

distribution. In this respect, preliminary results from affinity labelling studies on rat liver glucokinase (our unpublished observations) suggest that this enzyme differs even more markedly in its active-site geometry.

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B. A. Connolly and I. P. Trayer, Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham, Great Britain, B15 2TT

APPENDIX

Validity of a 'Steady-State' Treatment of Inactivation Kinetics

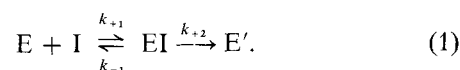
Athel CORNISH-BOWDEN

Department of Biochemistry, University of Birmingham

The method of Kitz and Wilson [1] for analysing the kinetics of inactivation and the more general variant described by Malcolm and Radda [2] are widely used and would require no special comment if it were not that they have been strongly criticized by Childs and Bardsley [3]. If this criticism were valid it would cast doubt on the analysis not only on our results but also those of many experiments in the literature. It seems important, therefore, to establish (a) that the criticisms by Childs and Bardsley [3] start from a premise that is certainly incredible in the specific cases

of the experiments of Kitz and Wilson [1] and those in the present paper, and probably many others also; and (b) that their treatment contains a subtle but serious error that introduces far worse problems than the relatively trivial ones that it sets out to solve.

The model of Kitz and Wilson [1] is similar to that of Michaelis and Menten [4] for enzymic catalysis in that it proposes that enzyme inactivation proceeds by a two-step mechanism in which equilibrium is maintained in the first step throughout the reaction:



According to Childs and Bardsley [3] these assumptions constitute a logical contradiction. They do not explain this view but presumably they mean that the net reaction can proceed only if there is flux through the first step, which cannot therefore be at equilibrium.

There is, however, an implied assumption in the model of Kitz and Wilson [1] that is used and understood in all branches of chemical kinetics and causes no difficulty unless the term equilibrium is defined in an excessively pedantic way; this is that provided k_{+2} is small compared with k_{-1} , the ratio $[E][I]/[EI]$ will not be detectably different during most of the reaction from the value k_{-1}/k_{+1} that it would have if the step were indeed an equilibrium. Kitz and Wilson [1] presented results for several specific inhibitors of acetylcholinesterase, of which those that most nearly merited the objections of Childs and Bardsley [3] were for 1-methyl-3-hydroxypyridinium iodide methane-sulphonate, for which Kitz and Wilson [1] found $k_{+2} = 5 \times 10^{-3} \text{ s}^{-1}$, $k_{-1}/k_{+1} = 0.1 \text{ mM}$. For these objections to have any substance, k_{-1} would have to be of the same order of magnitude as k_{+2} , or smaller, in which case k_{+1} would be of the order of $50 \text{ M}^{-1} \text{ s}^{-1}$. In other words, the binding of enzyme and specific inhibitor would have to proceed many orders of magnitude more slowly than a typical diffusion-limited process with rate constant about $10^7 \text{ M}^{-1} \text{ s}^{-1}$ [5]. This is very hard to believe, and for similar reasons we would reject analogous criticisms of the use of the method of Kitz and Wilson [1] in our own work.

Nonetheless, there may be circumstances in which the premise of Childs and Bardsley [3] has some merit, and we must therefore show that their main conclusions have none in any circumstances. Provided that the inhibitor is in large excess over enzyme, the inhibitor concentration is effectively constant, and the differential equations that describe (Eqn (1)) are readily soluble (see e.g. Fersht [6]). The process of inactivation follows a double exponential time course with relaxation times τ_1 and τ_2 as follows:

$$1/\tau_1 = 1/2 \{ k_{+1}[I] + k_{-1} + k_{+2} + \sqrt{(k_{+1}[I] + k_{-1} + k_{+2})^2 - 4k_{+1}k_{+2}[I]} \} \quad (2)$$

$$1/\tau_2 = 1/2 \{ k_{+1}[I] + k_{-1} + k_{+2} - \sqrt{(k_{+1}[I] + k_{-1} + k_{+2})^2 - 4k_{+1}k_{+2}[I]} \} \quad (3)$$

