A Kinetic Analysis of Coupled Enzyme Assays*

W. R. McClure†

ABSTRACT: Rate equations for consecutive irreversible reactions such as are encountered in coupled enzyme assays are solved. For an assay containing one auxiliary enzyme, an expression is derived relating the time required to produce a linear reaction velocity and the amount of coupling enzyme added.

The more complicated system in which two auxiliary enzymes are used required a graphical analysis. A nomogram is presented which shows the dependence of each coupling enzyme on the other in producing a linear reaction rate. Practical considerations for implementing these results are discussed with a view toward conserving auxiliary enzymes, saving the investigator's time, and increasing his confidence in results obtained from coupled assays.

An enzyme assay should, if possible, be accurate, sensitive, continuous, and convenient. Whereas only the first of these characteristics is essential, the last three are certainly to be desired. When the reaction of interest fails to meet these criteria, one solution to the problem is through the removal of a product by an auxiliary enzyme which provides sensitive and continuous monitoring of the resulting reaction rate. However, this approach has the following disadvantages: auxiliary substrates must be added, the efficiency of auxiliary enzymes to accurately reflect the true initial rate is sometimes doubtful, and a critical kinetic approach to the problem has never appeared.

To answer the above objections and others, I began an examination of coupled enzyme assays from a kinetic standpoint. From the theory derived, practical guidelines were sought which would conserve time in setting up an assay, and increase confidence in the results obtained.

In measuring a linear rate of conversion of A into B in an assay with one or more auxiliary enzymes, we will be interested in two main features: the concentration of intermediates in the steady state and secondly, the time required for the system to achieve this steady-state condition. The objective is not an accurate description of the transient states prior to a linear observed rate; instead I will emphasize relationships that will enable an investigator to efficiently minimize the lag time in coupled assays. In the same manner, expressions will be derived for the time necessary to attain a practical fraction of the steady state, since the theoretical criterion for this condition is that time approaches infinity.

Theory

One Auxiliary Enzyme. The simplest coupled assay is one in which a product of the primary reaction is removed by one auxiliary enzyme; represented schematically

$$A \xrightarrow[\text{primary enzyme}]{k_1} B \xrightarrow[\text{auxiliary enzyme}]{k_2} C$$

The following assumptions have been made for this system. (1) k_1 is the rate constant of a zero-order, irreversible reaction. This requires that all substrates of reaction 1 are effectively saturating or that only a small fraction of A is converted into B during the period of observation. Irreversibility is assumed since a product is continuously removed from the reaction; k_1 will be used with units of mM min^{-1}. (2) Reaction 2 is irreversible and first order with respect to (B). This assumption requires $(B) \ll K_B$, and that any other substrate for reaction 2 be essentially saturating. For those reactions where the equilibrium lies substantially to the right and/or only a small fraction of the reaction is followed, the irreversibility assumption is satisfactory. In cases where these conditions are met, the normal Michaelis–Menten equation for the conversion of B into C, $-d(B)/dt = V(B)/K_B + (B)$, reduces to $-d(B)/dt = (V/K_B)(B)$, and the first-order rate constant $k_2 = V_2/K_B$. At 25°, V_2 has units of mM min^{-1} or IU ml^{-1} and k_2 therefore is expressed in min^{-1}.

Since a linear initial rate is dependent upon B reaching a steady-state concentration, we will focus our attention on this intermediate. The rate equation for the above system based on the assumptions discussed is

$$\frac{d(B)}{dt} = k_1 - k_2(B) \tag{1}$$

which integrates to give

$$(B) = \frac{k_1}{k_2}(1 - e^{-k_2 t}) \tag{2}$$

At $t = 0$, $(B) = 0$, and as $t \rightarrow \infty$

$$(B)_{ss} = \frac{k_1}{k_2} \tag{3}$$

where the subscript refers to the steady-state concentration of B.

To obtain an expression for the time required to produce a practical percentage of the steady-state condition, we rearrange

* From the Department of Biochemistry and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin. Supported by grants from the National Institutes of Health (AM 10,334) and the National Science Foundation (GB 6676X).

