Biochem. J. (1993) 296, 423–433 (Printed in Great Britain)

Flux control coefficients determined by inhibitor titration: the design and analysis of experiments to minimize errors

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This paper is a study into the effects of experimental error on the estimated values of flux control coefficients obtained using specific inhibitors. Two possible techniques for analysing the experimental data are compared: a simple extrapolation method (the so-called graph method) and a non-linear function fitting method. For these techniques, the sources of systematic errors are identified and the effects of systematic and random errors are quantified, using both statistical analysis and numerical computation. It is shown that the graph method is very sensitive to random errors and, under all conditions studied, that the fitting method, even under conditions where the assumptions underlying the fitted function do not hold, outperformed the graph method. Possible ways of designing experiments to minimize the effects of experimental errors are analysed and discussed.

INTRODUCTION

In all biochemical experiments there will be a difference, or 'error', between a calculated or observed value and the true value. Even in the hands of an experienced experimenter with the 'best' equipment available, systematic errors (errors which are reproducible, such as those due to faulty calibration or biased observations) and random errors (i.e. fluctuations in observations which result in differences from experiment to experiment) will remain. Systematic errors will determine, in general, how accurate a measurement can be made, while random errors will determine how precise we can be in making the measurement [1]. If quantitative conclusions (as opposed to purely qualitative ones) are to be made from these experiments, the effects of systematic and random errors on these conclusions need to be carefully considered.

Metabolic control analysis (MCA) [2–4] is one such area where it is hoped that quantitative conclusions can be made. One of the fundamental concepts of MCA is the control coefficient, which gives a measure of the sensitivity of a systemic variable to a particular parameter. The flux control coefficient of enzyme E_i on flux J is given the symbol $C^J_{E_i}$. There have been many different techniques developed to determine experimentally the control coefficients [4]. One of these, developed by Groen et al. [5], involves titrating out an enzyme's activity using an inhibitor specific to that enzyme. It has been suggested that the traditional method (the so-called graph method) of analysing the experimental data to obtain an estimate of the value of the control coefficient may be highly sensitive to experimental errors [6–8]. It has also been suggested [8,9], that using a non-linear function to fit more of the data points than are used by the traditional method may help to reduce the effects of these errors. This alternative method, however, will also be subject to random, and particularly systematic, errors. This study, using numerical and statistical techniques, aims to identify the sources of the systematic error in both methods, and attempts to quantify the effects of random and systematic errors on the estimated values of the control coefficient obtained. Some conclusions for experimental design will be presented.

THE GRAPH METHOD

Groen et al. [5] derived a method to determine experimentally the flux control coefficients using titrations with specific enzyme inhibitors. As the amount of inhibitor tends to zero, then the response of the flux to the inhibitor can be expressed in MCA terms. For the case of an irreversible, specific, inhibitor, Groen et al. [5] have shown that an estimate of the value of the flux control coefficient of the inhibited enzyme is given by:

$$C^{J^o}_{E_i} = \left(\frac{\Delta J}{\Delta I}\right)_{I\to 0} \cdot \frac{I_{max.}}{J^o} \qquad (1)$$

where $(\Delta J/\Delta I)_{I\to 0}$ is the initial slope of the flux/inhibitor graph, J^o is the uninhibited flux value and $I_{max.}$ is the concentration of inhibitor which totally inhibits the enzyme. For a completely irreversible inhibitor, i.e. one with a dissociation constant, K_D, equal to zero, $I_{max.}$ can be determined easily from the flux–inhibitor graph. In most cases the dissociation constant, although very small, will not be exactly zero, and so a complete inhibition of enzyme activity does not occur. In this case, $I_{max.}$ must be determined by extrapolation. All three terms in eqn. (1) can be estimated solely from a plot of flux against inhibitor concentration (see Figure 1). Since for other types of inhibitors, e.g. competitive and non-competitive, the values of kinetic parameters (K_i and/or K_m) are also required [10], and these will probably be obtainable only from *in vitro* data, the irreversible (or low K_D) inhibitor is potentially the most accurate to use and so will be the focus of this study.

ERROR CONSIDERATIONS

To identify the source and effect of the two kinds of experimental errors, systematic error will be firstly considered in the absence of random error, and vice versa, before the combined effect of both kinds is analysed.

Systematic errors can arise from many sources, e.g. techniques used to quantify fluxes and/or inhibitor levels. However, we will only be concerned here with systematic errors which are peculiar

Abbreviation used: MCA, metabolic control analysis; abbreviations used only in Scheme 2 and Appendix 3 are defined there.

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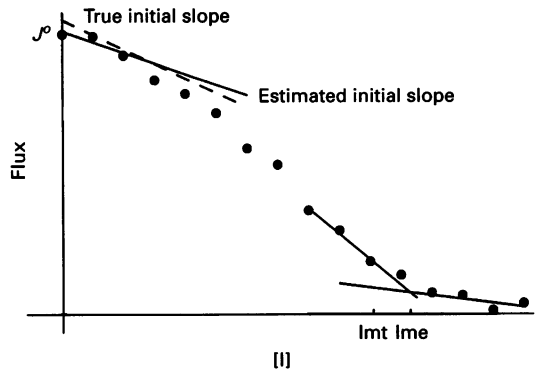


Figure 1 Determination of the parameters required in eqn. (1) to estimate the value of a flux control coefficient from a plot of flux against inhibitor concentration for an essentially irreversible (low K_D) inhibitor

Data points were generated using eqns. (3) and (4), and were randomized as described in the main paper. The 'experimental' initial slope (continuous line) estimate is determined by regression analysis to the first three data points. The true initial slope (broken line) is also shown. The estimate of $I_{max.}$, Ime, is determined by taking the intercept of two lines generated by linear regression of (a) the final three points and (b) an appropriate three points taken from the steepest part of the flux–inhibitor plot. Imt shows the true $I_{max.}$ value.

to the graph method. Owing to the extrapolation involved there will be inherent systematic errors associated with the graph method. The required 'initial slope' is, by definition, the slope obtained when an infinitesimal amount of inhibitor is added. Since this is impossible to do in practice, finite amounts of inhibitor must be used and the initial slope estimated by extrapolation back to zero inhibitor. The larger these finite changes are, the less likely that the estimate of the initial slope will be true. Owing to the convex nature of the flux–inhibitor slope, these estimates will tend to systematically overestimate the value of the initial slope. Experimentally, therefore, the best strategy will be to obtain estimates of the flux at very low levels of inhibitor (see, however, the following paragraph). In addition, the value of I_{max} is most likely to be obtained by extrapolation, and Gellerich et al. [8] have shown that this form of extrapolation may, with certain K_D values, introduce significant error into the estimate of the control coefficient.

On top of this systematic error, there will of course be random errors in the measurements of both the fluxes and inhibitor concentrations. The initial slope is particularly sensitive to this kind of error (see Figure 1), and this can have a dramatic affect on the final value of the control coefficient obtained. It can be shown (Appendix 1) that, if the first two inhibitor points and the zero inhibitor point (i.e. three points in total) are used to obtain an estimate of the initial slope by linear regression, then, in the absence of systematic errors, the confidence limits around the estimated value of the control coefficient can be approximated as follows:

$$C \pm \frac{I_{max.}}{\Delta I} \cdot \sqrt{2} \cdot \frac{\sigma_J}{J} \cdot \frac{t_{n-1}}{\sqrt{n}} \qquad (2)$$

where ΔI is the difference in concentration between the second inhibitor point and the zero inhibitor point, σ_J/J is the relative S.D. of the flux estimates, n is the number of repeat measurements of each flux at each inhibitor point and t_{n-1} is the Student t value for $n-1$ degrees of freedom. This equation assumes that each flux measurement has a constant relative S.D., $I_{max.}$ is determined accurately and no random error in the inhibitor values occurs.

