Kinetics of Coupled Enzyme Reactions†

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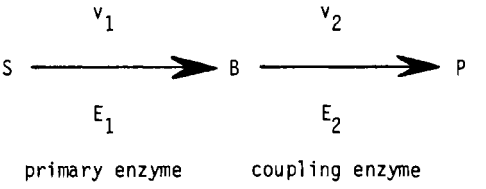
ABSTRACT: A theory has been developed for the kinetics of coupled enzyme reactions. This theory does not assume that the first reaction is irreversible. The validity of this theory is confirmed by a model system consisting of enoyl-CoA hydratase (EC 4.2.1.17) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) with 2,4-decadienoyl coenzyme A (CoA) as a substrate. This theory, in contrast to the conventional theory, proves to be indispensible for dealing with coupled enzyme systems where the equilibrium constant of the first reaction is small and/or the concentration of the coupling enzyme is higher than that of the intermediate. Equations derived on the basis of this theory can be used to calculate steady-state velocities of coupled enzyme reactions and to predict the time course of coupled enzyme reactions during the pre steady state.

Coupled enzyme systems, in which the product of one enzyme-catalyzed reaction serves as a substrate for another enzyme, are of great interest in biochemistry. For example, metabolic pathways are sequences of coupled enzyme reactions. In addition, coupled enzyme systems provide useful analytical tools for measuring the rates of enzyme-catalyzed reactions that are difficult or impossible to assay directly.

Haldane (1930) first began to analyze the kinetics of a coupled enzyme system shown in Scheme I. Further theoretical treatments based on this reaction scheme were provided by McClure (1969) and other authors (Hess & Wurster, 1970; Easterby, 1973). The basic assumptions made by McClure (1969) are as follows: (a) The reaction catalyzed by the primary enzyme (E_1) is irreversible and follows zero-order kinetics. To meet these conditions, the substrate of the primary enzyme must be saturating or must remain virtually constant by allowing only a small fraction of the substrate to react. The reaction is assumed to be irreversible because the intermediate (B) is continuously removed by the coupling reaction. (b) The coupled enzyme reaction is irreversible and first order with respect to the intermediate concentration. This requires that $[B] \ll K_{m2}$ [The Michaelis constant of the coupling enzyme (E_2) for B] and that the second substrate for the coupling enzyme be saturating or nearly so. The coupling reaction is assumed to be irreversible if its equilibrium is to the right or if the product is removed or not allowed to accumulate. Storer and Cornish-Bowden (1974), and subsequently other authors (Varfolomeev, 1977a,b; Rudolph et al., 1979; Easterby, 1981; Brooks et al., 1984; Kuchel et al., 1974; Kuchel, 1985), have published more general theoretical treatments without assuming that the second reaction follows first-order kinetics. They stressed that the steady-state concentration of the intermediate ($[B]_{ss}$) is a function of V_2 (the maximal velocity of coupling enzyme) and v_1 (the rate of the primary reaction). For example, $[B]_{ss}$ would be equal to K_{m2} when $V_2 = 2v_1$. Hence, the original assumption $[B]_{ss} \ll K_{m2}$ does not need to be met. Moreover, Varfolomeev (1977a,b) and other authors (Brooks et al., 1984; Kuchel et al., 1974; Kuchel, 1985) have derived equations for describing the time course of intermediate accumulation and product formation on the basis of the condition that the rate of the first reaction is a function of the substrate concentration and follows the Michaelis–Menten equation.

Scheme I



In the past decade, many of the assumptions underlying McClure's original analysis of coupled enzyme reactions have been dismissed except for the assumption that the reaction catalyzed by the primary enzyme be irreversible. In view of the well-established principles that (a) no reaction is absolutely irreversible and (b) any enzyme catalyzes simultaneously its forward and backward reactions, the assumed irreversibility of the primary reaction appears to be made mostly for mathematical convenience. If the equilibrium constant of the reaction of interest is high, the reverse reaction may be insignificant and previous theories work well (Hess & Wurster, 1970; Easterby, 1973; Storer & Cornish-Bowden, 1974; Cleland, 1979a; Garcia-Carmona et al., 1981; Yang et al., 1985). However, in a case where the equilibrium constant of the reaction of interest is very small, the reverse reaction is not negligible even though the coupling reaction is first order with respect to the intermediate concentration (Yang et al., 1986a). The conventional theories cannot be applied to such systems, because they have not considered the impact of the reverse reaction catalyzed by the primary enzyme.

In this paper, we develop a comprehensive kinetic theory of coupled enzyme reactions without assuming the first reaction to be irreversible and demonstrate that this theory is applicable to a greater variety of coupled enzyme systems than the conventional theories. The general expression derived from this theory can be used to compute the steady-state velocity of the overall reaction and to describe the time course of coupled enzyme reactions during the pre steady state.