† Predoctoral Fellow, National Institutes of Health.

eq 2, and take logarithms obtaining

$$\ln\left[1 - \frac{k_2}{k_1}(B)\right] = -k_2 t \tag{4}$$

Let F_B = fraction $(B)_{ss}$ (*i.e.*, fraction k_1/k_2 desired at time = t^*, and substitute into eq 4; rearranging we find

$$t^* = -\frac{\ln(1 - F_B)}{k_2} \tag{5}$$

Thus the time required to reach a fraction of the steady-state B concentration is inversely proportional to k_2.

Substitution of V_2/K_B transforms the above result into experimental terms, and we obtain

$$V_2 = -\frac{\ln(1 - F_B)K_B}{t^*} \tag{6}$$

All that remains in determining the necessary units of auxiliary enzyme for the assay is a knowledge of K_B under the experimental conditions and a decision as to the magnitude of F_B required at a desired time, t^*. For example, if K_B = 0.10 mM, t^* = 6 sec, and 0.99 $(B)_{ss}$ is chosen as being experimentally equivalent to the achievement of the steady state, eq 6 predicts that (2.303 × 2 × 0.10)/0.10 = 4.6 IU/ml of auxiliary enzyme is necessary. Thus under the above conditions, an essentially linear rate of conversion of B into C will be obtained within the time necessary to mix, manually, the initiating enzyme or substrate with the rest of the reaction solution.

The following features of eq 5 and 6 should be noted: (1) k_2 which represents the ability of the auxiliary enzyme to produce a linear initial rate is a quotient of V_2 (IU of auxiliary enzyme/ml)/K_B (not merely an "excess of auxiliary enzyme"). (2) The time required for steady-state production is independent of k_1. Therefore, once set up, a coupled assay is suitable for determining any activity of the primary enzyme, as long as $(B)_{ss} \ll K_B$ as determined by eq 3. (3) $(B)_{ss}$ is a function of k_1 and k_2. For example, if k_1 = 0.05 mM min^{-1} in the above example, then from eq 3, $(B)_{ss}$ = 0.0011 mM. We also learn from this result that our initial assumption requiring (B) ≪ K_B is valid, and that reaction 2 is indeed very nearly first order with respect to (B).

Figure 1 shows the time dependence of (B) and (C) for a hypothetical dehydrogenase reaction coupled to another enzyme using the kinetic parameters discussed above. The coincidence of a linear observed rate and 0.99 $(B)_{ss}$ is apparent at 6 sec. The right ordinate also indicates the decrease in absorbance which would be observed in a 10-mm cell.

This analysis also applies to a fixed-time assay where, for example, a radioactive substrate, A, is not available for the reaction of interest. However, if a product, B, can be coupled to a reaction where radioactive substrate, X*, is available, the primary enzyme can be conveniently assayed; this is represented schematically as

$$A \longrightarrow B \xrightarrow[\text{or}]{X^* \quad Y^*} C^*$$

where either labeled Y or C can be quantitated at fixed times.

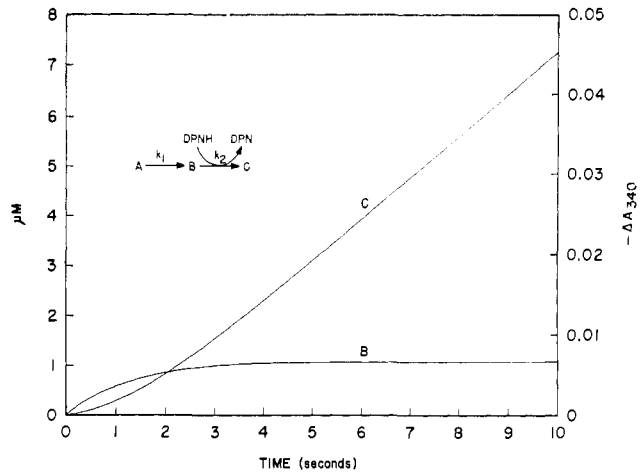


FIGURE 1: Concentrations of B and C are plotted *vs.* time for the coupled assay pictured above. The right ordinate shows the decrease in A_{340} accompanying the formation of C. k_1 = 0.05 mM min^{-1}, k_2 = 46 min^{-1}, and $(B)_{ss}$ = 1.09 μM.