The most important result from this expression is the fact that the confidence limits are inversely proportional to ΔI, i.e. the smaller the inhibitor concentrations used to determine the initial slope, the larger the confidence limits will be. This result illustrates why the use of the graph method is very sensitive to error. To reduce the effects of random error we must increase ΔI, i.e. use greater inhibitor concentrations in our estimate of the initial slope. However, as stated above, as we increase the amount of inhibitor we are increasing the amount of systematic error in the initial slope estimate. There is therefore a serious conflict of interest.

Eqn. (2) does suggest two possible ways of reducing the effects of random error. The first is to measure each point many times. If we assume that there is a constant relative error in all our flux measurements of 5% (i.e. $\sigma_J/J^o = 0.05$), and the largest inhibitor concentration which does not introduce any significant systematic error into the initial flux is 10% of $I_{max.}$, i.e. $I_{max.}/\Delta I = 10$, then to obtain 95% confidence limits of ± 0.1 around our estimate of C the following must be true:

$$0.1 \cdot \frac{\sqrt{n}}{t_{n-1}} = \frac{I_{max.}}{\Delta I} \cdot \sqrt{2} \cdot \frac{\sigma_J}{J^o}$$

$$\Rightarrow \frac{\sqrt{n}}{t_{n-1}} = 7.071$$

The solution to this equation is $n = 192$. In other words, each point must be measured more than 192 times to be certain of obtaining, under these (generous) conditions, 95% confidence limits better than $C \pm 0.1$. Clearly, this is not a feasible solution.

The second possibility is to use more precise methods of measuring the fluxes, such that the S.D.s of the measurements are reduced. In a similar manner to above, to obtain 95% confidence limits better than $C \pm 0.1$, with $I_{max.}/\Delta I = 10$, $n = 5$ (hence $t_4 = 2.776$), σ_J must be less than $0.0057 \times J^o$. Whether this level of precision can be obtained is dependent on the system under study and the techniques involved.

The above results are based on simplifying assumptions, and so to obtain a better understanding of the range of estimates of control coefficients which can result due to random and systematic error, a simulation study was carried out. Computer-generated flux–inhibitor data sets were produced using eqn. (3) below. To simulate the effects of random error in the flux measurements, an algorithm was written which, when given two values, $\bar{x}$ and s, generated a random number drawn from a set of numbers normally distributed around a mean $\bar{x}$ with a standard deviation of s. This algorithm was used on each computer-generated flux 'measurement', J_i, with J_i as the mean and a given S.D. of 5% of J_i. This was repeated five times for each J_i and an average taken of the resulting random numbers. Thus each of these averages ('experimental' J_i values) was assumed to be analogous to repeating the measurement of each flux, at each inhibitor point, five times under experimental conditions.

For each data set this whole process was repeated 100 times, and for each of these 100 experimental sets the graph method was used to estimate the value of the control coefficient under the given conditions. In most cases, the initial slope was obtained by linear regression using the first three (including $I_i = 0$) data points. In some cases, however, where using only three points resulted in a positive sign for the initial slope, further inhibitor points were also used. J^o, the uninhibited flux value, was also obtained from the same linear regression equation. An estimate of $I_{max.}$ was obtained as described in the legend to Figure 1. In the following results the combined effect of errors in initial slope, J^o and $I_{max.}$ will therefore be considered.

Table 1 Effect of experimental error on the estimate of flux control coefficients using the graphical method

Statistics are based on $n = 100$.

Case	K_D	$\Delta I / I_{max.}$	No error C^J	Experimental error Av. C^J	S.D. C^J	Min.	Max.	%±0.1	%±0.03
A	0.001	0.1	0.33	0.37	0.235	0.003	0.95	20	7
B	0.1	0.1	0.37	0.40	0.254	0.000	1.03	38	12
C	0.001	0.3	0.38	0.38	0.082	0.15	0.56	55	17
D	0.1	0.3	0.43	0.41	0.111	0.19	0.75	47	16

The results are shown for four situations in Table 1. In all cases, the true value of the control coefficient was set to equal 0.3. The effects of increasing K_D and $\Delta I/I_{max.}$ were investigated. Case A shows the best estimate of the control coefficient (0.33) in the absence of random error, illustrating that, under these conditions, there is little systematic error. However, when random error was included, a wide range of estimates was obtained (0–0.95). Only 20% of the estimates fell within ±0.1 of the true value. In fact, 13% of the estimates were $\leqslant 0.1$ and 11% of the estimates were $\geqslant 0.7$. Case B shows the effect of a larger K_D. There is an immediate increase in the systematic error, as shown in the value of the estimate of C^J when no random error is included. Again, when random error was included, a wide range of estimates was obtained.

By comparing the results for Case C and Case A, we can see the effect of increasing the range of inhibitor points used to estimate the initial slope. It is clear that this increase has introduced more systematic error, but on the other hand it has reduced the effect of random error. Case D, where both K_D and $\Delta I/I_{max.}$ have been increased, shows the greatest increase in systematic error, and also a reduction in the effect of random error.

Clearly, the estimated value of the control coefficient from the graph method is subject to a high degree of error. Even in the best case above (Case C), only 17% of the estimates fell within ±10% of the true value. The following section of this paper is concerned with an alternative method of estimating the control coefficient from the same data set.

THE FITTING METHOD

It is clear from the previous section that much of the error in value of the control coefficient obtained by the graph method is due to the high sensitivity of the initial slope to random error. To reduce the effect of systematic as well as random error, it would be desirable to use a non-linear function to fit to more (or all) of the flux–inhibitor data. Due to the general non-linear properties of enzyme rate equations, it is impossible to obtain an explicit algebraic equation which expresses the flux through a pathway solely in terms of system parameters. One alternative is to fit a polynomial to the data; however polynomials are generally not very good at approximating functions which are asymptotic in nature, which is what we may expect as I tends to zero. A simulation study did, however, show that some improvement was obtained by this method [9]. An alternative solution is to assume that the relationship between an enzyme, E_j and J, the flux it carries, is of a rectangular-hyperbolic nature. Using this assumption it can be shown [11] that:

$$J^I = J^o \cdot \frac{E_j^I}{E_j^I - C^J_{E_j} \cdot (E_j^I - E_j^o)} \tag{3}$$

where J^o is the flux at the uninhibited point, E_j^o is the enzyme concentration at the uninhibited point, E_j^I is the total concentration of enzyme not bound to the inhibitor at a concentration of inhibitor equal to I_t, and J^I is the resulting flux at that level of inhibition. To proceed any further, the relationship between the concentrations of free enzyme and total inhibitor must be known. For the type of inhibition we are considering, the enzyme and inhibitor are assumed to be subject to the equilibrium:

$$K_D = \frac{E \cdot I_t}{EI}$$

where K_D is the dissociation constant for the enzyme–inhibitor complex EI, EI is the concentration of the complex, E is the concentration of the non-inhibitor-bound enzyme and I_t is the free inhibitor concentration. This relationship assumes that the inhibitor binds non-competitively. The following relationship is therefore a consequence of this equilibrium:

$$E_j^I = 0.5 \cdot \{(E_j^o - I_t - K_D) + \sqrt{[(I_t - E_j^o + K_D)^2 + 4 \cdot K_D \cdot E_j^o]}\} \tag{4}$$

By substituting eqn. (4) into eqn. (3) we have a possible function to fit to the inhibitor–flux data (J^I versus I_t). There are four parameters whose best-fit values will be obtained by non-linear curve-fitting (J^o, E_j^o, K_D and $C^J_{E_j}$).

