thermore, the stringency of their numerical analysis is inadequate because K_x and K'_x have the same numerical value, which prevents detection of the error in their eqn. (7).

In conclusion and summary, it appears from careful analysis and correct application of the principle of microscopic reversibility that the treatment of the quasi-equilibrium situation for this mechanism given by Topham and Brocklehurst [2] is correct.

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- 1 Varón, R., García-Moreno, M., Garrido, C. and García-Cánovas, F. (1992) *Biochem. J.* **288**, 1072–1073
- 2 Topham, C. M. and Brocklehurst, K. (1992) *Biochem. J.* **282**, 261–265

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Some classical errors in the kinetic analysis of enzyme reactions

The appearance of the communication of Varón et al. (1992), which purports to identify errors in our analysis of modifier mechanisms (Topham and Brocklehurst, 1992), suggests that a brief summary of a number of classical pitfalls to be avoided when applying kinetic analysis to enzyme reactions might be useful. In our appraisal of both the well-known valuable method for the derivation of rate equations devised by Cha (1968) and its unwarranted criticism by Segel and Martin (1988), we derived the rate equation (eqn. 1) for the general modifier mechanism of Botts and Morales (1953) [Scheme 1 of the accompanying letter, Selwyn (1993)]:

$$v = \frac{(k_{+2} + k'_{+2}[X]/K'_x)[E]_T[S]}{(1 + [X]/K_x) + \frac{K_m^s(1 + [X]/K_x)}{(1 + [X]/K'_x)} + [S]} \quad (1)$$

where $[E]_T$ is the total enzyme concentration and $K_x (= k_{-x}/k_{+x})$ and $K'_x (= k'_{-x}/k'_{+x})$ are the equilibrium constants for the dissociation of modifier (X) from the binary (EX) and ternary (EXS) complexes respectively.

The general form of eqn. (1) is not in dispute (see Topham and Brocklehurst, 1992), but Varón et al. (1992) assert (wrongly) that our expression for K_m^s , the ratio $[E][S]/[ES]$, shown as eqn. (5) by Selwyn (1993), is incorrect, and attempt to support their own incorrect derivation by inappropriate numerical analysis. As demonstrated by Selwyn (1993) in his elegant dissection of the treatment offered by Varón et al. (1992), their derivation is fundamentally flawed by their failure to apply properly the principle of microscopic reversibility. Their failure to recognize that only seven of the eight rate constants of a thermodynamic box like that of the general modifier mechanism (Scheme 1 of Selwyn, 1993) are independent led them to assign arbitrary values to all eight rate constants and thus to calculate values of K_m^s that differ from those calculated by using our (correct) expression for K_m^s .

The flawed treatment of Varón et al. (1992), which led to their entirely incorrect assertion of error in eqn. (2) of Topham and

Brocklehurst (1992) (eqn. 5 of Selwyn, 1993) emphasizes the need in kinetic analysis (i) to distinguish rates from rate constants and (ii) to recognize the characteristics of kinetic models that include thermodynamic boxes and parallel pathways. By contrast with the view expressed by Varón et al. (1992), it was the failure of Segel and Martin (1988) to take account of fluxes in parallel pathways connecting two isolated groups of enzyme species existing in quasi-equilibrium with the modifier that led them to conclude (wrongly) that Cha's (1968) method requires constraints on the relative magnitudes of rate constants for slow steps outside the quasi-equilibrium segments of the kinetic model. More generally, failure to take proper account of (a) parallel pathways through thermodynamic boxes (see Plaut and Knowles, 1972; Brocklehurst and Dixon, 1976, 1977; Topham and Brocklehurst, 1992), (b) overlapping kinetically influential ionizations (see Brocklehurst et al., 1983; Bashford and Karplus, 1991; Mellor et al., 1993), (c) the conditions that define the state of quasi-equilibrium (see Brocklehurst and Cornish-Bowden, 1976; Brocklehurst, 1979) and (d) the relationship between steady-state and single-turnover kinetic data (see Brocklehurst and Topham, 1990) has sometimes confused or prevented proper appreciation of the significance of the important parameter k_{cat}/K_m (Bender and Kézdy, 1965; Brocklehurst et al., 1968; Fersht, 1977) and the form and interpretation of its pH-dependence.

A final point that is worthy of comment concerns the suggestion by Varón et al. (1992) that in the analysis of kinetic models for reactions where there is necessarily a net flux, the distinction between quasi-equilibrium and true thermodynamic equilibrium is substantive. This assertion, which is also implicit in the criticism by Childs and Bardsley (1975) of the methods of analysis of the kinetics of time-dependent enzyme inhibition (Kitz and Wilson, 1962; Malcolm and Radda, 1970), was shown to be without foundation by the analysis and argument reported by Cornish-Bowden (1979). Our hope is that, by collecting together the simple but fundamental points outlined above, we might help to prevent the appearance in the literature of flawed and confusing papers like that of Varón et al. (1992).

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Note: Varón and colleagues were given the opportunity to respond to the above Letters, but no acceptable reply has been received.

Differences in 2-oxoglutarate dehydrogenase regulation in liver and kidney

A paper with the above title by Smith et al. (1992) recently appeared in the *Biochemical Journal*. Its purpose was to seek an explanation for the different responses to acidosis of the liver, where gluconeogenesis and ammonia production for urea synthesis are decreased, compared with the kidney, where ammonia production and gluconeogenesis from glutamine are increased (see Smith et al., 1992). Its major conclusion was that this could be due to the differential pH-sensitivity, within these tissues, of what may be the key rate-controlling enzyme, the 2-oxoglutarate dehydrogenase complex (2-OGDH). This itself, on the basis of the data presented, was proposed to be a consequence of the different matrix environments of liver compared with kidney mitochondria. Thus it was proposed that the inherent H⁺-sensitivity of 2-OGDH (see below) in liver mitochondria is masked by the pH-sensitivity of glutamate-oxaloacetate transaminase (GOT), which is more active in liver than in kidney, and also the glutamate-aspartate carrier. In contrast, in kidney mitochondria the inherent pH-sensitivity of the enzyme is able to be expressed (Smith et al., 1992).