Both concentrations will show time dependencies as shown in Figure 1, and the important point is that the first aliquot of the reaction mixture not be taken until F_B = 0.99. Successive samplings at two or three fixed intervals will then determine the reaction velocity.

Although in most cases an equally satisfactory coupled assay could have been set up by adding successively larger amounts of auxiliary enzyme, an analytical expression containing all of the important parameters is preferable for interpreting any additional nonlinearity in an assay and for other reasons discussed below.

Two Auxiliary Enzymes. The addition of one more auxiliary enzyme to a coupled assay results in a system which is much more difficult to analyze intuitively. The empirical approach of adding increasing amounts of coupling enzymes can give satisfactory results for the one enzyme-coupled assay as noted above. However, when two auxiliary enzymes must be used, a large amount of time can be saved and greater confidence in a coupled assay obtained by considering the manner in which the two additional enzyme activities are dependent upon each other in producing a linear initial rate. The system treated is represented schematically as

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D$$

The same assumptions are made for this theory as were imposed on the simpler two-enzyme case: specifically, that k_1 is a zero-order rate constant and that k_2 and k_3 are the first-order rate constants, since (B) and (C) will each be much lower than their respective Michaelis constants. In addition all three reactions are considered irreversible.

As we are interested in the rate of $(C)_{ss}$ attainment, we will again examine the time dependence of this final intermediate concentration. The rate equation is

$$\frac{d(C)}{dt} = k_2(B) - k_3(C) \tag{7}$$

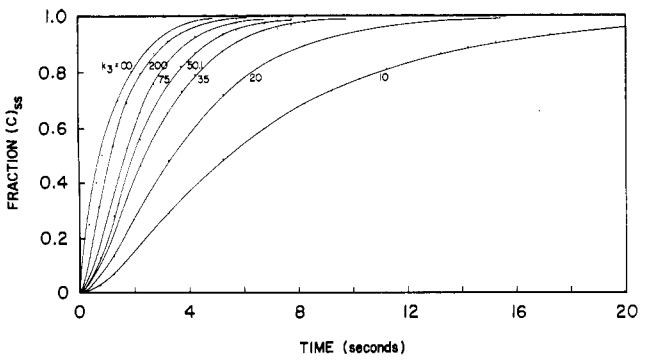


FIGURE 2: Computer solutions of eq 10 when $k_2 = 50$ min^{-1} and k_3 varies as indicated.

Substituting for (B) from eq 2, we obtain

$$\frac{d(C)}{dt} = k_1(1 - e^{-k_2t}) - k_3(C)$$

$$= k_1 - k_1e^{-k_2t} - k_3(C) \quad (8)$$

In the steady state $d(C)/dt = 0$ as $t \rightarrow \infty$. At this point

$$(C)_{ss} = \frac{k_1}{k_3} \quad (9)$$

Combining a particular solution to eq 8 with the general solution to the associated homogeneous equation and evaluating the constant for (C) = 0 at $t = 0$ and $(C)_{ss} = k_1/k_3$ as $t \rightarrow \infty$, we obtain

$$(C) = \frac{k_1}{k_3} - \frac{k_1}{k_3 - k_2}\left(e^{-k_2t} - \frac{k_2}{k_3}e^{-k_3t}\right) \quad (10)$$

