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The Kinetics of Coupled Enzyme Reactions

APPLICATIONS TO THE ASSAY OF GLUCOKINASE, WITH GLUCOSE 6-PHOSPHATE DEHYDROGENASE AS COUPLING ENZYME

By ANDREW C. STORER and ATHEL CORNISH-BOWDEN
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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1. A theory is developed to account for the kinetics of coupled-enzyme reactions without assuming that the second reaction follows first-order kinetics. 2. A simple procedure is described for applying the theory to the practical design of enzyme assays. 3. The validity of the theory is confirmed for the assay of glucokinase with glucose 6-phosphate dehydrogenase as coupling enzyme. 4. The possibility of extending the theory to three or more coupled reactions is discussed.

Many enzyme-catalysed reactions are difficult or impossible to assay directly. For these it is common practice to use a coupled assay, in which the product of the reaction of interest is made to react in a second reaction that is more amenable to assaying, as shown in the scheme:

$$A \xrightarrow{v_1} B \xrightarrow{v_2} C$$

where A → B is the reaction of interest, with velocity v_1, and B → C is the coupling reaction, with velocity v_2. Plainly the assay is valid only if the measured velocity v_2 becomes equal to v_1 under experimental conditions, and it is important to establish whether this is likely to be true in practice. There have been several theoretical studies of this scheme (Bergmeyer, 1953, 1963; Gutfreund, 1965; McClure, 1969; Barwell & Hess, 1970; Hart, 1970; Goldman & Katchalski, 1971; Easterby, 1973), some of which have treated more complex assays in which there are three or more consecutive reactions. In every case, the second and subsequent reactions have been assumed to follow first-order kinetics. But this appears to have been done more for mathematical convenience than because it is likely to be true; in reality, the conversion of B into C is almost invariably an enzyme-catalysed reaction, so that one would expect its velocity to be governed by the Michaelis–Menten equation:

$$v_2 = \frac{V_2 b}{K_2 + b} \qquad (1)$$

where b is the instantaneous concentration of B, and V_2 and K_2 are the maximum velocity and Michaelis constant respectively of the enzyme that catalyses the reaction B → C. This expression degenerates into a simple first-order dependence on b only if b is much smaller than K_2 at all times, and it has usually been assumed that a coupled assay can be valid only if this condition is met. Gutfreund (1965) suggests the looser condition b equal to or smaller than K_2 at all times, on the grounds that the first-order approximation does not become grossly incorrect until this condition is broken. The question is of practical importance in the design of enzyme assays, because if too little coupling enzyme is used the measured velocity bears an uncertain relationship to the velocity of interest, so that the assay is invalid. On the other hand, it is wasteful and expensive to use an unnecessarily large amount of coupling enzyme.

Our interest in this problem was stimulated by the observation that a previously satisfactory assay for glucokinase (EC 2.7.1.2) with glucose 6-phosphate dehydrogenase (EC 1.1.1.49) as coupling enzyme (Parry & Walker, 1966) displayed a significant lag period when used in the presence of free (i.e. uncomplexed) ATP. Any supposition that this might indicate a hysteretic property of glucokinase (Frieden, 1970) was made rather unlikely by the fact that Avigad (1966) had shown that free ATP was an inhibitor of glucose 6-phosphate dehydrogenase, competitive with glucose 6-phosphate, and that it caused a similar lag period when added to the analogous assay for hexokinase (EC 2.7.1.1). So the original purpose of the present investigation was to determine whether such inhibition of the coupling enzyme could account for these observations, and to delineate the kinetics of coupled-enzyme reactions more rigorously than had been done previously.

