Eur. J. Biochem. *161*, 171–176 (1986)

Allosteric character of the inhibition of rat-muscle hexokinase B by glucose 6-phosphate

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(Received June 17, 1986) – EJB 86 0620

We previously provided evidence from isotope-exchange measurements under non-equilibrium conditions that hexokinase B from rat muscle follows a compulsory-order mechanism with glucose binding before MgATP, and with both glucose 6-phosphate and MgATP capable of binding allosterically [Gregoriou, M., Trayer, I. P. & Cornish-Bowden, A. (1983) *Eur. J. Biochem. 134*, 283–288]. We have now re-examined this work in the light of recent criticisms [Ganson, N. J. & Fromm, H. J. (1985) *J. Biol. Chem. 260*, 12099–12105]. There is no difficulty in obtaining valid estimates of initial rates of isotope exchange when the equilibrium constant is unfavourable, if one uses highly radioactive reactants and low enzyme concentrations, as we did in the experiments we reported previously. However, our earlier suggestion that MgADP can be released within the inhibitory pathway, which was made for the sake of consistency with the catalytic pathway rather than because of any compelling experimental evidence, must be revised to avoid predicting that the rate must be zero in the absence of MgADP. Although our mechanism admits the possibility of substrate inhibition by MgATP, calculations show that there is no need for this to be observable under ordinary conditions. Indeed, with plausible values assumed for the kinetic constants one can calculate theoretical behaviour according to our model that closely resembles the experimental inhibition experiments that have been claimed as evidence against it.

Hexokinase occurs in mammalian tissues in several isoenzymic forms [1], of which the form predominant in muscle was designated as hexokinase B, though it has often been referred to also as hexokinase type II [2]. There has been controversy for many years about the nature of the powerful inhibition by glucose 6-phosphate of this isoenzyme and also of hexokinase A, the isoenzyme characteristic of brain. Crane and Sols [3] proposed that it arises from a specific inhibitory binding site for glucose 6-phosphate, i.e. an allosteric site in modern terminology, but others have maintained over almost as long a period that ordinary product inhibition can account for the phenomenon (e.g. [4]).

We [5] examined the properties of hexokinase B by the powerful flux-ratio method developed by Britton and co-workers [6, 7]. At low (largely non-inhibitory) concentrations of glucose 6-phosphate, the ratio of fluxes from glucose 6-phosphate to ATP and glucose behaved in the way characteristic of a compulsory-order mechanism in which ATP must be released (in the back reaction) before glucose. At inhibitory concentrations of glucose 6-phosphate, however, unprecedented behaviour was observed, whereby the flux ratio increased very steeply and with a positive curvature; in the most extreme case doubly-labelled glucose 6-phosphate generated labelled ATP more than 100 times as fast as labelled glucose. This behaviour was incompatible with simple mechanisms for the inhibition and appeared to require an inhibitory pathway in which transfer of label from glucose 6-phosphate to ATP could occur with release of two (or more) molecules of ATP before any glucose. Our results established both that the normal catalytic reaction proceeded by a compulsory-order mechanism with glucose binding before MgATP and that the inhibition by glucose 6-phosphate could not be solely due to ordinary product inhibition.

Ganson and Fromm [8] have recently discussed our work in detail, concluding in contrast to ourselves that the reaction catalysed by hexokinase B follows a random-order mechanism. They accept that an allosteric site for glucose 6-phosphate may exist, but they suggest that this is a 'low-affinity site compared to the active site', i.e. that it is not responsible for a major component of the inhibition. They make three main criticisms of our study of hexokinase B: first, they note that in some of our experiments chemical equilibrium would be reached after much less than 1% of reaction, and they suggest that it would not be possible to measure valid initial rates in such cases; second, they point out that if our proposed mechanism were correct the rate of reaction would be zero (in either direction) in the absence of ADP; third, they suggest that the fact that substrate inhibition by ATP has not been reported for hexokinase B although it is predicted by our mechanism proves that the mechanism is incorrect. We believe that Ganson and Fromm [8] are correct in only the second of these points, but that the difficulty can readily be avoided by modifying the mechanism in a way that does not alter its major features.