Note the following about eq 10. (1) The first term is $(C)_{ss}$; the remainder of the expression represents the time dependence of steady-state attainment. (2) The rate of steady-state production is symmetrical with respect to k_2 and k_3; that is, a pair of values for k_2 and k_3 can be interchanged without affecting the rate at which the steady state is obtained. This will be apparent below, when we take the limits of eq 11 as either k_2 or $k_3 \rightarrow \infty$. (3) $(C)_{ss} \neq f(k_2)$, and the time required to achieve a given fraction of $(C)_{ss}$ is not a function of k_1. (4) Equation 10 is impossible to solve analytically for t or t^* as we did so conveniently in the case of the two-enzyme-coupled assay. (5) There is an apparent singularity in eq 10 when $k_2 = k_3$. However, solving eq 8 for the special case of $k_2 = k_3$ yields the following

$$(C) = \frac{k_1}{k_3} - k_1te^{-k_2t} - \frac{k_1}{k_3}e^{-k_3t}$$

If we define F_C = fraction $(C)_{ss}$ at time t^* and substitute into eq 10, we obtain after rearrangement

$$(k_3 - k_2)(1 - F_C) = k_3e^{-k_2t^*} - k_2e^{-k_3t^*} \quad (11)$$

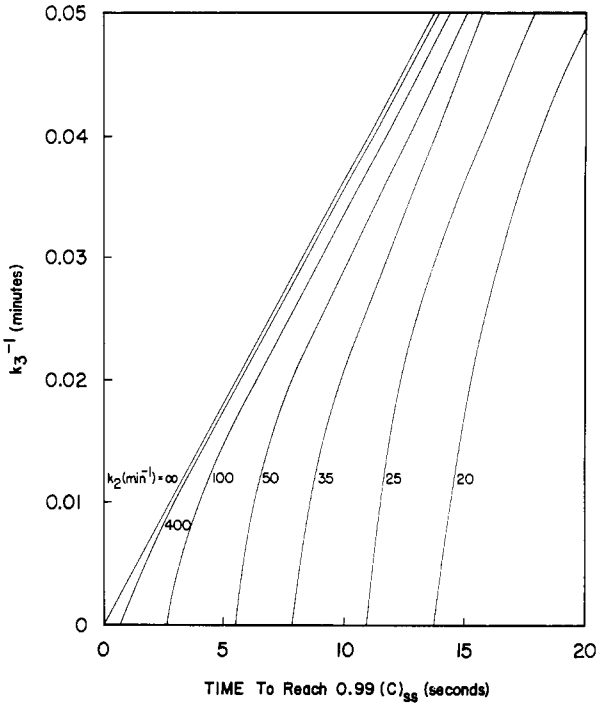


FIGURE 3: k_3^{-1} is plotted *vs.* time to reach 0.99 $(C)_{ss}$ for each of the indicated k_2 values. The slanted straight line is the theoretical limit for $k_2 = \infty$.

Dividing by k_3 and taking the limit as $k_3 \rightarrow \infty$

$$(1 - F_C) = e^{-k_2t^*} \text{ or}$$

$$t^* = -\frac{\ln(1 - F_C)}{k_2} \quad (12a)$$

This is exactly similar to eq 5 and means that as k_3 gets large in comparison with k_2, the concentration of C approaches a steady state as fast as does B and is limited only the the latter rate.

If instead we divide eq 11 by k_2 and take the limit as $k_2 \rightarrow \infty$, we find

$$t^* = -\frac{\ln(1 - F_C)}{k_3} \quad (12b)$$

In analogy with the above comments we say in this case that the rate of steady-state achievement is primarily dependent upon k_3, when k_2 is very large in comparison.

As seen in Figure 2, where fraction $(C)_{ss}$ is plotted *vs.* time for a fixed value of k_2 with varying values of k_3, it is not necessary for either k_3 or k_2 to approach their mathematical limit in order for the more simplified behavior expressed in eq 12a or 12b to obtain. This is due to the symmetry of eq 10 with respect to k_2 and k_3, and the fact that a large difference in the exponential terms will dominate the behavior of (C) as a function of time. If one constant is larger than the other by a factor of 4–5, the contribution of the corresponding exponential can essentially be neglected. For values of $k_2 \approx k_3$, eq 10 must be evaluated numerically. Although there is an apparent singularity in eq 10 when $k_2 = k_3$, the function is well

behaved when k_2 is very close to k_3 (*e.g.*, $k_2 = 100$ and $k_3 = 100.1$).