Theory

Consider a reactant B, generated at a constant velocity v_1 in a reaction A → B, and consumed in a second reaction B → C at a velocity v_2 given by eqn. (1). Then the rate of change of b is given by:

$$\frac{db}{dt} = v_1 - v_2 \qquad (2)$$

Table 1. *Values of ϕ at various values of v_1/V_2 and v_2/v_1*

The table provides a simple method of calculating the time required for v_2 to reach $0.9v_1$, $0.95v_1$ or $0.99v_1$, for various values of v_1/V_2. This time is given by $\phi K_2/v_1$.

	v_2/v_1		
v_1/V_2	0.9	0.95	0.99
0.00	0.00	0.00	0.00
0.05	0.12	0.16	0.25
0.10	0.26	0.35	0.54
0.15	0.42	0.56	0.89
0.20	0.60	0.81	1.31
0.25	0.81	1.11	1.81
0.30	1.06	1.46	2.42
0.35	1.35	1.88	3.18
0.40	1.67	2.39	4.12
0.45	2.10	3.02	5.32
0.50	2.59	3.80	6.86
0.55	3.20	4.79	8.91
0.60	3.96	6.08	11.7
0.65	4.93	7.80	15.6
0.70	6.20	10.2	21.4
0.75	7.91	13.6	30.3
0.80	10.3	18.7	45.5
0.85	13.8	27.2	74.3
0.90	19.4	42.8	141
0.95	29.2	77.9	377

Before this differential equation can be solved, one of the three variables b, t and v_2 must be eliminated. This may be done by rearranging eqn. (1) to give an expression for b in terms of v_2:

$$b = \frac{K_2 v_2}{V_2 - v_2} \tag{3}$$

which may be differentiated with respect to v_2 to give:

$$\frac{db}{dv_2} = \frac{V_2 K_2}{(V_2 - v_2)^2} \tag{4}$$

and so:

$$\frac{db}{dt} = \frac{V_2 K_2}{(V_2 - v_2)^2} \cdot \frac{dv_2}{dt} \tag{5}$$

After substituting this into eqn. (2) and rearranging, we have:

$$\int \frac{V_2 K_2 \, dv_2}{[(v_1 - v_2)(V_2 - v_2)^2]} = \int dt \tag{6}$$

which may be integrated to give:

$$\frac{-V_2 K_2}{V_2 - v_1}\left[\frac{1}{V_2 - v_2} + \frac{1}{V_2 - v_1} \cdot \ln\left(\frac{v_1 - v_2}{V_2 - v_2}\right)\right] = t + \alpha \tag{7}$$

where α is a constant of integration. The boundary condition $v_2 = 0$ when $t = 0$ gives the value of α, and, after rearranging, we have:

$$t = \frac{V_2 K_2}{(V_2 - v_1)^2} \cdot \ln\left[\frac{v_1(V_2 - v_2)}{V_2(v_1 - v_2)}\right] - \frac{K_2 v_2}{(V_2 - v_2)(V_2 - v_1)} \tag{8}$$

This equation shows, for any values of v_1, K_2 and V_2, the time required for v_2 to reach any value. For convenient application it may be written as:

$$t = \phi K_2 / v_1 \tag{9}$$

where ϕ is given by:

$$\phi = \frac{V_2 v_1}{(V_2 - v_1)^2} \cdot \ln\left[\frac{v_1(V_2 - v_2)}{V_2(v_1 - v_2)}\right] - \frac{v_1 v_2}{(V_2 - v_2)(V_2 - v_1)} \tag{10}$$

This expression shows that ϕ is a dimensionless number and a function of the ratios v_2/v_1 and v_1/V_2 only. Values of ϕ are given in Table 1 for various values of these ratios.

The value of v_2/v_1 that is appropriate to use in any given situation depends on the accuracy required in the assay: in a precise kinetic study, v_2/v_1 should be at least 0.99, but lower values may be adequate in more qualitative applications. Table 1 provides a simple way of calculating the smallest value of V_2 that can safely be used to obtain any desired accuracy, and an example will be given below.

Materials and Methods

Glucokinase was partially purified from rat liver by affinity chromatography, by the procedure outlined by Chesher *et al.* (1973).

NADP and yeast glucose 6-phosphate dehydrogenase were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A.