The matter is of general importance beyond the specific example of hexokinase B because, as we have discussed

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Abbreviations. $F(Q \rightarrow A)$, flux from Q to A; Glc6*P*, glucose 6-phosphate.

Enzyme. Hexokinase, ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1).

elsewhere [9], the flux-ratio method has important advantages over the conventional product-inhibition approach for studying the order of addition of substrates and release of products in an enzyme-catalysed reaction; if the criticisms of Ganson and Fromm [8] were sustained, the usefulness of the method would be severely curtailed.

THEORY AND RESULTS

Extent of reaction at equilibrium

In Fig. 2 of our previous paper [5] we showed the flux ratio $F(\text{Glc6}P \rightarrow \text{ATP})/F(\text{Glc6}P \rightarrow \text{Glc})$ as a function of the concentration of MgATP at three concentrations (0.085 mM, 2.85 mM and 3.00 mM) of glucose 6-phosphate, 5-mM MgADP, and MgATP concentrations from zero to 5 mM. Ganson and Fromm [8] have estimated that at the lowest concentration of glucose 6-phosphate and highest concentration of MgATP equilibrium would be reached after only 0.2% of reaction, and that in most of our experiments equilibrium would reached after less than 2% reaction. Their calculation was based on an assumption that the equilibrium constant was 490, whereas the equilibrium constant that we reported as applicable to our experiments at pH 6.5 was 234, but this does not affect the main point that our assay mixtures would reach equilibrium after very little reaction. The implication of this is not, however, that the flux-ratio method cannot be used in the study of reactions that reach equilibrium after a very small extent of reaction. Rather it is that one must take care to use reagents of very high specific radioactivity and to use very low enzyme concentrations, so that the fluxes are slow enough to be followed on a convenient time scale without attainment of equilibrium. It is also helpful to select experimental conditions that give as low an equilibrium constant as possible: this is why we worked at pH 6.5, where the equilibrium constant is 234 ± 14, rather than at the more usual pH 8.0, where it is 23500 [10].

Use of a low enzyme concentration to give a convenient time scale has long been a standard practice on steady-state enzyme kinetics and, equally, it is well known that the sensitivity of radioactive tracer experiments can be increased by using reagents of very high specific activity. Nonetheless, it would perhaps have been useful to have made this point explicitly in our earlier paper, and we now do so, to correct any impression that it was not possible to measure valid initial rates under our conditions.

Fig. 1 shows the time courses of the experiments that led to the $F(\text{Glc6}P \rightarrow \text{ATP})$ values used for calculating the flux ratios shown in Fig. 2 of our previous paper [5] at 2.85-mM glucose 6-phosphate. It may be seen that all of the lines are straight within experimental error, with no tendency to slow down as a consequence of approach to equilibrium. It is evident, therefore, that there was no difficulty in obtaining valid initial rates. That all of the reactions were far from equilibrium throughout the period of measurement is confirmed by actual amounts of radioactivity measured, which are compared with the values expected at chemical equilibrium in Table 1. For the experiment at 2.62-mM MgATP chemical equilibrium would be reached after conversion of 0.81% of the glucose 6-phosphate into glucose, but after 14.5 min the actual conversion was only 0.024%, i.e. the reaction had progressed only 3% of the way to equilibrium; this was the nearest approach to equilibrium at any of the MgATP concentrations used.