As noted above, an analytical expression giving the time dependence of reaching some desired fraction of the steady-state C concentration is not possible when $k_2 \approx k_3$. However, a series of graphs such as is shown in Figure 2 have been calculated using a CDC 1604 computer to evaluate eq 10 for selected values of k_1, k_2, k_3, and intervals of time such that 20–30 points were calculated for each curve. From these plots, the time, t^*, required to reach 0.99 $(C)_{ss}$ was obtained for each combination of k_2 and k_3 chosen. Note, however, that these graphs give relative concentrations of C as a fraction of $(C)_{ss}$ and not absolute (C) values.

In Figure 3, k_3^{-1} is plotted *vs.* t^*, the time required to produce 0.99 $(C)_{ss}$. Each curve thus represents data taken from a series of plots similar to Figure 2. The reciprocal plot was used so that higher values of k_3 could be presented and compared with the limiting value of $k_3 = \infty$ (*i.e.*, $k_3^{-1} = 0$). The limits imposed on t^* are bounded by the slanted line and the abscissa. For a given value of k_2, t^* approaches a minimum at $k_3^{-1} = 0$. This limit is computed from eq 12a and found on the abscissa. For example, if $k_2 = 50$ min^{-1}, $t^* > 5.5$ sec for any value of k_3. Similarly, for a given value of k_3, t^* approaches the minimum value of eq 12b as $k_2 \rightarrow \infty$; these values are found at the intercept of the slanted line and the corresponding k_3^{-1} value.

Any real values of these constants result in a longer t^* and can be found above and to the right of the theoretical limits for selected values of k_2 and k_3. Note that all the curves approach a limiting linear slope when $k_2 \gg k_3$. In addition k_2 and k_3 can be exchanged in Figure 3 if desired.

Another representation of the data taken from Figure 3 for discrete values of t^* and continuously varying values of k_2^{-1} and k_3^{-1} is shown in Figure 4.

The symmetry of the time dependence of $(C)_{ss}$ attainment with respect to k_2 and k_3 is apparent from this plot. In fact, for values of k_2 and $k_3 < 250$ min^{-1}, k_2^{-1} *vs.* k_3^{-1} is closely approximated by a quadrant of a circle. In this nomogram, combinations of k_2 and k_3 can be compared more readily for particular values of t^*. Some additional practical benefits of this representation will be discussed below.

Another obvious feature of Figure 4 is the linear increase in t^* as a function of k_2^{-1} and k_3^{-1}. For example, if k_2 and k_3 result in a particular value of t^*, then the combination of $2k_2$ and $2k_3$ will produce 0.99 $(C)_{ss}$ in $t^*/2$.

Application

Experimental Considerations. To implement these results into a working assay, a few practical considerations must be kept in mind. The following comments apply primarily to the two-auxiliary enzyme case, but application to the simpler coupled assay is apparent. Of primary importance is the determination of k_2 and k_3 under the conditions used to assay the primary enzyme. If commercially obtained enzymes are used, the IU/ml should be assayed routinely. It is a simple matter to assay each enzyme using the same buffer, pH, temperature, and substrate concentrations that will be employed to determine the primary enzyme activity. Equally convenient is the determination of an apparent Michaelis constant for substrates B and C under the same experimental conditions. Although the above-determined kinetic parameters may differ slightly from manufacturer or literature values, they will be appropriate for the use for which the investigator intends them.

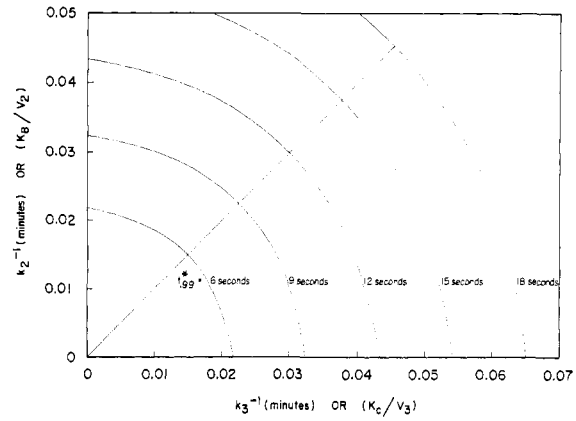


FIGURE 4: Nomogram relating combinations of k_2^{-1} and k_3^{-1} required to produce $F_C = 0.99$ in the times indicated.