Eqn. (4) is only valid for the case where the flux monitored is that which the inhibited enzyme catalyses. If a flux through another branch is monitored, then the following flux–enzyme relationship must be used [12]. For example, if enzyme E_a is the inhibited enzyme which catalyses the flux J_A but the monitored flux is J_B, then:

$$J_B^I = J_B^o \cdot \frac{E_a^I - (C^{J_A}_{E_a} - C^{J_B}_{E_a}) \cdot (E_a^I - E_a^o)}{E_a^I - C^{J_A}_{E_a} \cdot (E_a^I - E_a^o)} \tag{5}$$

where $C^{J_A}_{E_a}$ is the control coefficient of the enzyme with respect to the flux through its own branch, J_A, and $C^{J_B}_{E_a}$ is the control coefficient of the enzyme with respect to the monitored flux. By substitution of eqn. (4) into this equation, we again obtain a possible function to fit to our inhibitor titration data. Note that a non-linear curve-fit in this case will return best-fit estimates of five parameters, including the values of the two control coefficients, i.e. $C^{J_A}_{E_a}$, $C^{J_B}_{E_a}$, J_B^o, E_a^o and K_D. This equation is identical with that used by Gellerich et al. [8] when they considered this problem, except that they failed to notice that a second control coefficient could be estimated from the same data and instead they had an alternative fifth parameter, J_t, corresponding to the flux at complete inhibition of the enzyme. This is because, as I tends to infinity (i.e. $E_a \rightarrow 0$) in eqn. (5), the flux tends to:

$$J_B^o \cdot \left(1 - \frac{C^{J_B}_{E_a}}{C^{J_A}_{E_a}}\right) \tag{6}$$

The same raw 'experimental' data as were used in the study of the graph method were used in conjunction with eqns. (3) and (4) to obtain a best-fit estimate of the control coefficient in the presence and absence of random error. Since the original data had been generated using these equations, we will be considering, in this case, random error alone (i.e. zero systematic error). The

Table 2 Effect of experimental error on the estimate of flux control coefficients using the fitting method

Statistics are based on $n = 100$.

Case	K_D	$\Delta I / I_{max.}$	Experimental error: No error C^J	Av. C^J	S.D. C^J	Min.	Max.	%±0.1	%±0.03
A	0.001	0.1	0.30	0.30	0.014	0.27	0.33	100	96
B	0.1	0.1	0.30	0.30	0.028	0.22	0.36	100	75
C	0.001	0.3	0.30	0.30	0.023	0.25	0.34	100	77
D	0.1	0.3	0.30	0.30	0.034	0.22	0.38	100	59

Table 3 Influence of the true value of the control coefficient on the effects of experimental error

Statistics are based on $n = 20$. '%OK1' represents the percentage of the estimates which are ±0.1 of the true value, except for case E, where it represents the percentage of estimates < 0.15. '%OK2' represents the percentage of the estimates which are ±0.1% of the true value, except for case E, where it represents the percentage of estimates < 0.1.

Case	K_D	$\Delta I / I_{max.}$	True C^J	Experimental error: No error C^J	Av. C^J	S.D. C^J	Min.	Max.	%OK1	%OK2
E_{gra}	0.1	0.4	0.05	0.11	0.15	0.08	0.01	0.28	45	5
E_{fit}	0.1	0.4	0.05	0.05	0.05	0.01	0.02	0.07	100	100
F_{gra}	0.1	0.3	0.8	0.9	0.89	0.06	0.81	1.01	50	40
F_{fit}	0.1	0.3	0.8	0.8	0.8	0.06	0.67	0.91	90	75
G_{gra}	0.001	0.1	0.8	0.82	0.79	0.25	0.26	1.33	40	35

effects of systematic errors will be dealt with in the following sections. Table 2 gives the results for the four different cases. It is clear that the effect of random error in the flux measurements has much less effect on the control coefficient ascertained by this method, as opposed to the graphical method. In all cases the estimates of the control coefficient fell within ±0.1 of the true value of 0.3, and the spread of estimates, as summarized by the S.D., also decreased dramatically with the fitting method. It should be noted, however, that as K_D and/or $(\Delta I/I_{max.})$ increased, the fitting method became more susceptible to random error, although not as much as the graphical method.

To check whether the size of the true control coefficient value played a role in the effect of random error, further simulations were carried out using eqns. (3) and (4) with a low value and a high value for the control coefficient. Note that in these examples only 20 estimates (as opposed to 100 for the previous examples) were used to generate the statistics. Table 3 shows a summary of the results. For the graphical (*gra*) technique, comparing Case D in Table 1 ($C^J = 0.3$), with Case E_{gra} ($C^J = 0.05$) and Case F_{gra} ($C^J = 0.8$) in Table 3, where all three cases have a similar $\Delta I/I_{max.}$ ratio, indicates that the S.D.s in the 'experimental' data are similar in magnitude, no matter what the value of the coefficient. This resulted in a reasonable spread of estimates when the control coefficient was high (Case F_{gra}), but, owing to the systematic error resulting from using such a high $\Delta I/I_{max.}$ ratio, the estimates were clustered around 0.9 instead of 0.8, the true value. Attempts to decrease the systematic error (Case G_{gra}) resulted in a much larger S.D. and hence a large spread of estimates. Again, for case E_{fit} [where the fitting (*fit*) procedure was used], a much better spread of estimates was obtained. In the case of the high control coefficient value (F_{fit}) the graph method performed equally as well as the fitting method in terms of spread, but because the systematic error in the fitting method is set to zero, the estimates obtained by the fitting method were spread around the true value of the control coefficient.

Table 4 Effect of experimental error on the estimate of flux control coefficients where the monitored flux is not the flux carried by the inhibited enzyme and the ratio of the control coefficients of the enzyme with respect to the monitored flux and the flux it carries is equal to 1.2

Statistics are based on $n = 20$. '%OK1' represents the percentage of the estimates which are ±0.1 of the true value, except for estimate of the ratio, where it represents those estimates ±1. '%OK2' represents the percentage of the estimates which are ±0.1% of the true value in all four cases.

Control coefficient	Method	True C^J	Experimental error: No error C^J	Av. C^J	S.D. C^J	Min.	Max.	%OK1	%OK2
$C^{J_B}_{e_a}$	*gra*	0.25	0.36	0.35	0.119	0.18	0.62	60	10
$C^{J_B}_{e_a}$	*fit*	0.25	0.25	0.23	0.042	0.15	0.31	95	35
$C^{J_A}_{e_a}$	*fit*	0.30	0.30	0.28	0.052	0.18	0.38	95	35
$C^{J_A}_{e_a}/C^{J_B}_{e_a}$	*fit*	1.2	1.2	1.2	0.015	1.16	1.22	100	100

Table 5 Effect of experimental error on the estimate of flux control coefficients where the monitored flux is not the flux carried by the inhibited enzyme and the ratio of the control coefficients of the enzyme with respect to the monitored flux and the flux it carries is equal to 2

Statistics are based on $n = 20$. '%OK1' represents the percentage of the estimates which are ±0.1 of the true value, except for estimate of the ratio, where it represents those estimates ±1. '%OK2' represents the percentage of the estimates which are ±0.1% of the true value in all four cases.