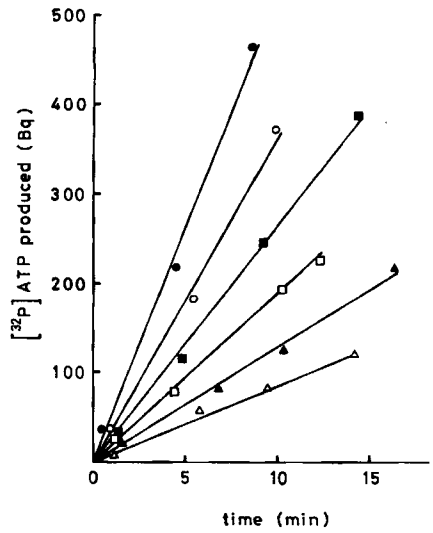


Fig. 1. *Progress curves for the transfer of ^{32}P from glucose 6-phosphate to ATP in the presence of 5-mM MgADP and 2.85-mM glucose 6-phosphate.* Measurements were made in the absence of glucose but in the presence of 4.67-mM (●), 3.51-mM (○), 2.62-mM (■), 1.98-mM (□), 1.34-mM (▲) and 0.67-mM (△) MgATP. The specific radioactivity (as ^{32}P) of the glucose 6-phosphate was 7.215×10^{11} Bq/mol. The slopes of the lines were used in calculating the flux ratios shown for 2.85-mM glucose 6-phosphate in Fig. 2 of [5]

It may at first appear surprising that none of the time courses in Fig. 1 show appreciable curvature, even though the radioactivity of ATP produced was, for example, about 90% of the radioactivity expected at chemical equilibrium after 8.6 min in the experiment at 4.67-mM MgATP. The reason for this is simple, but perhaps should be stated explicitly. The complete chemical reaction provides the only mechanism for conversion of glucose 6-phosphate into glucose, so the values for glucose represent true values for the extent of the chemical reaction. Glucose 6-phosphate and ATP, however, can exchange ^{32}P without completing the chemical reaction, both in the catalytic and the inhibitory pathways of our mechanism, and this exchange does not reach equilibrium until the specific radioactivities of glucose 6-phosphate and ATP are equal, with an equilibrium radioactivity of ATP of 69000 Bq; the measured radioactivity after 8.6 min was only 460 Bq, less than 0.7% of the value at exchange equilibrium.

We have discussed Fig. 2 of [5] in some detail because our main interest has been to explain the upward curvature of the flux-ratio plots given there, and so we should naturally be concerned if our technical approach that led to this result was shown to be invalid. Nonetheless, recognizing that much of the criticism of Ganson and Fromm [8] was directed at Fig. 1 B of our earlier paper, especially the result for the highest MgATP concentration, for which they commented that chemical equilibrium would be reached after only 0.2% of reaction, we have examined that experiment in a similar way. Under our conditions for this experiment equilibrium would be reached after 0.425% (not 0.2%) conversion of the glucose 6-phosphate into glucose: starting with 350000 Bq of ^{14}C-labelled glucose 6-phosphate, therefore, the radioactivity of glucose at equilibrium would be 1480 Bq. The measured rate was 0.18 Bq s^{-1}, and the reaction was followed for 4 min, with a final radioactivity of 43.1 Bq. As this is less than 3% of the value at equilibrium, it is evident that the criticisms of

Table 1. *Radioactivity values at equilibrium and during the period of measurement*
Experimental conditions were as described in the legend to Fig. 1

Initial MgATP concn	Equilibrium glucose concn	Glc6*P* converted at equilibrium	Equilibrium radioactivity		Initial fluxes measured	
			[^{32}P]ATP	[^{14}C]glucose	[^{32}P]ATP	[^{14}C]glucose
mM		%	Bq		Bq s^{-1}	
0.67	0.0793	2.78	3105	8263	0.178	0.223
1.34	0.0438	1.54	1716	4565	0.226	0.128
1.98	0.0304	1.07	1191	3169	0.376	0.076
2.62	0.0232	0.81	909	2418	0.456	0.084
3.51	0.0174	0.61	682	1815	0.630	0.040
4.67	0.0131	0.46	512	1363	0.891	0.039

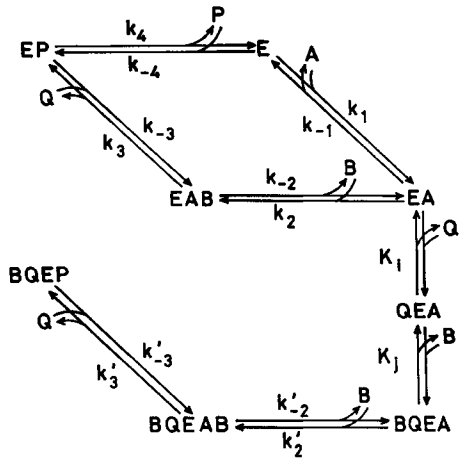
Ganson and Fromm [8] are as lacking in substance in this instance as in the other.