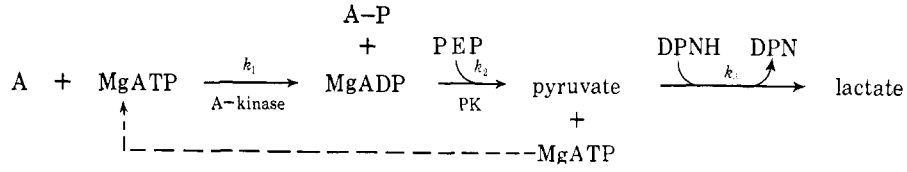
The effect of auxiliary substrates in addition to B and C on the primary enzyme must also be determined. This can be done by varying these concentrations and calculating an apparent inhibitor constant if any difference is observed. Presumably only uncompetitive or noncompetitive inhibitors (activators) could have an appreciable effect since all of the primary substrates are effectively saturating.

With the above information, a choice of t^* can be made using Figure 3 or Figure 4 on the basis of the cost and availability of the two enzymes needed. In most cases the steady-state concentrations of B or C will not be a factor in choosing the magnitude of k_2 or k_3. If necessary, however, $(B)_{ss}$ and $(C)_{ss}$ can be calculated from eq 3 and 9, respectively. The choice of k_2 and k_3 could then be made to keep $(B)_{ss}$ or $(C)_{ss}$ below some desired concentration.

The fact that k_2^{-1} *vs.* k_3^{-1} for a given value of t^* is closely approximated by a circle offers some practical insight on the choice of k_2 and k_3. Recall that $k_2^{-1} = K_B/V_2$ and that $k_3^{-1} = K_C/V_3$, where the V's refer to IU/ml of the respective auxiliary enzymes. To minimize the total number of coupling enzyme units used, we want to maximize the sum: $K_B/V_2 + K_C/V_3$. From the analytical geometry of a circle this sum is greatest when $K_B/V_2 = K_C/V_3$ or $k_2 = k_3$. The line of unit slope in Figure 4 intersects these points on each of the t^* circles.

Specific Example. As an illustration of the theoretical results obtained, consider the following generalized kinase reaction in which the substrate A is phosphorylated by MgATP, the resulting MgADP is rephosphorylated by phosphoenolpyruvate and pyruvate kinase. The pyruvate produced in the second reaction is reduced to lactate with the oxidation of DPNH and a corresponding decrease in absorbance monitored at 340 nm.

Let $k_1 = 0.05$ mM min^{-1}. In 10-mm cell, this will result in a $\Delta A_{340} = 0.310$ min^{-1}, which is a convenient optical density change to measure on a recording spectrophotometer. For this example the following literature values will be used: $K_{ADP} = 0.21$ mM (Reynard *et al.*, 1961) and therefore $k_2 =$ (IU of PK/ml)/0.21 mM. Similarly, $K_{Pyr} = 0.14$ mM (Boyer *et al.*, 1963) and $k_3 =$ (IU of LDH/ml)/0.14 mM. If t^* is chosen equal to 12 sec, we see from Figure 4 that k_2 and k_3 must be larger than ≈ 23 min^{-1}.



If the availability of the two enzymes is of concern, k_2 and k_3 should be chosen approximately equal. From Figure 4 we see that the appropriate values are $k_2 = k_3 = 33$ min^{-1}. This would require: 0.21 mM × 33 min^{-1} = 6.9 IU/ml of PK and 0.14 mM × 33 min^{-1} = 4.6 IU/ml of LDH.

If, however, the price of the required enzymes were important, the ratio of k_2 and k_3 could be appropriately adjusted to minimize the cost of an assay rather than the total units used.