Experimental Procedures

The kinetic parameters of individual enzymes, i.e., bovine liver enoyl-CoA hydratase (crotonase) and pig heart 3-hydroxyacyl-CoA dehydrogenase, were determined by the least-squares method of fitting data to rate equations (Cleland, 1979b). The catalytic rate constants and the Michaelis constants are listed in Table I. Experimental details of the preparation of substrates and the enzyme assays are given in

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Table I: Some Kinetic Parameters of Bovine Liver Enoyl-CoA Hydratase and Pig Heart 3-Hydroxyacyl-CoA Dehydrogenase

enzyme	substrate	apparent Michaelis constant		catalytic rate constant	
		symbol	μM	symbol	min^{-1}
enoyl-CoA hydratase	2-*trans*,4-*trans*-decadienoyl-CoA	K_{m1}	16.9 ± 1.6	k_{cat1}	535 ± 18
enoyl-CoA hydratase	L-3-hydroxy-4-*trans*-decenoyl-CoA	K_{m-1}	12.1 ± 1.3	k_{cat-1}	129 250 ± 4757
3-hydroxyacyl-CoA dehydrogenase[a]	L-3-hydroxy-4-*trans*-decenoyl-CoA	K_{m2}	1.5 ± 0.1	K_{cat2}	2489 ± 32

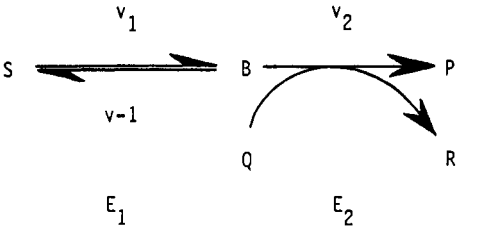
[a] The apparent Michaelis constant for NAD^+ is 6.8 ± 0.6 μM.

a recent paper (Yang et al., 1986b). The steady-state velocity of 2-*trans*,4-*trans*-decadienoyl coenzyme A (CoA) degradation were determined spectrophotometrically at 25 °C by measuring the increase in absorbance at 360 nm. The assay mixture of 0.6 mL contained 0.2 M KP_i (pH 8.0), 1.8 mM NAD^+, 0.25 mM CoASH, 20 μM 2-*trans*,4-*trans*-decadienoyl-CoA, pig heart 3-ketoacyl-CoA thiolase (33 milliunits, measured with acetoacetyl-CoA as a substrate), pig heart 3-hydroxyacyl-CoA dehydrogenase (648 milliunits unless otherwise indicated), and bovine liver crotonase. The reaction was started by the addition of crotonase. 3-Ketoacyl-CoA thio esters, products of 3-hydroxyacyl-CoA dehydrogenase, were instantly cleaved due to the presence of a large excess of pig heart 3-ketoacyl-CoA thiolase and CoASH in the assay system. The removal of 3-ketoacyl-CoA thio esters prevents the inhibition of 3-hydroxyacyl-CoA dehydrogenase by them and also prevents the backward reaction catalyzed by 3-hydroxyacyl-CoA dehydrogenase. Since 2-*trans*-octenoyl-CoA passes rapidly through a second β-oxidation cycle, the observed rate of NADH formation is twice the steady-state velocity of 2-*trans*,4-*trans*-decadienoyl-CoA degradation. A unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per minute.