Steady-state rate in the absence of ADP

In Scheme 2 of [5] we proposed a mechanism in which the inhibition by glucose 6-phosphate was a consequence of a five-step dead-end pathway in which binding of glucose 6-phosphate and two molecules of MgATP was followed by release of MgADP and glucose 6-phosphate. Ganson and Fromm [8] have correctly pointed out, however, that the presence of a MgADP-release step in this pathway predicts a zero rate in the absence of ADP. Their Eqn (1) also implies, misleadingly, a zero rate in the absence of glucose 6-phosphate. This is because they neglected to cancel between numerator and denominator the concentration of glucose 6-phosphate, a common factor of all 38 terms.

Ganson and Fromm [8] carried out experiments in the presence of phospho*enol*pyruvate and pyruvate kinase (to remove any ADP that might be present as a contaminant of their ATP stock) that demonstrated that the rate of the forward direction is not, in fact, zero in the absence of ADP. This experiment is not strictly relevant to our mechanism, because it was carried out in the absence not only of ADP but also of glucose 6-phosphate, whereas according to the proposed mechanism there is no entry into the inhibitory pathway in the absence of glucose 6-phosphate. Nonetheless, we do not claim that the rate is zero in the absence of ADP; we included the MgADP-release step in the mechanism to avoid arbitrary differences between the catalytic and inhibitory tiers of the mechanism, and the order of release of products in the catalytic pathway was drawn by analogy with hexokinase D [11] rather than because of any experimental need to show release of MgADP before glucose 6-phosphate. In trying to find a mechanism that would account for the unprecedented flux-ratio behaviour we overlooked this prediction of a zero rate in the absence of ADP.

As seen in Fig. 3 of [5], the fluxes from ATP to ADP and glucose 6-phosphate were equal within experimental error at all concentrations of glucose 6-phosphate and MgADP considered. Moreover, we were unable to trap enzyme–glucose-6-phosphate or enzyme–MgADP binary complexes. Both of these experiments indicate that release of products is close to concerted: whichever is released first, the second follows very rapidly afterwards. Accordingly, therefore, a revised mechanism (Scheme 1) in which there is no release of MgADP in the inhibitory pathway and glucose 6-phosphate is released before MgADP in the catalytic pathway is no less in accord with the flux-ratio data than the mechanism suggested previously, but now there is no prediction of a zero rate in the absence of ADP.



Scheme 1. *Proposed mechanism for hexokinase B from rat muscle.* The mechanism is based on that proposed previously [5], but differs from it in that no MgADP-release step is shown in the inhibitory pathway and the product-release steps are reversed in the catalytic pathway. Note, however, that the experimental results do not permit a decision about the order of these steps

Substrate inhibition by MgATP

Our proposed mechanism for hexokinase B, both as proposed originally and as shown in its revised form in Scheme 1, allows substrate inhibition by MgATP at high concentrations of MgATP and inhibitory concentrations of glucose 6-phosphate. We were aware that no such substrate inhibition had been reported, for example by Grossbard and Schimke [12] or by Lueck and Fromm [13], and we attributed this to a supposition that under normal assay conditions insufficient complexes with two molecules of MgATP bound would occur to produce any readily observable effects [5]. Ganson and Fromm [8] do not comment on our suggestion, but instead interpret the lack of reports of substrate inhibition by MgATP as evidence that our mechanism cannot be correct.

Fig. 5 of [13] shows plots of reciprocal rate against reciprocal MgATP concentration at various concentrations of glucose 6-phosphate. Our contention is that even at the highest

concentrations of MgATP (1 mM) and glucose 6-phosphate (0.15 mM) substrate inhibition could have been too slight to be perceived. Before discussing this, however, we should note an apparent inconsistency in the concentrations of glucose 6-phosphate reported in the legend to Fig. 5 of [13], which were 0, 0.003 mM, 0.0075 mM and 0.015 mM. These values indicate a far higher sensitivity to glucose 6-phosphate than is evident from other reports, such as [12], or even from Fig. 11 of the same paper [13]. If the legends to Figs. 5 and 11 of [13] are correct as given, the presence of 1-mM ADP has the effect of raising more than tenfold the concentration of glucose 6-phosphate needed to decrease the rate 50%, for 0.1-mM glucose and 1-mM MgATP. We shall assume that the interpretation of this anomaly is that the actual concentrations of glucose 6-phosphate used in Fig. 5 of [13] were tenfold higher than the values stated. Note, however, that if the concentrations were actually as small as stated, our contention that substrate inhibition by MgATP would not have been observable would be strengthened, not weakened.