Control coefficient	Method	True C^J	Experimental error: No error C^J	Av. C^J	S.D. C^J	Min.	Max.	%OK1	%OK2
$C^{J_B}_{e_a}$	*gra*	0.15	0.22	0.22	0.137	0.03	0.46	40	5
$C^{J_B}_{e_a}$	*fit*	0.15	0.15	0.16	0.067	0.06	0.30	85	20
$C^{J_A}_{e_a}$	*fit*	0.30	0.30	0.31	0.137	0.12	0.61	55	20
$C^{J_A}_{e_a}/C^{J_B}_{e_a}$	*fit*	2.0	2.0	1.99	0.077	1.82	2.12	100	100

The use of eqn. (5) (when the monitored flux is in a branch other than the branch in which the inhibited enzyme lies) in order to obtain estimates of two flux control coefficients was also tested. Tables 4 and 5 give summaries of the results for the example where an enzyme E_a is inhibited and the flux monitored is J_B, assuming the relationships shown in eqns. (4) and (5). The two control coefficients which can be determined by such an experiment are therefore $C^{J_A}_{E_a}$, the control coefficient for the flux through the branch in which the enzyme lies, and $C^{J_B}_{E_a}$, the control coefficient for the monitored branch flux. Note that, using the graph method, only $C^{J_B}_{E_a}$ can be determined.

As was the case for the unbranched case, the curve-fitting procedure appears to be less sensitive to random error than the graph method. The curve-fitting procedure has the added advantage of providing estimates to the values of two control coefficients. In the first example (Table 4) the effect of random error appears to be the same for the estimates of both the coefficients. In the second example (Table 5), however, the effect of random error is greater for the second, additional, control

coefficient. This is despite the fact that, under the same conditions, the fitting-procedure estimate of the ratio of the coefficients ($R = C^{J_A}_{E_a}/C^{J_B}_{E_a}$) is not as sensitive to this error. The fact that σ_R is much less than expected from the individual S.D. suggests that the estimates of the two control coefficients are correlated; in fact, the estimates are highly correlated [correlation coefficient (r) > 0.997 in both cases]. This positive correlation means that:

$$\sigma_R \leqslant \sigma_{C^{J_A}} + \sigma_{C^{J_B}} \quad (7)$$

and the fact that the correlation is approximately 1 means that

$$\sigma_{C^{J_A}} \approx R \cdot \sigma_{C^{J_B}} \quad (8)$$

(see Appendix 2). These results are both reflected in Tables 4 and 5.

SYSTEMATIC ERROR AND THE FITTING METHOD

The results presented above suggest that, with the correct choice

Table 6 Effect of saturation in a branched system on the estimates of the control coefficient values obtained by both the graphical (*gra*) and the fitting (*fit*) methods

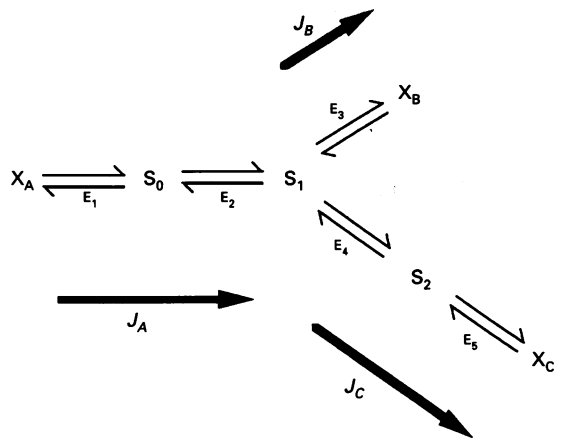
(a) Δ Saturation is the change in the saturation function, $(1 + S_1/K_{m_{4f}} + S_2/K_{m_{4r}})$, for enzyme E_4. The initial conditions in this case were low flux control and high initial saturation (saturation function equals 15.51). The parameter values were as described in the legend to Scheme 1 and, in addition, $V_{max_4} = 500$, $K_{m_{4f}} = 50$ and $K_{m_{4r}} = 75$. The value of the dissociation constant, K_D, was set to be 0.01 times the $V_{max.}$ of the inhibited enzyme. Statistics are based on $n = 20$. (b) Δ Saturation is the change in the saturation function, $(1 + S_1/K_{m_{4f}} + S_2/K_{m_{4r}})$, for enzyme E_4. The initial conditions in this case were low flux control and medium initial saturation (saturation function equals 2.47). The parameter values were as described in the legend to Scheme 1, and, in addition, $V_{max_4} = 500$, $K_{m_{4f}} = 50$ and $K_{m_{4r}} = 75$. The value of the dissociation constant, K_D, was set to be 0.01 times the $V_{max.}$ of the inhibited enzyme. Statistics are based on $n = 20$.

(a)

Enzyme inhibited	Δ Saturation	Flux monitored	Control coefficient	Method	True value	No error C^J	Experimental error			
							Av. C^J	S.D. C^J	Min.	Max.
E_1	−14.28	J_A	$C^{J_A}_{E_1}$	*gra*	0.61	0.73	0.72	0.09	0.58	0.89
				fit	0.61	0.61	0.60	0.04	0.52	0.69
		J_B	$C^{J_B}_{E_1}$	*gra*	0.66	0.78	0.79	0.11	0.62	0.97
				fit	0.66	0.66	0.67	0.09	0.51	0.81
			$C^{J_A}_{E_1}$	*fit*	0.61	0.66	0.67	0.10	0.50	0.81
		J_C	$C^{J_C}_{E_1}$	*gra*	0.57	0.69	0.68	0.13	0.47	0.97
				fit	0.57	0.57	0.56	0.08	0.40	0.68
			$C^{J_A}_{E_1}$	*fit*	0.61	0.57	0.56	0.08	0.39	0.70
E_4	3.25	J_B	$C^{J_B}_{E_4}$	*gra*	−0.08	−0.14	−0.26	0.14	−0.04	−0.56
				fit	−0.08	−0.09	−0.10	0.04	−0.03	−0.56
			$C^{J_C}_{E_4}$	*fit*	0.08	0.08	0.09	0.04	0.03	0.20
		J_C	$C^{J_C}_{E_4}$	*gra*	0.08	0.13	0.17	0.11	0.01	0.32
				fit	0.08	0.08	0.08	0.01	0.06	0.10
E_5	17.62	J_B	$C^{J_B}_{E_5}$	*gra*	−0.48	−0.64	−0.61	0.19	−0.24	−0.93
				fit	−0.48	−0.47	−0.51	0.14	−0.27	0.83
			$C^{J_C}_{E_5}$	*fit*	0.44	0.44	0.47	0.13	0.23	0.75
		J_C	$C^{J_C}_{E_5}$	*gra*	0.44	0.59	0.61	0.11	0.45	0.85
				fit	0.44	0.43	0.45	0.06	0.36	0.53

(b)