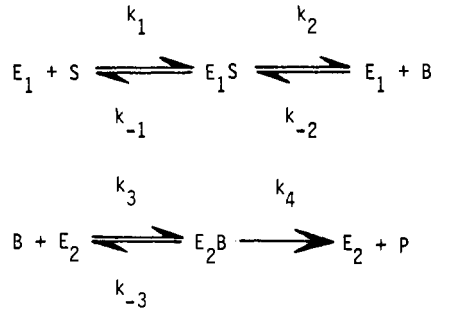
Results and Discussion

Limitations of Conventional Theory of Coupled Enzyme Reactions. Previous authors have developed equations describing the relationship between F_B (i.e., the desired fraction of $[B]_{ss}$), t (i.e., the desired time to reach a given F_B), V_2 (i.e., the amount of coupling enzyme needed), K_{m2}, and v_1 by simply assuming that the first reaction is irreversible. However, the limitations of the existing theories are illustrated by the hydration of 2-*trans*,4-*trans*-decadienoyl-CoA to L-3-hydroxy-4-*trans*-decenoyl-CoA followed by its dehydrogenation to 3-keto-4-*trans*-decenoyl-CoA. These two reactions of the pathway by which polyunsaturated fatty acids are degraded are catalyzed by enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, respectively. Calculations according to Storer and Cornish-Bowden's theory, which is reported (Rudolph et al., 1979) to yield results similar to those obtained by McClure's equation, resulted in the theoretical predictions that at v_1 = 1.67 milliunits/mL a 3-hydroxyacyl-CoA dehydrogenase activity of 48 milliunits/mL (V_2) would be adequate for the ratio of v_2/v_1 to reach 0.95 within 6 s and to attain a value of 0.99 after 9.5 s. However, after 48 milliunits/mL of 3-hydroxyacyl-CoA dehydrogenase was added to an assay containing 20 μM substrate and a near saturating concentration of NAD^+ and 6 s or longer passed, only 57% of the expected rate was actually observed. This finding suggests that the first reaction was not irreversible although the product of the first reaction was continuously removed from the reaction by the addition of coupling enzyme that should have been adequate according to the conventional theory. Since the equilibrium constant of 2-*trans*,4-*trans*-decadienoyl-CoA hydration is only 3×10^{-3} (Yang et al., 1986b), the equilibrium concentration of L-3-hydroxy-4-*trans*-decenoyl-CoA would be 60 nM in the absence of the coupling enzyme.

Scheme II



Scheme III



Moreover, the steady-state concentration of free intermediate in a coupled enzyme assay cannot rise to this limit because of the presence of coupling enzyme 3-hydroxyacyl-CoA dehydrogenase. In fact, the steady-state concentration of free intermediate is much lower than the K_m value of coupling enzyme, and not more than 3 s are needed for this coupled enzyme system to reach the steady state. The first reaction does obey the initial velocity condition because only 0.4% of the substrate is consumed when the steady state is reached. Evidently, the conventional theory can predict neither the steady-state velocity nor the lag time of coupled enzyme systems where the primary reaction has a small equilibrium constant.

Theory. The inadequacy of the conventional theory when applied to some coupled enzyme reactions (Yang et al., 1986a,b) prompted us to consider a more general and realistic model of coupled enzyme systems as shown in Scheme II, where E_1 is the primary enzyme of interest and E_2 is the coupling enzyme, usually a dehydrogenase. The dehydrogenase is assumed to catalyze an unireactant reaction as long as its second substrate (Q) is nearly saturating (McClure, 1969; Hess & Wurster, 1970; Easterby, 1973, 1981; Storer & Cornish-Bowden, 1974; Rudolph et al., 1979; Cleland, 1979a; Brooks et al., 1984). Thus, the simplest representation of the overall reaction (Segel, 1975) is as shown in Scheme III. According to Scheme III the rate of the overall reaction, or the observed rate of the coupled enzyme system, at any moment is given by

$$v_2 = \frac{d[P]}{dt} = k_4[E_2B] \quad (1)$$

A general expression of this rate equation in terms of the total coupling enzyme concentration ($[E_2]_t = [E_2] + [E_2B]$) and the total intermediate concentration ($[B]_t = [B] + [E_2B]$), no matter whether or not $[B]_t$ is in great excess of $[E_2]_t$, is

given (Reiner, 1968; Cha, 1970; Cornish-Bowden, 1976; Griffiths, 1979) as eq 2, where $K_{m2} = (k_{-3} + k_4)/k_3$ and $k_{cat2} = k_4$. The observed rate gradually increases in the pre-steady-state phase until the concentration of total intermediate ($[B]_t$) reaches its maximal value ($[B]_{ss}$). At steady state, $[B]_{ss}$ can be substituted for $[B]_t$ in eq 2, and the steady-state rates of coupled enzyme reactions can be calculated.

$$v_2 = (1/2)k_{cat2}(K_{m2} + [B]_t + [E_2]_t)\{1 - [1 - 4[E_2]_t[B]_t/(K_{m2} + [B]_t + [E_2]_t)^2]^{1/2}\} = (1/2)k_{cat2}\{k_{m2} + [B]_t + [E_2]_t - [(K_{m2} + [B]_t + [E_2]_t)^2 - 4[E_2]_t[B]_t]^{1/2}\} \quad (2)$$

