in which two hydrogen bonds I and II link the two molecules A and B. If we confine our attention to only one of the two symmetrically arranged molecules, say A, the effects of the hydrogen bonding on its electronic structure will be the sum of two independent effects contributed by I and II. As mentioned in previous section, the hydrogen bond I causes the absorption spectrum of molecule A to shift toward the blue, while the hydrogen bond II shifts it toward the red. The observed red shift of the absorption of benzoic acid on formation of dimer indicates that perturbing interactions on the π-electronic structure of molecule A due to the bond II predominates over that due to the bond I. When the hydrogen bond is strong enough, as with benzoic acid dimer, the magnitude of the red shift due to the bond II is sufficiently large to overwhelm the blue shift due to the bond I. Since the hydrogen bond energies of I and II must be identical, the above result means that the amount of the spectral shift of absorption bands depends mainly on the type and arrangement of hydrogen bonds rather than the magnitude of their energies.

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The Relaxation Spectra of Simple Enzymatic Mechanisms[1,2]

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When a reaction is close to equilibrium, the approach to equilibrium may be characterized by a spectrum of relaxation times which can be related to the rate constants involved in the mechanism.[4] The relaxation spectrum is discussed for the n-intermediate mechanism for a simple enzymatic reaction $S \rightleftarrows P$; such a reaction is characterized by $n + 1$ relaxation times. The inclusion of competitive inhibition in this mechanism is discussed. If the initial substrate concentration is much greater than the initial concentration of enzymatic sites, one relaxation time is characteristic of the steady state and it may be evaluated in terms of the Michaelis constants and maximum velocities. For an n-intermediate mechanism, the steady state relaxation time is

$$\tau_{ss} = [1 + (\bar{s}/K_S)(1 + V_S/V_P)]/(V_S/K_S + V_P/K_P)$$

where $\bar{s}$ is the equilibrium concentration of substrate, K_S and K_P are the Michaelis constants and V_S and V_P are the maximum velocities. If competitive inhibition is included in the mechanism and $i_0 >> e_0$

$$\tau_{ss} = [1 + (\bar{s}/K_S)(1 + V_S/V_P) + i_0/K_I]/(V_S/K_S + V_P/K_P)$$

where i_0 is the total inhibitor concentration and K_I is the competitive inhibition constant. The competitive inhibition constant K_I can be obtained by determining the steady state relaxation time as a function of total substrate concentration and inhibitor concentration. Experimental results for the fumarase reaction at two pH's are presented which show the expected dependence of the long relaxation time on s_0, e_0 and i_0. In addition, one of the Michaelis constants was determined through initial velocity measurements, thus allowing calculation of K_S, V_S/V_P and K_I.

Introduction

Reaction rates usually are measured on systems far from equilibrium. However, Eigen[4] has shown how the study of reactions very close to equilibrium may be used for determining the rate constants of reactions in solution which are too rapid to permit the use of mixing methods. In this manner, Eigen and his co-workers have measured reaction rates with half times as short as 10^{-8} sec. In this method, a reaction mixture is displaced slightly from equilibrium by changing an independent variable such as pressure, electric field strength or temperature. Alternatively, if the reaction rates are sufficiently slow, one may start with a reaction mixture containing concentration ratios slightly different from those of the equilibrium mixture. When close to equilibrium, the return to equilibrium is characterized by a spectrum of relaxation times which are related to the rate constants involved in any particular mechanism. Several discussions of this type of phenomenom are now available.[5-7] This experimental approach is applicable, in principle, to the study of all types of reactions. The purpose of this paper is to describe the relaxation spectrum for an n-intermediate enzymatic reaction; the effect of including inhibition in this mechanism is discussed and detailed equations are given for the case of competitive inhibition. Experimental measurements of the steady state relaxation time for the fumarase reaction are described.

Relaxation Spectrum of an n-Intermediate Enzyme Reaction.—The familiar n-intermediate reaction mechanism to be considered is given in

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \ldots X_i \underset{k_{-(i+1)}}{\overset{k_{i+1}}{\rightleftarrows}} \ldots X_n \underset{k_{-(n+1)}}{\overset{k_{n+1}}{\rightleftarrows}} E + P \quad (1)$$

At equilibrium:	$\bar{e}$	$\bar{s}$	$\bar{x}_1$	$\bar{x}_i$	$\bar{x}_n$	$\bar{e}$	$\bar{p}$
At any time t:	$\bar{e} + \Delta e$	$\bar{s} + \Delta s$	$\bar{x}_1 + \Delta x_1$	$\bar{x}_i + \Delta x_i$	$\bar{x}_n + \Delta x_n$	$\bar{e} + \Delta e$	$\bar{p} + \Delta p$
At any time t:	e	s	x_1	x_i	x_n	e	p

(1) Presented at the American Chemical Society Meeting in Boston, April 6, 1959.