Enzyme inhibited	Δ Saturation	Flux monitored	Control coefficient	Method	True value	No error C^J	Experimental error			
							Av. C^J	S.D. C^J	Min.	Max.
E_1	−1.44	J_A	$C^{J_A}_{E_1}$	*gra*	0.61	0.72	0.71	0.09	0.57	0.90
				fit	0.61	0.6	0.6	0.06	0.49	0.72
		J_B	$C^{J_B}_{E_1}$	*gra*	0.65	0.77	0.79	0.10	0.65	1.00
				fit	0.65	0.65	0.68	0.09	0.54	0.81
			$C^{J_A}_{E_1}$	*fit*	0.60	0.65	0.68	0.10	0.53	0.82
		J_C	$C^{J_C}_{E_1}$	*gra*	0.57	0.69	0.67	0.13	0.42	0.92
				fit	0.57	0.57	0.56	0.09	0.36	0.73
			$C^{J_A}_{E_1}$	*fit*	0.61	0.57	0.56	0.10	0.35	0.74
E_4	0.32	J_B	$C^{J_B}_{E_4}$	*gra*	−0.12	−0.19	−0.22	0.15	−0.01	−0.49
				fit	−0.12	0.65	0.68	0.09	0.54	0.81
			$C^{J_C}_{E_4}$	*fit*	0.12	0.12	0.12	0.04	0.05	0.23
		J_C	$C^{J_C}_{E_4}$	*gra*	0.12	0.19	0.28	0.11	0.06	0.54
				fit	0.12	0.12	0.12	0.02	0.07	0.16
E_5	1.75	J_B	$C^{J_B}_{E_5}$	*gra*	−0.42	−0.55	−0.50	0.13	−0.23	−0.78
				fit	−0.42	−0.41	−0.40	0.11	−0.22	−0.65
			$C^{J_C}_{E_5}$	*fit*	0.42	0.42	0.41	0.12	0.22	0.71
		J_C	$C^{J_C}_{E_5}$	*gra*	0.42	0.55	0.54	0.12	0.31	0.77
				fit	0.42	0.41	0.42	0.05	0.35	0.55



Scheme 1 A simple branched pathway

All enzymes show linear kinetics, $v_i = (V_{max_i}/K_{m_i})\cdot(S_i - S_j/K_i)$, except enzyme E_4 whose rate equation is $v_4 = (V_{max_4}/K_{m_{4f}})\cdot(S_1 - S_2/K_4)/(1 + S_1/K_{m_{4f}} + S_2/K_{m_{4r}})$. Pools of X_A, X_B and X_c are considered to be constant. The initial values of the parameters were set as follows: $X_A = 500$; $X_B = 0.01$; $X_C = 0.01$; $V_{max_1} = 250$; $K_{m_1} = 1000$; $K_1 = 0.82$; $V_{max_2} = 625$; $K_{m_2} = 1000$; $K_2 = 1.15$; $V_{max_3} = 750$; $K_{m_3} = 1000$; $K_3 = 2.5$; $K_4 = 1.2$; $V_{max_5} = 1000$; $K_{m_5} = 1000$; $K_5 = 1.1$. V_{max_4}, $K_{m_{4f}}$ and $K_{m_{4r}}$ were set to achieve the initial conditions required.

of functions, the fitting method is more precise and accurate than the graphical one. In this section, the effect of systematic error in the fitting method will be examined. If the functions chosen do not accurately reflect the true relationships then this will introduce systematic error into the determination of the control coefficient value. Widely inaccurate functions should be indicated by a poor goodness-of-fit, but a more serious problem may arise if the function is consistent with the data, in that a reasonable fit is obtained, but the interpretation of what the best-fit parameter values represent is flawed.

Eqns. (3) and (5) are derived on the assumption that either linear kinetics hold, or that if any non-linearities do occur (e.g. saturability), they do not change significantly in response to the change in inhibitor concentration. It is likely, however, that these assumptions will not be valid throughout the whole range of inhibitor values. Any deviation from expectation will introduce a component of systematic error. Since it is not possible to obtain exact algebraic solutions for these non-linear conditions, it is necessary to resort to computer simulation to assess the relative size of this error component and its effect on the estimates obtained. The following test data were generated using a metabolic simulation package known as SCAMP [13].

Previous simulation studies [12] have suggested that eqns. (3) and (7) may be reasonably robust with respect to saturation. To test this idea further, and its implication for determining control coefficient values, the five-step branched pathway shown in Scheme 1 was modelled. It was assumed that step 4 was subject to substrate and product saturation, while the remaining steps showed linear kinetics. The effect of inhibiting three of the enzymes independently was investigated.

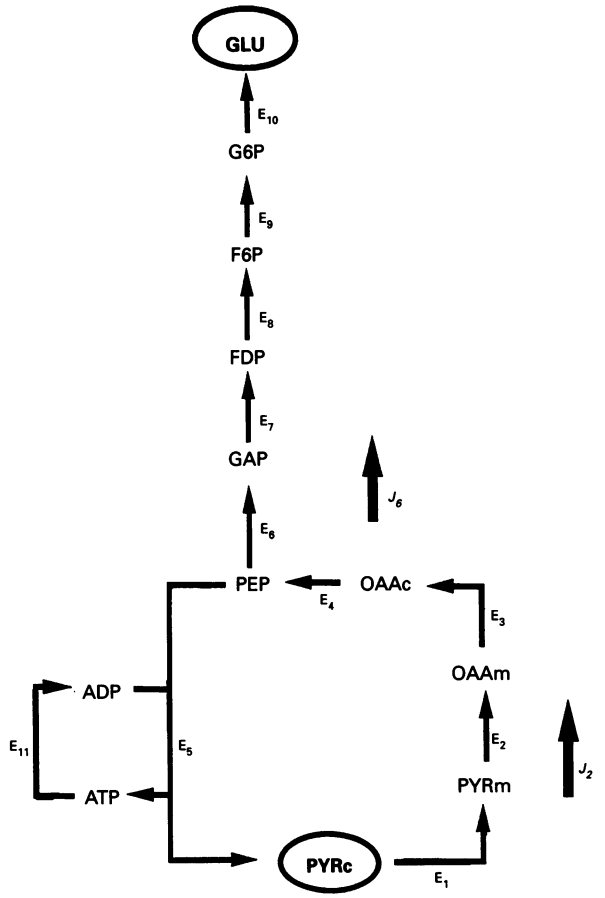
Three different initial conditions were chosen: condition 1 (results summarized in Table 6a), where the saturable enzyme had a low flux control coefficient for the flux it carries and the initial degree of saturation $(1 + S_1/K_{m_{4f}} + S_2/K_{m_{4r}})$ is high (15.51); condition 2 (results summarized in Table 6b), where the saturable enzyme also had a low flux control coefficient for the flux it carries, but the initial degree of saturation was less extreme (2.47); and condition 3, where the saturable enzyme had a high flux control coefficient for the flux it carries and the initial degree of saturation was 2.65.

The results of Tables 6(a), 6(b) and 7 show that estimates obtained by the fitting procedure for the monitored flux control coefficient $C^{J\,x}_{E_i}$ remain consistently better than the graphical technique, despite the large changes in saturation seen. In the absence of random error, the fitting procedure estimates showed little or no systematic error under these conditions. The introduction of random error (statistics based on 20 repeats) introduced little further systematic error (as seen by the good fit of the average estimate to the true value). The spread of estimates was similar to those found when the effect of random error alone was investigated. In the cases where the value of a second control coefficient could be estimated, the situation was different. Although the estimates of the monitored flux control coefficient showed little systematic error, the estimates of the second

Table 7 Effect of saturation in a branched system on the estimates of the control coefficient values obtained by both the graphical (*gra*) and the fitting (*fit*) method

Δ Saturation is the change in the saturation function, $(1 + S_1/K_{m_{4f}} + S_2/K_{m_{4r}})$ for enzyme E_4. The initial conditions in this case were high flux control and medium initial saturation (saturation function equals 2.65). The parameter values were as in the legend to Scheme 2 and, in addition, $V_{max_4} = 50$, $K_{m_{4f}} = 50$ and $K_{m_{4r}} = 75$. The value of the dissociation constant, K_D, was set to be 0.01 times the V_{max} of the inhibited enzyme. Statistics are based on $n = 20$.