If the first reaction cannot a priori be assumed to be irreversible, i.e., the value of k_{-2} is not negligibly small, as has been done in deriving published theories (McClure, 1969; Hess & Wurster, 1970; Easterby, 1973, 1981; Storer & Cornish-Bowden, 1974; Varfolomeev, 1979a,b; Rudolph et al., 1979; Brooks et al., 1984; Kuchel et al., 1974; Kuchel, 1985), the appropriate rate equation for the consumption of substrate when $[E_1S]$ is already in a steady state is (Segel, 1975)

$$\frac{d[s]}{dt} = v_{1,net} = \frac{\frac{V_1}{K_{m1}}[S] - \frac{V_{-1}}{K_{m-1}}[B]}{1 + \frac{[S]}{K_{m1}} + \frac{[B]}{K_{m-1}}} \quad (3)$$

where $K_{m1} = (k_2 + k_{-1})/k_1$, $K_{m-1} = (k_2 + k_{-1})/k_{-2}$, $V_1 = k_2[E_1]_t$, $V_{-1} = k_{-1}[E_1]_t$, and $[E_1]_t = [E_1] + [E_1S]$ (see Scheme III). According to the Haldane relationship (Segel, 1975), $V_1/K_{m1} = (V_{-1}/K_{m-1})K_{eq}$. In a coupled enzyme assay, [S] is usually much higher than [B] when the steady state is reached. Hence, [S] is approximately equal to its original value $[S]_t$. If K_{eq} (i.e., the equilibrium constant of the first reaction) is very large, the effect of the reverse reaction is negligible. If so, eq 3 simplifies to the Michaelis–Menten equation used in the conventional theory (Varfolomeev, 1977a,b; Brooks et al., 1984; Kuchel et al., 1974; Kuchel, 1985):

$$v_1 = v_{1,net} = \frac{V_1[S]_t}{K_{m1} + [S]_t} \quad (4)$$

When the coupled enzyme reaction reaches a steady state, the rate of substrate consumption should be equal to that of product formation, i.e., $v_{1,net} = v_2$ and $d[B]_t/dt = 0$. Thus, we have

$$\frac{k_{cat1}[E_1]_tK_{m-1}[S]_t - k_{cat-1}[E_1]_tk_{m1}[B]_tf}{K_{m1}K_{m-1} + k_{m-1}[S]_t + K_{m1}[B]_tf} = \frac{1}{2}k_{cat2}(K_{m2} + [B]_t + [E_2]_t)\{1 - [1 - 4[E_2]_t[B]_t/(K_{m2} + [B]_t + [E_2]_t)^2]^{1/2}\} \quad (5)$$

where $f = [B]/[B]_t$ is a coefficient that will be discussed in more detail.

The differential equation describing changes of $[E_2B]$ with time (see Scheme III) is

$$\frac{d[E_2B]}{dt} = k_3[E_2][B] - (k_{-3} + k_4)[E_2B] \quad (6)$$

At steady state, $d[E_2B]/dt = 0$ and eq 6 yields

$$\frac{[E_2][B]}{[E_2B]} = K_{m2} \quad (7)$$

The rearrangement of eq 7 yields

$$f = \frac{[B]}{[B]_t} = \frac{K_{m2}}{K_{m2} + [E_2]} = \frac{K_{m2}}{K_{m2} + [E_2]_t(1 - v_2/V_2)} \quad (8)$$

and

$$v_2 = \frac{V_2[E_2B]}{[E_2]_t} = \frac{V_2[B]}{K_{m2} + [B]} = \frac{V_2[B]_tf}{K_{m2} + [B]_tf} \quad (9)$$

Table II: Approximations of Coefficient f under Different Conditions

condition	f	rate eq[a]	eq no.
(A) $[B]_t \gg [E_2]_t$ or $[E_2]_t \ll K_{m2}$	1	$v_2 = V_2[B]_t/(K_{m2} + [B]_t)$	11
(B) $[B]_t \approx [E_2]_t$	$K_{m2}/[K_{m2} + (1/2)[E_2]_t]$	$v_2 = V_2[B]_t/[K_{m2} + (1/2)[E_2]_t + [B]_t]$	12
(C) $[B]_t \ll [E_2]_t$	$K_{m2}/(K_{m2} + [E_2]_t)$	$v_2 = V_2[B]_t/(K_{m2} + [E_2]_t)$	13

[a] These equations were obtained by substituting the f values into eq 9. They are suitable for describing the kinetics of ZONE A, ZONE B, and ZONE C of enzyme behavior, respectively (Goldstein, 1944; Webb, 1963; Griffiths, 1979).

Equation 9 is, in fact, a modified form of eq 2, in which the influence of substrate depletion due to E_2B formation is taken into account (Cha, 1970; Griffiths, 1979). Derivations of this equation differ from the original analysis made by Briggs and Haldane (1925) in two aspects: (a) it is not assumed that the total concentration of the substrate for the coupling enzyme ($[B]_t$) is much greater than the total enzyme concentration ($[E_2]_t$); (b) a distinction is being made by use of the coefficient f between the total concentration of substrate for the coupling enzyme ($[B]_t$) and the concentration of its free form ($[B]$). If the first two terms of the binomial expansion of the square-root term in eq 2 are taken for estimating the ratio of $[E_2B]$ to $[E_2]_t$ as described previously (Cha, 1970), an expression of f is obtained by rearranging eq 9:

$$f = \frac{K_{m2}\left[1 + \frac{[B]_t[E_2]_t}{(K_{m2} + [B]_t + [E_2]_t)^2}\right]}{K_{m2} + [E_2]_t\left[1 - \left(\frac{[B]_t}{K_{m2} + [B]_t + [E_2]_t}\right)^2\right]} \quad (10)$$