The rate equation generated by Scheme 1 for the forward reaction in the absence of P (MgADP) is as follows:

$$v = k_1k_2k_3e_oab/[k_{-1}(k_{-2} + k_3) + k_1(k_{-2} + k_3)\,a \cdot G(b, q) + k_2k_3b + k_1k_2ab] \quad (1)$$

where e_o is the total concentration of enzyme, a, b and c are the concentrations of A (glucose), B (MgATP) and Q (glucose 6-phosphate) respectively, and

$$G(b, q) = 1 + (q/K_i) \left(1 + (b/K_j)\{1 + (k_2'b/k'_{-2})[1 + (k_3'/k'_{-3}\,q)]\}\right). \quad (2)$$

The rate constant k_4 does not appear in Eqn (1) because, as discussed above, the flux-ratio experiments indicate that the second product-release step follows the first so rapidly that in effect k_3 can be regarded as the rate constant for conversion of the ternary complex to the free enzyme and both products.

As Eqn (1) contains terms in ab^2q and ab^2q/q it allows substrate inhibition by MgATP, as noted previously [5] and emphasized by Ganson and Fromm [8]. We describe this last term as a term in ab^2q/q rather than in ab^2 because in the absence of Q there is no entry into the inhibitory pathway. Thus although q/q can be written as 1 for finite values of q, it should be taken as zero when $q = 0$. For very small but non-zero values of q this last term contributes to the steady-state rate only in a rather abstract sense, because the true steady state would be reached so slowly that it would be irrelevant to a real experiment. (Although one usually assumes that a steady state will be reached within much less than 1 s after the start of reaction, and in simple cases this is clearly valid [4], it is by no means necessarily true in reactions that include slow steps that are not on the main reaction pathway.) Ganson and Fromm [8] appear to have overlooked this point, and to avoid any suggestion that we are biasing the argument in favour of our point of view we shall also effectively overlook it in the discussion that follows, i.e. we shall take $q/q = 1$ for all values of q.

To determine whether Eqn (1) predicts observable substrate inhibition by MgATP it is necessary to decide on reasonable numerical values for the kinetic constants; one cannot conclude from the mere presence of a term in a rate equation that it will generate observable effects. A considerable amount of information can be deduced from the details of the flux-ratio experiments [5], but first we must revise Eqn (4) of that paper to express the flux ratio for the revised mechanism as shown in Scheme 1:

$$\frac{F(Q \rightarrow B)}{F(Q \rightarrow A)} = 1 + \frac{k_2k_3b}{k_{-1}(k_{-2} + k_3)} + \frac{k_2'k_3'b^2q}{k_{-1}(k'_{-2} + k_3')\,K_iK_j} \quad (3)$$

Assuming that this equation describes the lines plotted in Figs 2 and 3 of [5], we can calculate (using the data for 2.85-mM glucose 6-phosphate in the case of Fig. 3) that the coefficients of b and b^2q approximate to the following values:

$$k_2k_3/k_{-1}(k_{-2} + k_3) = 0.6\ \text{mM}^{-1} \quad (4)$$

$$k_2'k_3'/k_{-1}(k'_{-2} + k_3')\,K_iK_j = 1\ \text{mM}^{-3}. \quad (5)$$

As we have no further information about the values of the rate constants in the inhibitory pathway we cannot proceed further without a hypothesis: the most economical is to suppose that steps that occur in both tiers of the mechanism have equal rate constants, i.e. primed and unprimed rate constants are equal. With this hypothesis, and noting that a value of $K_i = 0.03$ mM is of the right order to give an appropriate sensitivity to glucose 6-phosphate, we can estimate K_j as 20 mM by dividing Eqn (4) by Eqn (5).