Enzyme inhibited	Δ Saturation	Flux monitored	Control coefficient	Method	True value	No error C^J	Experimental error			
							Av. C^J	S.D. C^J	Min.	Max.
E_1	−1.62	J_A	$C^{J_A}_{E_1}$	*gra*	0.56	0.69	0.69	0.12	0.48	0.86
				fit	0.56	0.56	0.57	0.05	0.49	0.66
		J_B	$C^{J_B}_{E_1}$	*gra*	0.64	0.76	0.78	0.07	0.66	0.89
				fit	0.64	0.64	0.66	0.07	0.66	0.89
			$C^{J_A}_{E_1}$	*fit*	0.56	0.64	0.67	0.07	0.53	0.78
		J_C	$C^{J_C}_{E_1}$	*gra*	0.36	0.49	0.50	0.16	0.12	0.85
				fit	0.36	0.36	0.37	0.04	0.30	0.44
			$C^{J_A}_{E_1}$	*fit*	0.56	0.36	0.37	0.04	0.30	0.45
E_4	0.16	J_C	$C^{J_C}_{E_4}$	*gra*	0.60	0.73	0.75	0.12	0.59	0.92
				fit	0.60	0.60	0.60	0.07	0.43	0.69
E_5	1.51	J_C	$C^{J_C}_{E_5}$	*gra*	0.25	0.37	0.38	0.12	0.16	0.54
				fit	0.25	0.24	0.24	0.02	0.21	0.29



Scheme 2 A complex model system showing various non-linearities

GLU and PYRc are considered to be constant pools. The pools of the remaining metabolites are variables. Abbreviations used: PYRc, cytoplasmic pyruvate; PYRm, mitochondrial pyruvate; OAAm, mitochondrial oxaloacetate; OAAc, cytoplasmic oxaloacetate; PEP, phosphoenolpyruvate; GAP, glyceraldehyde 3-phosphate; FDP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; GLU, glucose.

coefficient were generally poor. The reason for this differing response of the two estimates could lie in the fact that the initial part of the flux–inhibitor relationship will be highly dependent on the value of the control coefficient with respect to the monitored flux. Since the change in saturation will be significantly less at the lower inhibitor levels, the predictions based on linear assumptions remain reasonable. A good estimate of the second control coefficient depends on predicting accurately the flux at very large inhibitor levels (see above) and hence the full effect of the large changes in the saturation function may be brought to bear.

The above model included only one of many forms of non-linearity. To gain a little insight into what may occur in reality, a much more complex model was studied. The structure of the model, loosely based on gluconeogenesis in rat hepatocytes, is shown in Scheme 2. Details of the reaction mechanisms and parameter values can be found in Appendix 3. The reaction mechanisms contain examples of the many forms of non-linearity (saturability of most enzymes, bimolecular reactions, cofactors, sigmoidal kinetics and feedback inhibition). This model has only been slightly modified from a model used for different purposes before [9], where the parameter values were not chosen to reduce the effects of non-linearity. Most of these values were set before the large change relationships were derived [11,12], and the changes made to the model (making ATP and ADP free variables) will have had the effect of increasing the non-linearities. Despite the highly non-linear nature of this model, the results of two inhibitor 'experiments' (Table 8) show that, as was the case with the simple model, the estimates for the control coefficient with respect to the monitored flux were very good; however, when eqn. (5) was used, the estimate of the value of the second coefficient was affected by the non-linearities.

A second source of systematic error in the fitting method may come from an inappropriate function assumed for the relationship between enzyme activity and inhibitor concentration. Without knowing what this error is, the effects cannot be quantitatively evaluated. In some cases these effects may not be large. For example if the equation used throughout this paper (eqn. 4) were accurate at low, but not at higher, levels of inhibitor, then, as was seen for the non-linearity cases above, the estimate obtained by the fitting method may still be reliable. If the errors occurred at the lower levels of inhibitor, then the estimate may be inaccurate using the fitting method. Note that the already inaccurate graph method will be even more inaccurate under these conditions.

DISCUSSION

In this paper it has been shown that there are a number of problems involved in estimating control coefficient values using specific inhibitor titrations. Evidence presented suggests that the graphical method can be extremely sensitive to random error. This is on top of the inherent systematic error of this method. Attempts to reduce the systematic error by using lower inhibitor points to estimate the initial slope will increase the sensitivity of the method to random errors and will probably be

Table 8 Effect of various non-linearities in a complex system on the estimates of the control coefficient values obtained by both the graphical (*gra*) and the fitting (*fit*) method

Statistics are based on $n = 20$.

Enzyme inhibited	Flux monitored	Control coefficient	Method	True value	No error C^J	Experimental error			
						Av. C^J	S.D. C^J	Min.	Max.
E_{PC}	J_2	$C^{J_2}_{PC}$	*gra*	0.88	0.95	1.00	0.06	0.90	1.09
			fit	0.88	0.89	0.93	0.05	0.84	1.02
	J_6	$C^{J_6}_{PC}$	*gra*	0.62	0.75	0.79	0.12	0.57	1.05
			fit	0.62	0.63	0.66	0.10	0.47	0.85
		$C^{J_2}_{PC}$	*fit*	0.88	0.62	0.66	0.11	0.46	0.86
E_{COM}	J_6	$C^{J_6}_{COM}$	*gra*	0.31	0.44	0.45	0.13	0.24	0.65
			fit	0.31	0.32	0.32	0.04	0.23	0.39

counterproductive. Improving the precision of flux measurements would, of course, reduce the effect of random errors on the estimate of the control coefficient, but statistical analysis suggests that, to obtain reasonable confidence limits, the error in the flux measurements will have to be very small. Statistical evidence (not shown) also suggests that increasing the number of points used to determine the initial slope, or having a geometric distribution of points within the 'initial' range, will have only a limited effect. This is backed up by numerical studies. Statistics were determined for a modification of Case A, Table 1, where either (a) a geometric distribution of the first three points was used to determine the initial slope or (b) extra points were added between the first and third point such that ten points were used to determine the initial slope. The statistics generated, based on 20 repeats, were (a) average estimate of $C^J = 0.41$, S.D. = 0.26 and (b) average estimate of $C^J = 0.40$, S.D. = 0.23. These results show no significant difference from those shown in Table 1.

Although systematic error will tend to overestimate the estimated value of the control coefficient, random error can, in certain cases, overwhelm this error so that a significant proportion of estimates may underestimate the true value. Hence, from a single experiment, it cannot be claimed with any degree of certainty that the estimate obtained is greater than the true value. Repeating the experiment an increasing number of times, and averaging the estimates, will cause this mean estimate to tend towards the estimated value which would be obtained in the absence of random error. This estimate will, however, still reflect the systematic error. Note, however, that the mean estimate may fluctuate widely at low numbers of repeats. For Case A in Table 1 it took 18 repeats before the mean estimate fluctuated within $\pm 10\%$ of the mean based on 100 repeats. It is clear that the graphical method must be used with extreme care, and there should be some attempt to provide confidence limits either empirically (as was done for eqn. 2) or experimentally, by repeating the experiment a number of times.