Practically, approximations of the coefficient f are summarized in Table II. At steady state where $[B]_t = [B]_{ss}$, eq 11 gives eq 14. This simplified version of eq 9 was usually used by

$$v_2 = \frac{V_2[B]_{ss}}{K_{m2} + [B]_{ss}} \quad (14)$$

others to describe the steady-state kinetics of coupled enzyme reactions. When $[B]_{ss} \ll K_{m2}$, eq 14 simplifies to

$$v_2 = V_2[B]_{ss}/K_{m2} \quad (15)$$

In contrast to eq 9, eq 14 and 15 cannot be used at conditions that are characteristic of ZONE B or ZONE C (Goldstein, 1944; Webb, 1963). As a result, the conventional theories are restricted to the condition $[B]_t \gg [E_2]_t$ or $[E_2]_t \ll K_{m2}$. Evidently, existing theories are covered by the theory presented here.

Once the kinetic parameters of individual enzymes (i.e., the primary enzyme and the coupling enzyme) have been determined, one can compute the steady-state rates of the overall reaction (v_{ss}) as a function of $[S]_t$, $[E_1]_t$, or $[E_2]_t$. Since at steady-state $0 < [B]_tf \leq [S]_t(k_{cat1}K_{m-1})/(k_{cat-1}K_{m1})$, the steady-state concentration of the intermediate ($[B]_{ss}$) can be calculated by successively refining the value of $[B]_t$ in an iterative process to obtain a best fit for eq 5. Then, v_{ss} is obtained by substituting the value of $[B]_{ss}$ into eq 2.

If it is assumed that each of the enzyme–substrate complexes, but not the intermediate, is already in a steady-state, a differential equation for the accumulation of intermediate

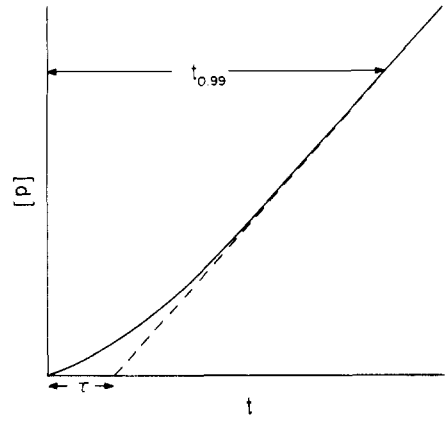


FIGURE 1: Time course of product formation in a coupled enzyme reaction. The steady-state asymptote to the progress curve shown as a dashed line intersects the time axis at τ, the transition time, and converges with the progress curve at $t_{0.99}$, the lag time required for the total intermediate concentration to reach 99% of its steady-state concentration.

with time is set up by a treatment similar to that of Kuchel et al. (1974, 1975) and Kuchel (1985):

$$\frac{d[B]_t}{dt} = \frac{k_{cat1}[E_1]_t K_{m-1}[S] - k_{cat-1}[E_1]_t K_{m1}[B]_t f}{K_{m1}K_{m-1} + K_{m-1}[S] + K_{m1}[B]_t f} - \frac{k_{cat2}[E_2]_t[B]_t f}{K_{m2} + [B]_t f} \quad (16)$$

The initial condition is $[S] = [S]_t$, $[B]_t = 0$, and $[P] = 0$. If only a small fraction of the substrate is "used up" during the pre-steady-state period, the substrate concentration [S] is assumed to be constant and equal to $[S]_t$. When the intermediate concentration reaches steady state, $d[B]_t/dt = 0$ and eq 16 gives

$$\frac{k_{cat1}[E_1]_t K_{m-1}[S]_t - k_{cat-1}[E_1]_t K_{m1}[B]_t f}{K_{m1}K_{m-1} + K_{m-1}[S]_t + K_{m1}[B]_t f} = \frac{k_{cat2}[E_2]_t[B]_t f}{K_{m2} + [B]_t f} \quad (17)$$

Equation 17 shows that $[B]_t f$ is an implicit function of $[S]_t$ and both enzyme concentrations. In consequence, for a given coupled enzyme assay system the steady-state concentration of free intermediate and thus the steady-state rates of the overall reaction (see eq 9) can be calculated.