In addition to these values we have the Michaelis constants for glucose and MgATP as 0.2 mM and 0.8 mM respectively under our assay conditions [5]. In addition, Lueck and Fromm [13] reported that double-reciprocal plots in the absence of products intersect on the abscissa, which means that the constant term in the denominator of the rate equation, after dividing all terms by k_1k_2, the coefficient of ab in Eqn (1), is equal to the product of Michaelis constants, i.e. 0.16 mM^2.

As a digression, but one that allows a check on the consistency of our experiments, we may note that $1/K_m$ for MgATP, which was found experimentally to be $1/0.8 = 1.25\ \text{mM}^{-1}$, may be written as $k_2k_3/k_{-1}(k_{-2} + k_3)$, i.e. it is the same quantity that was found to have a value of $0.6\ \text{mM}^{-1}$ from the flux-ratio measurements, as given in Eqn (4) above. Given that one of these values is derived from ordinary kinetic measurements in the forward direction, and the other from flux-

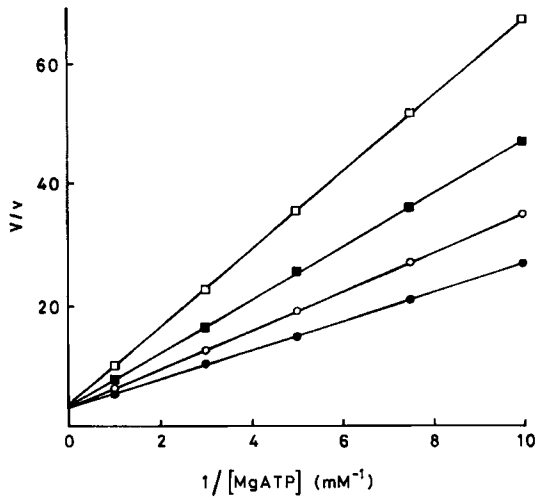


Fig. 2. *Inhibition by glucose 6-phosphate as calculated from the proposed model.* The concentrations of glucose 6-phosphate were 0 (●), 0.03 mM (○), 0.075 mM (■) and 0.15 mM (□). The design points are the same as those plotted in Fig. 5 of [13], but the v values are calculated from Eqn (1) of the present paper with the kinetic constants given in the text

ratio measurements in the reverse direction, we regard this agreement as reasonable.

Finally, we require values for k'_{-2}/k'_2 and k'_3/k'_{-3}. On the basis of the hypothesis above that primed and unprimed rate constants can be equated, k'_{-2}/k'_2 should be smaller than the Michaelis constant for MgATP, so a value of 0.5 mM is not unreasonable, and if we assume that glucose 6-phosphate binds equally well to EA and to BQEP, we have $k'_3/k'_{-3} = K_i = 0.03$ mM.

As many assumptions have been needed to arrive at these numerical values we should not expect them to predict the behaviour of the enzyme with any exactness. Nonetheless, if we use them to calculate the expected appearance of Fig. 5 of [13] the result, shown in Fig. 2, is remarkably similar in appearance to the original. Two points should be noted: first, the curvature expected by Ganson and Fromm is barely perceptible, even at the highest concentrations of MgATP and glucose 6-phosphate, and the deviations from linearity are much smaller than the experimental error evident in the original. Thus it is in no way surprising that Lueck and Fromm [13] did not report substrate inhibition by MgATP. We may note in passing that Ganson and Fromm [8] did not comment on the much more striking curvature visible in all five plots in their Fig. 1. That this curvature is highly significant may be demonstrated without any assumptions about the error distribution or possible physical interpretations of the curvature by noting that, for each of the five plots, a straight line drawn through the first and fourth points passes under the second and third. For any one plot such an arrangement is as likely as any other, but the probability of finding the same arrangement by chance in a second plot is 0.25, and that of finding it in all of four other plots is 0.25^4, or about 0.4%.

The second point to note about Fig. 2 is that despite the presence of terms in *bq* in Eqn (1) the plots agree closely with the appearance expected for competitive inhibition, rather more closely, indeed, than the original data [13].