Although this is an attractive proposition, there are, however, a number of earlier reports (see below) which offer evidence that may be contradictory to these findings, or which offer alternative approaches with which different results may have been obtained. Indeed, it is surprising that these earlier reports, which clearly contain material very relevant to this work, and often contain precedent findings, were not quoted in Smith et al. (1992).

To begin with, Smith et al. (1992) eliminated the possibility that the extracted 2-OGDH activities from the two different tissues have different pH- or Ca²⁺-regulatory properties. (Ca²⁺ regulation is also thought to be important in mediating hormonal effects on ammonia production and gluconeogenesis in both tissues; increases in either [Ca²⁺] or [H⁺] within their physiological ranges each activate the enzyme by decreasing its *K_m* for 2-oxoglutarate.) However, this information could have been obtained from the literature, where extensive studies of the regulatory properties of both liver (McCormack, 1985) and kidney (McCormack et al., 1988) 2-OGDH have been described, and in which they were shown to be essentially similar to those found with the initially characterized pig heart enzyme (McCormack and Denton, 1979).

The second major finding of Smith et al. (1992), and on which their hypothesis is largely based, is that, whereas in intact kidney mitochondria increases in H⁺ concentration still stimulated the enzyme, in their studies with intact liver mitochondria they were unable to demonstrate any activation of the enzyme by increasing H⁺ concentration, and in fact reported some inhibition. Their third approach was then to measure transport and GOT activities, and hence, together with the apparent inability of H⁺ to regulate

2-OGDH within the intact liver mitochondrial matrix, to arrive at the conclusions outlined above.

However, a first criticism to be raised is that Smith et al. (1992) used a rather indirect method of calculating 2-OGDH flux (see their paper). In contrast, using a direct assay in which ¹⁴CO₂ production from 2-oxo[1-¹⁴C]glutarate was monitored, McCormack (1985) demonstrated that the enzyme did show pH-sensitivity when located within intact liver mitochondria. Although the pH differences (7.2 versus 7.0) and incubation conditions were different from those used in Smith et al. (7 versus 6.6), this contrary finding must surely put in doubt the major conclusions of the latter work, or at least the work should maybe be repeated using the more direct assay system and perhaps also more suitable incubation conditions (see below).

McCormack (1985) also obtained similar results using the ¹⁴CO₂ technique, but with [1-¹⁴C]glutamate, except that the observed *K_m* values were around 5 times those obtained with 2-oxoglutarate. This would be consistent with the expected physiological equilibrium status of these two substrates through glutamate dehydrogenase. However, this finding is again at variance with the suggestion by Smith et al. (1992), based on their data and methodology with exogenous glutamate, where a preference for this as a substrate was shown, that 2-oxoglutarate generated by transamination has preferential access to 2-OGDH.

Smith et al. (1992) also reported modest activatory effects of Ca²⁺ on 2-OGDH in intact mitochondria from both tissues under some incubation conditions. Again, it is surprising that earlier reports were not quoted, where very marked demonstrations of the Ca²⁺-sensitivity of this enzyme, and indeed the other two matrix Ca²⁺-sensitive dehydrogenases (NAD⁺-isocitrate dehydrogenase and pyruvate dehydrogenase) were fully described within intact liver (McCormack, 1985; Johnston and Brand, 1987) and kidney (Tullson and Goldstein, 1982; McCormack et al., 1988) mitochondria under many different conditions. Another unquoted work of relevance was the observations by Johnston and Brand (1989) that Ca²⁺ also stimulated citrulline synthesis by intact liver mitochondria.

Consultation of these early works and adoption of some of the approaches used could clearly have helped the work of Smith et al. (1992) and may have led to different conclusions. For instance, the latter workers could observe no effects of Ca²⁺ in the presence of ADP or using the oxygen electrode, and therefore concluded that at high ADP/ATP ratios neither Ca²⁺ nor H⁺ could regulate the enzyme. In contrast, McCormack et al. (1988) clearly demonstrated effects of Ca²⁺ using the oxygen electrode and other techniques with kidney mitochondria incubated with ADP, although McCormack (1985) did report difficulty in demonstrating such effects with liver mitochondria under these conditions; nevertheless, effects were clearly evident under other conditions using several other techniques, including a 'State 3.5' respiration condition.

However, Johnston and Brand (1987) were able to show clear effects of Ca²⁺ on liver mitochondrial ADP-stimulated respiration, by simply using an oxygen electrode, and with flux through all three of the matrix Ca²⁺-sensitive dehydrogenases. The critical difference here was that the temperature of incubation was the physiological 37 °C, and this clearly influences what step becomes rate-limiting for the overall process. The experiments of Smith et al. (1992) were carried out at 28 °C, and it is important to ask what would have been found, and what would be the resultant hypothesis, if they had been carried out at 37 °C. It may also be useful to make further use of the observations of Johnston and Brand (1987) on oxygen uptake with isocitrate as substrate, as NAD⁺-isocitrate dehydrogenase is also regulated by pH in a similar manner to 2-OGDH (McCormack and Denton, 1981),