It is difficult to integrate eq 16 except numerically. However, if the concentration of intermediate is much lower than K_{m-1} as well as K_{m2}, eq 16 can be simplified to a linear differential equation. After integrating it, the following analytical solution is obtained:

$$[B]_t = \frac{k_{cat1}[E_1]_t[S](K_{m2} + [E_2]_t)K_{m-1}}{(K_{m1} + [S])k_{cat2}[E_2]_t K_{m-1} + k_{cat-1}[E_1]_t K_{m1}K_{m2}}\left(1 - \exp\left\{-\left[\frac{k_{cat2}[E_2]_t}{K_{m2}} + \frac{k_{cat-1}[E_1]_t}{K_{m-1}}\frac{K_{m1}}{(K_{m1} + [S])}\right]\frac{K_{m2}}{K_{m2} + [E_2]_t}t\right\}\right) \quad (18)$$

By the end of the lag period (i.e., $t_{0.99}$ as shown in Figure 1), the intermediate concentration approaches its maximal value. Since the exponential term of eq 18 disappears as the steady state is approached, we have

$$[B]_t = [B]_{ss} = \frac{k_{cat1}[E_1]_t[S]K_{m-1}(K_{m2} + [E_2]_t)}{k_{cat2}[E_2]_t K_{m-1}(K_{m1} + [S]) + k_{cat-1}[E_1]_t K_{m1}K_{m2}} \quad (19)$$

Since at steady state v_2 is equal to v_{ss}, eq 19 can be substituted into eq 13 to yield eq 20. The latter equation clearly shows

$$v_{ss} = \frac{k_{cat1}[E_1]_t[S]_t/K_{m1}}{1 + \frac{[S]_t}{K_{m1}} + \frac{k_{cat-1}[E_1]_t K_{m2}}{k_{cat2}[E_2]_t K_{m-1}}} \quad (20)$$

how the values of v_{ss} are depressed due to the backward reaction catalyzed by the primary enzyme. When the equilibrium constant of the first reaction is much larger than $K_{m2}/[S]_t$, eq 20 simplifies to $v_{ss} = V_1[S]_t/(K_{m1} + [S]_t) = v_1$, the equation used in the conventional theory.

If the fraction of v_1 attained at the steady state is $F_v = v_{ss}/v_1$, substitution of eq 20 and 4 into this expression followed by rearrangement yields

$$V_2 = \frac{k_{cat-1}[E_1]_t K_{m1}K_{m2}F_v}{K_{m-1}(K_{m1} + [S])(1 - F_v)} \quad (21)$$

The amount of coupling enzyme necessary to obtain any desired F_v can be easily calculated. According to the conventional theories, the ratio of v_1/V_2 is only related to the length of the lag time but does not affect the steady-state rate of the overall reaction as long as v_1 is not larger than V_2 (Storer & Cornish-Bowden, 1974; Varfolomeev, 1977a,b; Brooks et al., 1984; Kuchel, 1985). However, the present theory predicts that if the primary reaction has a small equilibrium constant, the observed rate will not approach v_1 unless the reverse reaction catalyzed by the primary enzyme is suppressed by coupling enzyme at concentrations much higher than predicted by the conventional theories. When $[E_2]_t$ is increased to more than $0.01K_{m2}$ and $[B]_{ss}$ is lower than $[E_2]_t$, the rate equation of the conventional theory for coupled enzyme systems cannot be used because of the large error associated with the use of the Michaelis–Menten equation (Cha, 1970; Griffiths, 1979). Obviously, the lag time calculated by use of the equations of the conventional theories (McClure, 1969; Hess & Wurster, 1970; Easterby, 1973; Storer & Cornish-Bowden, 1974; Varfolomeev, 1977b; Brooks et al., 1984) would significantly deviate from the actual value under the above circumstances. Recently, Easterby (1981) has published a corrected equation for calculating the transition time (τ) for systems in which the coupling enzyme concentration is high. However, the reverse reaction catalyzed by the primary enzyme was not considered. A general expression that describes the time course of product formation even if the primary reaction has a small equilibrium constant and/or the system contains high concentrations of coupling enzyme can be derived by substituting eq 18 into eq 13, and integrating the resultant equation we find

$$[P] = v_{ss}t + \frac{v_{ss}}{\left[\frac{k_{cat2}[E_2]_t}{K_{m2}} + \frac{k_{cat-1}[E_1]_t}{K_{m-1}}\frac{K_{m1}}{(K_{m1} + [S])}\right]\frac{K_{m2}}{K_{m2} + [E_2]_t}}\left\{\exp\left(-\left[\frac{k_{cat2}[E_2]_t}{K_{m2}} + \frac{k_{cat-1}[E_1]_t}{K_{m-1}}\frac{K_{m1}}{(K_{m1} + [S])}\right]\times\frac{K_{m2}}{K_{m2} + [E_2]_t}t\right) - 1\right\} \quad (22)$$

