± 0.025	1.00	—	0.00
Triose-phosphate isomerase*	6.66 ± 0.15	1.00	—	0.00
Glycerol-30-phosphate dehydrogenase*	4.00 ± 0.17	1.00	—	0.00
Summation of Flux Control Coefficients (ΣC^J_E)...				1.01 ± 0.015

* Activities of fructose-bisphosphate aldolase, triose-phosphate isomerase and glycerol-3-phosphate dehydrogenase are those of the experimental system, after addition of commercial enzymes to make then 'auxiliaries'. Their activities before this addition were respectively 0.33, 1.66 and 1.00 units/mg of protein.

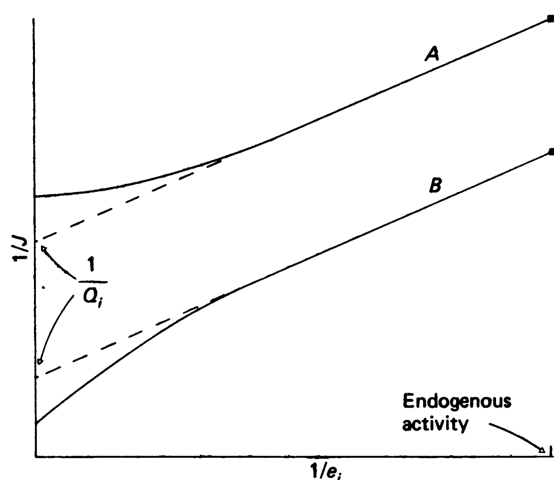


Fig. 3. Effects of changes in saturation during titration

The Figure shows a computer simulation of changes in pathway flux where the degree of saturation of one or several enzymes changes significantly during titration. Curve *A*, increase in saturation; curve *B*, decrease in saturation.

procedure owing to the movement of the pools. If such changes in saturation do occur, the inverse plot will deviate in the manner given in Fig. 3. $J_{max.}$ values so obtained by extrapolation to $1/e_i = 0$ will therefore either over- or under-estimate the Q_1 parameter (J. S. Hofmeyr & H. Kacser, unpublished work). Titration results will nevertheless be usable if extrapolation is carried out by using the initial points as indicated in the Figure. This is the equivalent of estimating the slope of the J-versus-e_i relationship by using small differences around the steady state (modulation). Reciprocal plots (or any other linear transformation, such as the equivalent of the Eadie–Hofstee plot) will give a better estimate than the estimated tangent of the direct plot.

If the unsaturated conditions apply, the 'shortening' of the pathway will leave the ratios of the Control Coefficients undisturbed although their absolute values will have increased. This will make it practicable to measure the relative values of the coefficients, which in the whole system may be too small to measure with any accuracy. By a series of overlapping shortened segments all the relative values of the Control Coefficients can be estimated. The determination of the absolute value of any one (by whatever method) will then give the absolute value of all.

The relevance of these considerations to the analysis of systems *in vivo* will depend on some information of the saturation status of the pathway enzymes. A similar deviation from hyperbolicity will occur if negative feedbacks (or other non-linear interactions) exist and change substantially during titration. It is further obvious that if extraction destroys important structural aspects, such as membrane attachments or compartmentations, the behaviour of the system *in vitro* will not give true estimates of the control relations in the system *in vivo*. The method may, however, be of some value, particularly if genetic variation or specific inhibitors are not readily available.

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