DISCUSSION

Conventional methods of analysing patterns of inhibition by products and by analogues of substrates and products to determine the orders of addition of substrates and release of products do not by themselves yield information of the relative contributions of alternative pathways. The reasons for this have been discussed by Britton and Dann [7]. When supplemented with data on the dependence of flux ratios on product concentrations, inhibition studies can, however, lead to reliable mechanistic conclusions. The method of using flux ratios measured under non-equilibrium conditions was developed by Britton and Dann [7] from earlier work of Britton [6], and they commented that 'the equilibrium method would only seem appropriate when the non-equilibrium method is impractical for technical reasons'.

Nonetheless, in their study of pyruvate kinase Dann and Britton [15] did work at chemical equilibrium for determining the flux ratio for the fluxes from ATP to ADP and phospho*enol*pyruvate. They did not explicitly state the reason for this decision, but presumably they considered the equilibrium constant for pyruvate kinase, 2125 ± 81 under their conditions, to be excessively unfavourable. This is much higher than the equilibrium constant for hexokinase at pH 6.5, and it does not follow that the non-equilibrium method was inappropriate and invalid in our study of hexokinase B, as argued by Ganson and Fromm [8]. On the contrary, the time courses shown in Fig. 1 and the details of the radioactivity measurements shown in Table 1 demonstrate that in our experiments there was no difficulty in measuring valid initial fluxes uncomplicated by approach to equilibrium. Indeed, as none of the reaction mixtures used in our analysis proceeded more than 3% towards equilibrium, it would probably have been possible to obtain usable information even if the equilibrium constant were as high as 1000, for the same specific radioactivities of reactants and the same enzyme concentrations that we used; with more highly radioactive reactants and lower enzyme concentrations one could tolerate even less favourable equilibrium constants.

We were aware, of course, that a mechanism in which MgATP was capable of acting as an allosteric inhibitor and in which exchange of ^{32}P between glucose 6-phosphate and ATP could occur in the inhibitory pathway was a novel proposal that was not clearly suggested by previous evidence. Nonetheless, we considered that the unequivocally curved flux-ratio plots that we obtained did not admit of a simpler interpretation. It is unfortunate that Ganson and Fromm [8], despite rejecting our interpretation, have offered no alternative explanation of upwardly curved flux-ratio plots.

In conclusion, and to correct any impression that the flux-ratio method is fraught with practical difficulties, we shall summarize what we believe to be its principal advantages over more conventional approaches to steady-state enzyme kinetics. As it is not in essence an extrapolation technique, it is free from arguments about whether lines are parallel or are converging to a distant point of intersection, or whether an extrapolated point of intersection should be placed on or off a particular axis. Contrast, for example, the unequivocal differences between the dependence of the flux ratios on the two substrates of the forward reaction seen in Fig. 1 of [5] with the argument of Ganson and Fromm [8] that Fig. 3A of [16] 'could easily be drawn' to give whatever conclusions are required, i.e. mixed inhibition in 1967, but competitive inhibition in 1985!

The non-equilibrium flux-ratio method also allows attention to be focused on whatever part of the mechanism is of interest: the order of addition of substrates can be investigated independently of the order of release of products, and vice versa. Moreover, dead-end inhibition, which can create havoc in the interpretation of product-inhibition experiments, has no effect on a flux ratio (because there is no net flux through a dead-end pathway), unless, as in the rather unusual situation illustrated in Scheme 1, the dead-end pathway provides a means of transfer of label separate from the normal reaction. In general, flux-ratio measurements are unaffected by any mechanistic complications that exist outside the part of the mechanism that allows exchange of isotopic labels.

Finally, the flux-ratio method can be applied to enzymes that do not follow Michaelis-Menten kinetics. This was a useful and important point when we used the method to study the reaction catalysed by hexokinase D [11].

This work was supported by a grant from the Medical Research Council to I.P.T., A. C.-B. and D. G. Walker. We are grateful to J. E. Wilson and M. L. Cárdenas for discussion of this work, and also to Dr T. Ureta for the use of facilities at the Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, during preparation of the manuscript.

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