When the steady-state rate approaches $0.99v_1$, $k_{cat2}[E_2]_t/K_{m2}$ will be much larger than $k_{cat-1}[E_1]_t/K_{m-1}$. Thus, eq 18 can be simplified and rearranged to its logarithmic form, eq 23, where $F_B = [B]_t/[B]_{ss}$. This expression permits calculation of the time required to reach a fraction of the steady-state intermediate concentration even if the primary reaction has

$$t_{F_B} = \frac{-\ln (1 - F_B)}{\left[\frac{k_{cat2}[E_2]_t}{K_{m2}} + \frac{k_{cat-1}[E_1]_t}{K_{m-1}} \frac{K_{m1}}{(K_{m1} + [S])} \right] \frac{K_{m2}}{K_{m2} + [E_2]_t}} \quad (23)$$

a small equilibrium constant and the concentration of coupling enzyme is very high. The relationship between $[P]_{t_{0.99}}$ (i.e., the amount of product formed at $t_{0.99}$), the lag time $t_{0.99}$, and the transition time τ is given by eq 24 and illustrated in Figure 1. After substituting eq 22 and 23 into eq 24, we obtain

$$[P]_{t_{0.99}} = v_{ss}(t_{0.99} - \tau) \quad (24)$$

$$\tau = \frac{K_{m2} + [E_2]_t}{k_{cat2}[E_2]_t + \frac{k_{cat-1}[E_1]_t}{K_{m-1}} \frac{K_{m1}K_{m2}}{K_{m1} + [S]}} \quad (25)$$

If $k_{-2} = 0$ (i.e., $K_{m-1} = \infty$) or $k_{cat-1}[E_1]_t K_{m1}/(K_{m1} + [S])$ is close to zero and $[E_2]_t < 0.01K_{m2}$, eq 23 simplifies to

$$t_{F_B} = \frac{-K_{m2} \ln (1 - F_B)}{k_{cat2}[E_2]_t} \quad (26)$$

and eq 25 to

$$\tau = \frac{K_{m2}}{k_{cat2}[E_2]_t} \quad (27)$$

Equations 26 and 27 are the key equations of the conventional theories (McClure, 1969; Hess & Wurster, 1970; Easterby, 1973; Rudolph et al., 1979). They are valid only under the above-mentioned conditions.

Applications. The degradation of 2-*trans*,4-*trans*-decadienoyl-CoA, a key metabolite of trans ω-6 unsaturated fatty acids, catalyzed by a reconstituted mitochondrial β-oxidation system was studied to test the accuracy of the theoretical predictions made by the extended theory. If the general model and the steady-state rate equations of coupled enzyme systems are correct, the corresponding equations that describe the pre steady state should also be correct because they are derived from an appropriate rate equation by well-established procedures (Kuchel, 1985). Since the pre steady state of 2-*trans*,-4-*trans*-decadienoyl-CoA degradation is very short (<0.5 s under the experimental conditions used in this study) and the amount of products formed during this period of time is very small, we have not attempted to make measurements during the pre steady state. Brooks et al. (1984) and other authors (Storer & Cornish-Bowden, 1974; Varfolomeev, 1977a,b; Rudolph et al., 1979; Kuchel, 1985) have pointed out that when a coupled enzyme reaction reaches the steady state, the observed rate will be that of the primary enzyme and a plot of the observed rate vs. v_1 will give a straight line with a slope of 1 as long as the rate of the primary enzyme is no more than the maximal velocity of the coupling enzyme; if v_1 is larger than V_2, the observed rate equals V_2. However, these theoretical predictions did not agree with the experimental data, which showed the rate approaching a limiting value even when V_2 was much higher than v_1 in the assay system (see Figure 2). In addition, it was generally assumed (Easterby, 1973; Varfolomeev, 1977a; Kuchel, 1985) that if the reaction catalyzed by the primary enzyme is rate limiting, the steady-state velocity is determined by the primary enzyme, whereas the lag phase is controlled by the coupling enzyme. However, it was observed that the steady-state velocity is a function of the amount of coupling enzyme in the assay system even when v_1 was much smaller than V_2 (see Figure 3). All these findings suggest that the conventional theory is inappropriate for describing the kinetics of coupled enzyme systems, where the primary enzyme reaction will not proceed to a substantial extent without being driven by a coupling reaction. A number of important metabolic reactions, e.g., the dehydrogenation of malate catalyzed by malate dehydrogenase (EC 1.1.1.37), belong to this type of reaction (Lehninger, 1982).