The 'fitting' technique has been shown to be, under the conditions studied, less susceptible than the graph method to random errors. In addition, when the conditions were such that there was potential for systematic error (due to the erroneous use of eqn. 3 or 5) being introduced, the technique remained robust, in that the estimate of the monitored flux control coefficient showed very little systematic error. However, when eqn. (5) was used under these same conditions, the estimate of the value of the non-monitored flux control coefficient was not as robust. For this reason, the confidence in this estimate will rely heavily on the confidence in which it is believed that the assumptions used in the derivation of eqn. (5) hold (or are only relaxed to a small degree).

The use of inhibitors to determine flux control coefficients must be done with great care. The inhibitor must be specific to one enzyme, and measurements must be done extremely accurately and/or appropriate functions used. Owing to these inherent difficulties, it may be suggested that there are other ways to use these inhibitors to determine control coefficients. A better option may be to use the inhibitors to perturb a system and measure the relative responses of fluxes and metabolites and determine the value of the metabolite elasticity coefficients [9,15,16], or control coefficients directly, as suggested in a recent publication [17]. These methods, of course, will again rely on extrapolation and will also be subject to experimental error. The effects of these errors will have to be determined to see whether these alternative methods will provide more accurate and/or precise results. A simulation study [9] has suggested that this may be the case. In addition, another study [18] has suggested that the control coefficient values obtained via elasticity values may be insensitive to errors in the values of many of these elasticities.

Only one type of inhibition was investigated in this study. Other types, e.g. competitive, mixed, etc., require, in order to use the graph method, either a knowledge of kinetic parameter values and/or metabolite concentrations or a method of obtaining the response of the enzyme to the inhibitor in isolation from the system, but under *in vivo* conditions. Both these approaches will increase the effects of both random and systematic error, in the former because most kinetic parameters are determined *in vitro* and hence may be inappropriate, and in the latter case because this will involve extrapolation to an initial slope and hence will suffer the same consequences as the extrapolation to the initial slope of the flux–inhibitor relationship. Whether relationships between flux and enzyme activity used in this investigation will be as robust under non-linear conditions when competitive and/or mixed inhibition is used remains to be discovered.

I thank Dr. David Fell and Dr. Henrik Kacser for their comments on previous drafts of this manuscript. I am also grateful for funding by the Wellcome Trust (Grant number 032342/Z/90).

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APPENDIX 1

Derivation of eqn. (2) of the main paper

In the following derivations, y will stand for the flux measurements and x the inhibitor concentrations. The estimated value of the control coefficient, C, using linear regression on the initial inhibitor points, can be expressed as:

$$C = -b \cdot \frac{I_{max.}}{a}$$

where a and b are the best-fit estimates of the intercept and slope respectively. If we assume that $I_{max.}$ is known exactly, then the standard error (se) of C can be related to the standard error of the flux measurements as follows [1]:

$$\frac{se_C^2}{C^2} \approx \frac{se_b^2}{b^2} + \frac{se_a^2}{a^2}$$

$$\approx \Sigma\left(\frac{se_{y_i}}{y_i} \cdot \frac{\partial a}{\partial y_i} \cdot \frac{y_i}{a}\right)^2 + \Sigma\left(\frac{se_{y_i}}{y_i} \cdot \frac{\partial b}{\partial y_i} \cdot \frac{y_i}{b}\right)^2 \quad \text{(A1)}$$

Using standard linear-regression equations (see [2]), it is possible to obtain an expression for eqn. (A1) in terms of individual x and y values. Using the assumptions that the relative standard errors of all the flux measurements are equal in magnitude i.e. $se_{y_i}/y_i = se_y/y$ for all i used in the initial slope and intercept estimation, that the inhibitor values (x values) are equally (arithmetically) distributed, three points are used for the linear regression and that the middle flux value is approximately half the difference of the first and third, then we obtain:

$$\frac{se_C^2}{C^2} \approx \frac{se_y^2}{y^2} \cdot \left[\frac{2 + 2 \cdot \Delta y/y_1 + (\Delta y/y_1)^2}{(\Delta y/y_1)^2} + \frac{30 + 2 \cdot (\Delta y/y_1)^2 + 6 \cdot \Delta y/y_1}{64 + 4 \cdot (\Delta y/y_1)^2 + 32 \cdot \Delta y/y_1}\right]$$

In the inhibitor titration experiments, to minimize systematic errors, $\Delta y/y_1 \ll 1$. If this is true, then the first term inside the square brackets of the above equation will totally dominate the value of expression and hence, after rearrangement, an estimate of the standard error of C is:

$$se_C \approx \frac{se_y}{y} \cdot \sqrt{2} \cdot \frac{y_1}{\Delta y} \cdot C \quad \text{(A2)}$$

The value of C is approximately equal to $(\Delta y/y_1) \cdot I_{max.}/\Delta x$, where Δx is the largest concentration of the inhibitor used in the regression (i.e. in the case x_3). Substituting this expression into eqn. (A2) gives:

$$se_C \approx \frac{se_y}{y} \cdot \sqrt{2} \cdot \frac{I_{max.}}{\Delta x} \quad \text{(A3)}$$

The relative standard error of y, (se_y/y), is related to the relative standard deviation of y, σ_y/y, by:

$$\frac{se_y}{y} = \frac{\sigma_y}{y} \cdot \frac{t_{n-1}}{\sqrt{n}}$$

where n is the number of repeat measurements of each inhibitor point and t is the Student t value for the desired confidence limits. Using standard statistical techniques to obtain confidence limits, eqn. (2) of the main paper can thus be generated, i.e.:

$$C \pm \frac{I_{max.}}{\Delta I} \cdot \sqrt{2} \cdot \frac{\sigma_J}{J} \cdot \frac{t_{n-1}}{\sqrt{n}} \quad \text{(A4)}$$

where $\sigma_J/J \equiv \sigma_y/y$.

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APPENDIX 2

Derivation of eqns. (7) and (8) of the main paper

For the purpose of this derivation, let $A \equiv C^{J_A}_{E_a}$, $B \equiv C^{J_B}_{E_a}$ and $R = A/B$. From [1] the standard deviation of R, σ_R, can be approximately expressed as:

$$(\sigma_R/R)^2 \approx (\sigma_A/A)^2 + (\sigma_B/B)^2 - 2\sigma_{AB}/(A \cdot B)$$

where σ_{AB} is the covariance of A and B. Since the correlation between A and B is positive, $R > 1$ and both A and B are less than 1, then (eqn. 7 of the main paper):

$$\sigma_R \leqslant \sigma_A + \sigma_B$$

In addition, since the correlation coefficient is very close to unity, [2]:

$$\sigma_{AB} \approx \sigma_A \cdot \sigma_B$$

Since we are dealing with only random errors, the fitted initial slope and the fitted final slope of the inhibitor titration should deviate from the true values by a similar amount. Since the initial slope error will be reflected in the error in B, and the final slope error in the error of R, σ_B and σ_R should be similar in value. Using these assumptions, the following (eqn. 8 of the main paper) is a consequence:

$$\sigma_A \approx R \cdot \sigma_B$$

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APPENDIX 3

Reaction mechanisms and parameter values for Scheme 2 of the main paper

Some metabolite abbreviations are defined in the legend to Scheme 2 of the main paper. Others are as follows: PT, mitochondrial pyruvate translocator; PC, pyruvate carboxylase; $V_{max.}t$, $V_{max.}$ of oxaloacetate transport, t, across the mitochondrial membrane; $K_m tm$, K_m of OAAm with respect to t; $K_m tc$, K_m of OAAc with respect to t; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; L_{PK}, allosteric constant for pyruvate kinase; COM, combined steps; $V_{max.}FBP$, $V_{max.}$ of fructose-1,6-bisphosphatase; PGI,

phosphoglucoisomerase; GP, glucose-6-phosphatase; $V_{max.}ATPc$, $V_{max.}$ of non-specified ATP-consuming reactions; $K_{eq.}c$, equilibrium constant of non-specified ATP-consuming reactions; DHAP, dihydroxyacetone phosphate; TPI, triosephosphate isomerase; CIT(R), citrate.