Closing Remarks. Several features of this treatment recommend it to the investigator who wishes to set up an effective coupled assay. First, the experimenter and reader are both assured that reported activities accurately reflect the conversion of A into B. The familiar "lag time" can be computed and chosen at will by the worker, so that any other nonlinearity (*e.g.*, enzyme destruction, activation by product, etc.) occurring in the assay can be correctly assigned to its proper cause. This approach allows a conservation of (in some cases) expensive auxiliary enzymes. Mimimizing the amount of auxiliary enzyme in an assay also has the advantage of reducing the activities of contaminating enzymes that could interfere with the assay. In addition, one can tell at a glance whether a theoretically possible coupled assay is going to be practical, by varying k_2 and k_3 on Figure 4 instead of spending valuable time working up an assay at the lab bench. Finally, a quantitative method of expressing the concentrations of intermediates, the time required to produce a specific fraction of their steady-state concentration, and the effect of varying the activity of the two coupling enzymes is to be preferred over the former practice of adding "excess" coupling enzymes, and obtaining a "short lag time."

An examination of the validity of the initial assumptions for this treatment is in order now that we have seen how the results are applied to a practical assay. For typical values of t^*, the magnitude of k_2 and k_3 is large enough to keep the steady-state concentrations of intermediates well into the first-order range of the auxiliary enzymes. These very low concentrations also serve to keep all but the last reaction essentially irreversible. The irreversibility of the last reaction and the zero-order rate assumed for the first are guaranteed if one important experimental condition is met: measure <3–5% of the last reaction if it does approach some moderate equilibrium value.

An objection to our treatment of the two-auxiliary enzyme assay could be raised on the basis that our use of $F_C = 0.99$ is too stringent a criterion for attainment of a linear rate. From Figure 1 some may conclude that if the last intermediate is 0.95 steady state, a rate indistinguishable from linear is produced. A practical way of using a different fraction steady state is to use Figure 4 to obtain the desired ratio of k_2 and k_3, but to choose t^* longer by a factor of log (1 − 0.99)/log (1 − F_C). While not exact, this procedure will yield values very close to the desired t^* for $0.90 \leq F_C \leq 0.99$.

Although the emphasis in this paper has been on assaying the primary enzyme using saturating substrates, the fact that t^* never depends upon k_1 means that the results obtained here can be applied to the case of the primary enzyme operating under limiting substrate concentrations. The necessary conditions are that only a few per cent of the primary reaction be measured and that t^* be a small part of the total assay time; this assures a pseudo-zero-order rate for the period of observation. Since the above criterion is necessary in order to obtain valid initial velocities during a kinetic study, it does not limit the applicability of this approach with respect to this important type of enzyme assay.

Much of the doubt as to the effectiveness of coupled assays found in the literature could be eliminated by reporting the kinetic parameters discussed here as well as the IU of coupling enzymes added. The author concludes that the following information is the minimum possible to characterize a coupled assay adequately: (1) units of auxiliary enzyme added per milliliter as determined under the assay conditions used to measure the primary enzyme activity; (2) apparent Michaelis constants for B and C also determined using the anticipated assay conditions; (3) t^* and F_B or F_C chosen on the basis of k_2 and k_3 computed from the above information; and (4) effect of additional auxiliary substrates on primary enzyme activity.

In most cases these results will probably substantiate the assumption that most of the coupled assays in the literature set up by careful workers are indeed accurately measuring the activity of the primary enzyme. It is my intent, however, that assays employing auxiliary enzymes will be formulated in the future using less of the researcher's time and resulting in more confidence for all those evaluating the results obtained.

Acknowledgment

It is a pleasure to acknowledge my indebtedness to Dr. H. A. Lardy in whose laboratory this work was done, and whose many suggestions during all stages in the preparation of this work provided needed guidance and encouragement to the author. I am also grateful to Professor W. W. Cleland for a very helpful discussion of the manuscript which included some suggestions for implementing the theory into practical enzyme assays; and to Mr. D. J. McClure for checking the mathematics in the Theory section.

References

Boyer, P. D., Lardy, H. A., and Myrbäck, K. (1963), *Enzymes 7*, 125.

Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. (1961), *J. Biol. Chem. 236*, 2277.