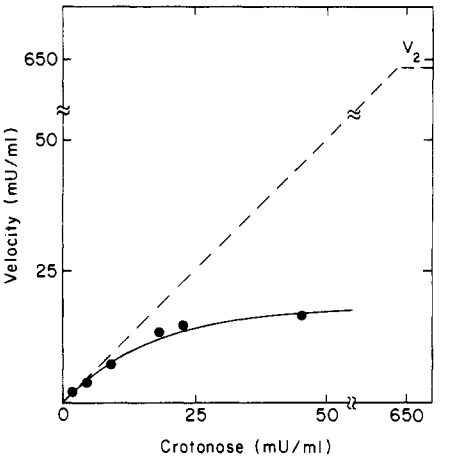


FIGURE 2: Steady-state velocities of 2-*trans*,4-*trans*-decadienoyl-CoA degradation catalyzed by a β-oxidation enzyme system as a function of the primary enzyme enoyl-CoA hydratase (crotonase) activity. For experimental details, see Experimental Procedures. Theoretical predictions according to the conventional theory and the theory presented in this paper are shown as a dashed line and a solid line, respectively. The maximal velocity of coupling enzyme is marked by V_2. Experimental data are indicated by solid circles.

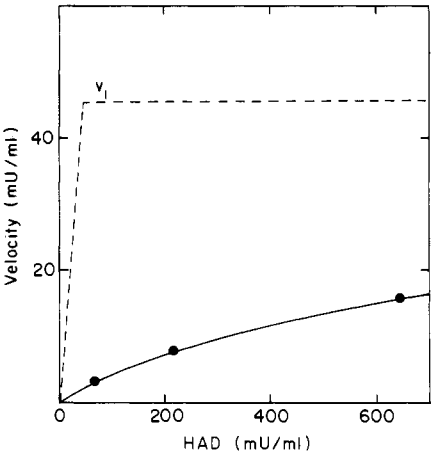


FIGURE 3: Steady-state velocities of 2-*trans*,4-*trans*-decadienoyl-CoA degradation catalyzed by a β-oxidation enzyme system as a function of the maximal velocity of the coupling enzyme 3-hydroxyacyl-CoA dehydrogenase (HAD). For experimental details, see Experimental Procedures. Theoretical predictions according to the conventional theory (dashed line) and the theory presented in this paper (solid line) are presented while experimental data are indicated by solid circles. The activity of the primary enzyme is marked as v_1.

The effect of the substrate concentration on the steady-state velocity is shown in the Hanes–Woolf plot (Segel, 1975) (see Figure 4). At a low concentration of primary enzyme, the observed values agree with the calculated values obtained by use of the conventional theory. However, at a 10-fold higher concentration of primary enzyme, the experimental data are lower than the calculated rates as if a competitive inhibitor were present in the reaction mixture (see Figure 4). This deviation reflects the backward reaction catalyzed by the primary enzyme. The influence of the backward reaction is only negligible at very high concentrations of substrate or at very low concentrations of primary enzyme. However, when equations of the extended theory are used, values calculated for various concentrations of substrate, primary enzyme, or

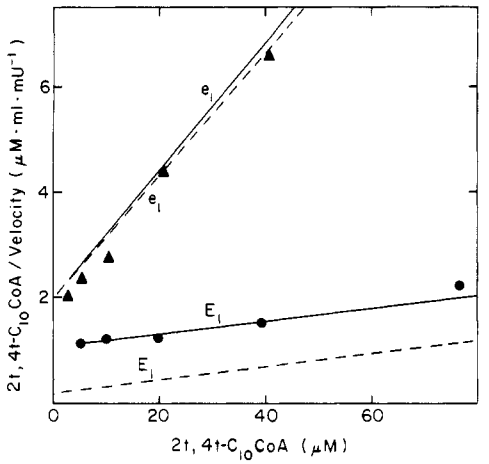


FIGURE 4: Ratios of 2-*trans*,4-*trans*-decadienoyl-CoA concentration to the steady-state velocity of its degradation catalyzed by a β-oxidation enzyme system as a function of 2-*trans*,4-*trans*-decadienoyl-CoA (2t,4t-C_{10}CoA) concentration. The β-oxidation enzyme system contained either 2.57 (▲) or 25.7 μg/mL (●) enoyl-CoA hydratase (primary enzyme). For experimental details, see Experimental Procedures. Theoretical predictions according to the conventional theory and the theory presented in this paper are shown as dashed lines and solid lines, respectively. These lines are labeled e_1 when the primary enzyme concentration was 2.57 μg/mL or E_1 when it was 25.7 μg/mL.

coupling enzyme agree well with the experimental data (see Figures 2–4). Evidently, the theory presented here is better suited than the conventional theories for kinetic investigations of metabolic reactions with small equilibrium constants and for dealing with coupled enzyme systems in which the concentrations of coupling enzyme exceed intermediate concentration.

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