Glucose 6-phosphate was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Reactions were carried out at 30°C in 0.05M-tetramethylammonium glycylglycinate buffer at pH8.0, containing 1mM-NADP, 20mM-glucose, 5mM-ATP, 5mM-$MgCl_2$, 0.1M-KCl, glucokinase and glucose 6-phosphate dehydrogenase, in a total volume of 0.75ml. The glucose 6-phosphate dehydrogenase had been dialysed overnight against 0.02M-Tris–HCl buffer at pH7.5, to remove $(NH_4)_2SO_4$.

Production of NADPH was followed with a Gilford recording spectrophotometer model 2400-S with digital print-out, which was purchased from Gilford Instruments Ltd., Morden, Surrey, U.K.

Kinetic parameters for glucose 6-phosphate dehydrogenase under the conditions of the assay were determined from the direct linear plot of Eisenthal & Cornish-Bowden (1974).

Results

Glucokinase and glucose 6-phosphate dehydrogenase provide a convenient example for illustrating the kinetics of coupled-enzyme reactions. In order to apply the theory to the design of an assay for glucokinase, it is necessary to know K_2, i.e. the Michaelis constant for glucose 6-phosphate in the dehydrogenase reaction, and to specify the largest value of v_1 to be measured, and the time t within which v_2 must

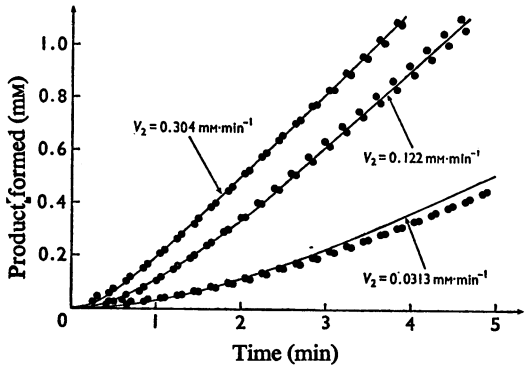

Fig. 1. *Comparison of theory with experimental results for the assay of glucokinase with glucose 6-phosphate dehydrogenase as coupling enzyme*

Experimental points were obtained in duplicate under the conditions described in the Materials and Methods section. Six different concentrations of coupling enzyme were used, of which the results of three are shown here, with the values of V_2 indicated on the curves. The curves were calculated by numerical integration of eqn. (8), after inserting the values $v_1 = 0.0309$ mM·min^{-1}, $K_2 = 0.11$ mM, and the appropriate values of V_2.

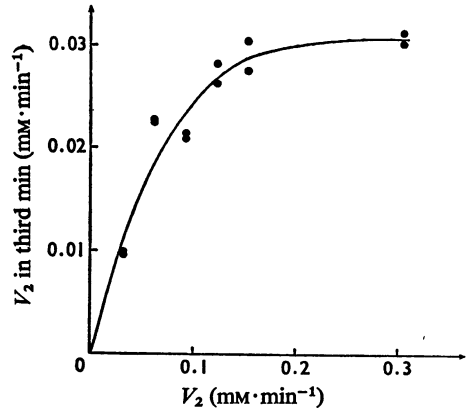

Fig. 2. *Velocities estimated from the 'linear' portions of glucokinase assays*

Values of v_2 were estimated for the apparently linear period from $t = 2.0$ to $t = 3.0$ min for each of twelve assays, and are plotted against V_2. The curve is a theoretical line, and shows the instantaneous velocities for $t = 2.5$ min, calculated as described in the text.

reach a given fraction of v_1. K_2 was found in a preliminary experiment to be 0.11 mM under the conditions of the assay; and, for example, let us assume that v_1 will not exceed 0.04 mM·min^{-1} and that v_2 is to reach $0.99v_1$ within 1 min. Substitution of these values of K_2, v_1 and t into eqn. (9) shows ϕ to be $1.0(0.04/0.11) = 0.36$. Table 1 shows that $\phi = 0.25$ for $v_1/V_2 = 0.05$ and $v_2/v_1 = 0.99$, and $\phi = 0.54$ for $v_1/V_2 = 0.1$ and $v_2/v_1 = 0.99$; so the required value of v_1/V_2 is between 0.05 and 0.1. For most practical purposes, a rough interpolation between these values, e.g. $V_2 = v_1/0.08 = 0.5$ mM·min^{-1}, is sufficiently accurate; but more precise interpolation can be used if greater accuracy is needed.