Reaction mechanisms

E_1: PYRc $\rightleftharpoons$ PYRm

$$V_{max.}PT \cdot \frac{PYRc - PYRm/K_{eq.}PT}{PYRm + PYRc}$$

E_2: PYRm $\rightleftharpoons$ OAAm

$$\frac{V_{max.}PC}{K_m PYRm} \cdot \frac{PYRm - OAAm/K_{eq.}PC}{1 + PYRm/K_m PYRm + OAAm/K_m OAAm}$$

E_3: OAAm $\rightleftharpoons$ OAAc

$$\frac{VOAAt}{K_m tm} \cdot \frac{OAAm - OAAc/K_{eq.}t}{1 + OAAm/K_m tm + OAAc/K_m tc}$$

E_4: OAAc $\rightleftharpoons$ PEP

$$\frac{V_{max.}PEPCK}{K_m OAAc \cdot K_m GTP} \cdot \frac{OAAc \cdot GTP - PEP \cdot GDP/K_{eq.}PEPCK}{\Theta_4}$$

where

$$\Theta_4 = 1 + \frac{OAAc}{K_m OAAc} + \frac{GTP}{K_m GTP} + \frac{PEP}{K_m PEP} + \frac{GDP}{K_m GDP} + \frac{OAAc \cdot GTP}{K_m GTP \cdot K_m OAAc} + \frac{OAAc \cdot PEP}{K_m OAAc \cdot K_m PEP} + \frac{PEP \cdot GDP}{K_m PEP \cdot K_m GDP}$$
$$+ \frac{GDP \cdot GTP}{K_m GDP \cdot K_m GTPK_m} \frac{OAAc \cdot PEP \cdot GTP}{OAAc \cdot K_m PEP \cdot K_m GTP} + \frac{PEP \cdot GDP \cdot GTP}{K_m GTP \cdot K_m GDP \cdot K_m PEP}$$

E_5: PEP + ADP $\rightleftharpoons$ PYRc + ATP

$$V_{max.}PK \cdot \frac{\frac{ADP}{ADP + K_m ADP_{PK}} \cdot \frac{PEP}{K_m PEP_{PK}} \cdot (1 + PEP/K_m PEP_{PK})^2}{L_{PK} \cdot \left(\frac{1 + ATP/K_m ATP_{PK}}{1 + FDP/K_m FDP_{PK}}\right)^3 + (1 + PEP/K_m PEP_{PK})^3}$$

E_6: PEP $\rightleftharpoons$ GAP

$$V_{max.}COM \cdot (PEP - GAP/K_{eq.}COM)$$

E_7: 2 GAP $\rightleftharpoons$ FDP

$$\frac{V_{max.}ALD}{K_m GAP \cdot K_m DHAP} \cdot \frac{GAP \cdot GAP \cdot K_{eq.}TPI - FDP/K_{eq.}ALD}{\Theta_7}$$

where

$$\Theta_7 = 1 + \frac{GAP}{K_m GAP} + \frac{GAP \cdot K_{eq.}TPI}{K_m DHAP} + \frac{FDP}{K_m FDP_{ALD}} + \frac{GAP \cdot K_{eq.}TPI \cdot GAP}{K_m GAP \cdot K_m DHAP} + \frac{GAP \cdot FDP}{K_m FDP_{ALD} \cdot K_i GAP}$$

E_8: FDP $\rightleftharpoons$ F6P

$$\frac{V_{max.}FBP \cdot FDP}{FDP + K_m FDP \cdot (1 + AMP/K_i AMP)}$$

E_9: F6P $\rightleftharpoons$ G6P

$$V_{max.}PGI \cdot (F6P - G6P/K_{eq.}PGI)$$

E_{10}: G6P $\rightleftharpoons$ GLU

$$\frac{V_{max.}GP \cdot G6P}{(1 + GLU/K_i GLU) \cdot [G6P + K_m G6P \cdot (1 + P_i/K_i/P_i + CITR/K_i CIT)]}$$

E_{11}: ATP $\rightleftharpoons$ ADP

$$V_{max.}ATPc \cdot (ATP - ADP/K_{eq.}c)$$

Parameter values

$V_{max.}PT = 11.27$, $K_{eq.}PT = 2.77$, $PYRc = 1.5$.

$V_{max.}PC = 0.515$, $K_m PYRm = 4.4 \times 10^{-2}$, $K_{eq.}PC = 0.222$, $K_m OAAm = 5 \times 10^{-2}$.

$VOAAt = 1.1$, $K_m tm = 0.011$, $K_m tc = 0.021$, $K_{eq.}t = 0.88$.

$V_{max.}PEPCK = 50$, $K_m OAAc = 1.5 \times 10^{-1}$, $K_m GTP = 1.6 \times 10^{-1}$, $K_m PEP = 1.2$, $K_m GDP = 6.3 \times 10^{-2}$, $K_{eq.}PEPCK = 0.372$, $GTP = 0.475$, $GDP = 4.5 \times 10^{-3}$.

$V_{max.}PK = 52.89$, $K_m PEP_{PK} = 0.19$, $K_m FDP_{PK} = 0.002$, $K_m ATP_{PK} = 9.3$, $K_m ADP_{PK} = 0.3$, $L_{PK} = 3400$.

$V_{max.}COM = 1$, $K_{eq.}COM = 0.03125$.

$V_{max.}ALD = 200$, $K_{eq.}ALD = 300$, $K_m GAP = 0.3$, $K_m DHAP = 1 \times 10^{-4}$, $K_m FDP_{ALD} = 3 \times 10^{-3}$, $K_i GAP = 3 \times 10^{-1}$, $K_{eq.}TPI = 0.045$.

$V_{max.}FBP = 1.5$, $K_m FDP = 1.2 \times 10^{-3}$, $K_i AMP = 0.2$, $AMP = 0.3$.

$V_{max.}PGI = 100$, $K_{eq.}PGI = 3.333333$.

$V_{max.}GP = 6$, $K_i GLU = 88$, $K_m G6P = 2$, $K_i P_i = 6 \times 10^{-3}$, $K_i CIT = 13$, $P_i = 0.01$, $CITR = 13$, $GLU = 1$.

$V_{max.}ATPc = 0.2483$, $K_{eq.}c = 0.1$,

Conserved metabolites: $(ATP + ADP) = 8.31$.

Received 20 May 1993/13 July 1993; accepted 16 July 1993