It is obvious from inspection that τ_2 is the larger of τ_1 and τ_2 for any values of the rate constants. Consequently, if apparent first-order kinetics are observed after decay of the first exponential, the apparent first-order rate constant k_{app} is identical with $1/\tau_2$. Childs and Bardsley [3], however, give a different expression for k_{app} , their Eqn (8), which takes the following form when the second step in the reaction is irreversible ($k_{-2} = 0$ in their symbolism):

$$k_{app} = 1/2 \{ k_{+1}[I] + k_{-2} + k_{+2} - (k_{+1}[I] + k_{-1} - k_{+2}) \times \sqrt{1 + \frac{4k_{-1}k_{+2}}{(k_{+1}[I] + k_{-1} - k_{+2})^2}} \} \quad (4)$$

Table 1 A. Values of k_{app} calculated in various ways

For various values of k_{-1} and k_{+2} , with $k_{+1}[I]$ assigned a value of 1 arbitrary unit, the table shows the values of k_{app} given by the correct expression [Eqn (3)], the expression of Childs & Bardsley [3] [Eqn (4)] and an approximate 'steady-state' expression [Eqn (5)]

k_{-1}	k_{+2}	k_{app} obtained using		
		Eqn (3)	Eqn (4)	Eqn (5)
0.01	0.1	0.0989	0.0989	0.0901
0.1	0.01	0.00908	0.00908	0.00901
0.1	0.1	0.0901	0.0901	0.0833
0.1	1	0.730	0.730	0.476
0.1	10	0.989	10.1	0.901
1	0.1	0.0488	0.0488	0.0476
1	1	0.382	0.382	0.333
1	10	0.901	11.1	0.833
10	0.1	0.00902	0.00902	0.00901
10	1	0.0839	0.0840	0.0833
10	10	0.488	0.488	0.476
10	100	0.908	110	0.901
100	10	0.0902	0.0901	0.0901

Although this expression is indeed equivalent to that for $1/\tau_2$ if $k_{+1}[I] + k_{-1} \geq k_{+2}$, it is equivalent to that for $1/\tau_1$ if $k_{+1}[I] + k_{-1} \leq k_{+2}$. This difference arises because Eqn (4), unlike Eqn (3), does not necessarily refer to the longer relaxation time. Thus, Eqn (4) is not necessarily a correct expression for k_{app} and it is precisely when the premise of Childs and Bardsley [3] is correct, i.e. when k_{+2} cannot be assumed small compared with k_{-1} , that their own analysis is most seriously in error.

It remains to show a less rigorous but simpler analysis, the steady-state treatment of Malcolm and Radda [2], gives nearly correct results in most circumstances. By analogy with the steady-state treatment of the catalytic analogue of Eqn (1) [7], one might suppose that the apparent first-order rate constant would be given by an expression of the form

$$k_{app} = k_{+1}k_{+2}[I]/(k_{+1}[I] + k_{-1} + k_{+2}) \quad (5)$$

regardless of the relative magnitudes of k_{-1} and k_{+2} . This equation can be obtained from Eqn (3) by assuming that $(k_{+2} + k_{-1} + k_{+1}[I])^2$ is large compared with $4k_{+1}k_{+2}[I]$, which can be regarded as the condition for a steady state in this system. In fact Eqn (5) is not quite correct but the error involved is small in most circumstances, as may be judged from the representative values calculated in Table 1 A. Only when $k_{+2} \approx k_{+1}[I] \gg k_{-1}$ do the errors exceed about 10% and even then they are trivial by comparison with the errors one risks by using the expression for k_{app} given by Childs and Bardsley [3]. This rather special case is readily recognizable experimentally because if $k_{+2} \approx k_{+1}[I] \gg k_{-1}$ the two relaxation times defined by Eqns (2) and (3) are of similar magnitude and consequently one would observe large deviations from first-order kinetics in any time scale.

The slight extension of the model of Malcolm and Radda [2] to accommodate competitive inhibition by substrate (Scheme 1 of main paper) is analogous to competitive inhibition in a catalytic reaction and does not require any special discussion.

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A. Cornish-Bowden, Department of Biochemistry, University of Birmingham,
P.O. Box 363, Birmingham, Great Britain, B15 2TT