A calculation of this type allows no margin of error, and does not allow higher values of v_1 to be measured accurately. It is wise therefore to allow for values of v_1 appreciably higher than one expects to encounter. In addition, no allowance was made in the calculation for product inhibition of glucokinase by glucose 6-phosphate, or, in the general case, for inhibition of the first enzyme by reactant B. This possibility can be checked by calculating the steady-state concentration of reactant B from eqn. (3), remembering that $v_2 = v_1$ at the limit, so that, in the example given, the steady-state concentration of reactant B, i.e. glucose 6-phosphate, is $0.11(0.04)/(0.5-0.04) = 0.0096$ mM. This value is trivial by comparison with the value of $K_i = 65$ mM for inhibition of glucokinase by glucose 6-phosphate (Parry & Walker, 1966), so that product inhibition should cause utterly negligible errors in this assay. But the situation would be different if the same assay were to be applied to brain hexokinase instead of glucokinase: in this case, with $K_i = 0.4$ mM (Crane & Sols, 1954), significant, though still small, errors would result from inhibition by glucose 6-phosphate.

To test the validity of the theory experimentally, assays were done in duplicate with six different concentrations of glucose 6-phosphate dehydrogenase, chosen so that V_2 would vary in the range $1v_1$ to $10v_1$. These values of V_2 were deliberately made rather smaller than that calculated above to provide conditions where the simpler first-order theory would certainly fail. The results for three values of V_2 are shown in Fig. 1. The value of v_1 was estimated as 0.0309 mM·min^{-1} from the mean of the limiting slopes of the two assays at the highest values of V_2. This value of v_1 was used, with the appropriate values of K_2 and V_2, in calculating the three theoretical lines from eqn. (8). It may be seen that the observations agreed very well with the theory during the first 2½ min of each assay, and that the subsequent deviations were quite small and within experimental error.

For each of the assays done, the observations were found to lie approximately on a straight line during the third minute, i.e. from $t = 2.0$ to $t = 3.0$ min. This may be seen in Fig. 1 for the six assays illustrated, though it is clear from the entire plots that in four of the six the apparent linearity in the third minute

is misleading and does not provide a correct impression of the limiting velocity. Thus the apparent linearity of a recorder trace is a very unreliable criterion for determining whether a coupled assay is valid, unless the apparently linear period is at least ten times as long as the period of obvious acceleration. To develop this point further, the mean velocities during the third minute were determined for all twelve experiments, and are plotted in Fig. 2. The curve was calculated by solving eqn. (8) for v_2, with $t = 2.5$min. Although the results agree with the theory within experimental error, they also emphasize that apparently constant rates can greatly underestimate the steady-state rates.

Discussion

The theory we have described is as simple to apply as earlier treatments, notably that of McClure (1969), but it is valid under much more general conditions, and shows that appreciable deviations from first-order kinetics can be tolerated in coupled assays, and can be described quantitatively. This conclusion must be applied cautiously, however, because it is easy to be misled into supposing a reaction to have reached its limiting velocity when it has not. It is advisable therefore to calculate the time required for v_2 to reach $0.99v_1$, and not to rely on the apparent linearity of the reaction. The calculation requires a rough maximum estimate of v_1, but this is likely to be known in advance with sufficient accuracy in most practical situations. In other cases a preliminary experiment along the lines of those described in this paper may be required.

A disadvantage of the first-order treatment of coupled assays is that the kinetic constants V_2 and K_2 must be coalesced into the first-order rate constant V_2/K_2. So environmental effects that alter the apparent values of these constants cannot be separated; but eqn.(8) does permit them to be separated. It shows, for example, that the time required for v_2 to reach any given fraction of v_1 is directly proportional to K_2, so that competitive inhibitors of the coupling enzyme should decrease the effectiveness of an assay considerably. This agrees with the observations that the lag period before a steady state is reached in assaying glucokinase or hexokinase is markedly increased by the addition of free ATP, a competitive inhibitor of the coupling enzyme, glucose 6-phosphate dehydrogenase (Avigad, 1966). Newsholme *et al.* (1970) have reported very similar observations with the assay of phosphofructokinase.

The first-order treatment also implies that if K_2 and V_2 are perturbed in constant ratio, e.g. by the presence of an uncompetitive inhibitor, the lag-time should be unaffected. But eqn. (8) shows that the behaviour is not as simple as this, and that a decrease in V_2 is not exactly compensated by a proportionate decrease in K_2. Only if V_2 is at least $10v_1$ does the simpler theory provide an adequate approximation. But in favourable cases, i.e. if v_1 is constant for a long period and product inhibition is slight, it is possible to have a valid assay when V_2 is appreciably less than $10v_1$, with a considerable saving of coupling enzyme.

It is not merely wasteful to use an unnecessarily high concentration of coupling enzyme; it can also lead to complications because of non-specific reactions. For example, glucose 6-phosphate dehydrogenase can use glucose as a substrate instead of glucose 6-phosphate (Kuby & Noltmann, 1966), so that there is a small blank rate in the absence of glucokinase. At the concentrations used in our experiments, this blank rate was negligible, but it would not have been negligible if the concentration of glucose 6-phosphate dehydrogenase had been much higher than the highest concentration actually used. In this case, blank-rate corrections would have been needed; but such corrections are undesirable, not only because of the inconvenience, but also because they require an assumption that the blank rate is the same in the complete system as in the incomplete system. There can rarely be any certainty that such an assumption is correct.

The theory presented here cannot readily be extended to assays in which there are three or more reactions in sequence, because the differential equations that describe such systems are much more complex than eqn. (6), and cannot be solved simply by integration. But an extension of the theory for two reactions does provide a simple way of calculating upper and lower limits for the time required for the observed velocity to reach a given fraction of v_1. Consider the following scheme:

$$A \xrightarrow{v_1} B \xrightarrow{v_2} C \xrightarrow{v_3} D$$

where all three reactions are irreversible, v_1 is constant, v_2 is governed by eqn. (1), and v_3 is governed by a similar equation with constants V_3 and K_3. The time t_1 required for v_2 to reach $0.99v_1$ (for example) may be found from eqn. (9) and Table 1, exactly as for the simpler system, since the third reaction does not affect v_2. Then, by treating v_2 as a constant, the time t_2 required for v_3 to reach $0.99v_2$ may be estimated in the same way, substituting K_3 for K_2, v_2 for v_1 and V_3 for V_2. This calculation overestimates t_2, because v_3 begins to increase long before v_2 reaches $0.99v_1$, and so the total time $t_{tot.}$ for v_3 to reach $0.98v_1$ (i.e. 0.99^2v_1) is less than (t_1+t_2). But $t_{tot.}$ cannot be less than the greater of t_1 and t_2, and must therefore be bounded by:

$$t_1, t_2 < t_{tot.} < (t_1 + t_2)$$

These limits define $t_{tot.}$ precisely enough for most practical purposes. This approach can be extended in

an obvious way to systems of four or more coupled reactions.

We have been primarily concerned in this paper with coupled-enzyme reactions in the context of enzyme assay. But some workers, such as Barwell & Hess (1970) and Goldman & Katchalski (1971), have been more concerned with the interaction of consecutive reactions under metabolic conditions, where the concentrations of substrates and enzymes are not directly under the control of the experimenter. It is by no means certain that any of the reactions obey first-order kinetics under such conditions (Gutfreund, 1965); instead, it may well be possible for efficient flow of reactants through a metabolic pathway to be achieved with much lower enzyme concentrations than would be needed to give first-order kinetics. Thus it seems likely that the theory presented in the present paper may prove helpful in the analysis of metabolic systems.

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