(2) This work was supported by the National Science Foundation and by the Research Committee of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

(3) General Electric Fellow, 1958–1959.

(4) M. Eigen, *Discussions Faraday Soc.*, **17**, 194 (1954).

(5) J. Meixner, *Kolloid Z.*, **134**, 3 (1953).

(6) M. Eigen, *Discussions Faraday Soc.*, **24**, 25 (1957).

(7) G. G. Hammes and L. E. Erickson, *J. Chem. Ed.*, **35**, 611 (1958).

equation 1; directly below the equation are the symbols which are used to represent the various concentration variables.

This mechanism can be completely characterized by $n + 1$ differential equations and two conservation equations of the types

$$\frac{ds}{dt} = -k_1 se + k_{-1}x_1$$

$$\frac{dx_1}{dt} = k_1 es + k_{-2}x_2 - (k_{-1} + k_2)x_1 \qquad (2)$$

$$\frac{dx_i}{dt} = k_i x_{i-1} + k_{-(i+1)}x_{i+1} - (k_{-1} + k_{i+1})x_i$$

$$\frac{dx_n}{dt} = k_n x_{n-1} + k_{-(n+1)}ep + (k_{-n} + k_{n+1})x_n$$

Conservation equations

$$e_0 = e + \sum_{j=1}^{n} x_i \qquad (3a)$$

$$s_0 = s + p + \sum_{j=1}^{n} x_i \qquad (3b)$$

Here e_0 is the total enzyme concentration and s_0 is the total substrate concentration. The differential equations 2 can now be simplified if the assumption is made that the reactant concentrations are only slightly different from their equilibrium values, so that Δ^2 terms in the concentration variables are negligible. Utilizing the facts that the time derivatives of the concentration variables are identically zero at equilibrium and that $\Delta e = -\sum_{j=1}^{n} \Delta x_j$ and $\Delta p = -\Delta s - \sum_{j=1}^{n} \Delta x_j$, the first order linear differential equations obtained are

$$\frac{d\Delta s}{dt} = -k_1\bar{e}\Delta s + (k_{-1} + k_1\bar{s})\Delta x_1 + k_1\bar{s}\sum_{j=2}^{n}\Delta x_j \qquad (4)$$

$$\frac{d\Delta x_1}{dt} = k_1\bar{e}\Delta s - (k_1\bar{s} + k_{-1} + k_2)\Delta x_1 - (k_1\bar{e} - k_{-2})\Delta x_2 - k_1\bar{s}\sum_{j=3}^{n}\Delta x_j$$

$$\cdots$$

$$\frac{d\Delta x_i}{dt} = k_i\Delta x_{i-1} + k_{-(i+1)}\Delta x_{i+1} - (k_{-1} + k_{i+1})\Delta x_i$$

$$\cdots$$

$$\frac{d\Delta x_n}{dt} = -k_{-(n+1)}\bar{e}\Delta s + k_n\Delta x_{n-1} - (k_{-n} + k_{n+1})\Delta x_n - k_{-(n+1)}(\bar{e} + \bar{p})\sum_{j=1}^{n}\Delta x_j$$

These equations can be written in the form

$$\frac{d\Delta s}{dt} = a_{11}\Delta s + \sum_{j=1}^{n} a_{1,j+1}\Delta x_j \qquad (5)$$

$$\frac{d\Delta x_i}{dt} = a_{i1}\Delta s + \sum_{j=1}^{n} a_{i,j+1}\Delta x_j$$

where the a's are constant coefficients defined by equations 4. The condition for solutions of these simultaneous differential equations of the type

$$\Delta s = \sum_{j=1}^{n+1} A_j e^{\lambda_j t} \qquad (6)$$

$$\Delta x_i = \sum_{j=1}^{n+1} B_{ij} e^{\lambda_j t}$$

is that the following determinant of order $n + 1$ vanish.

$$\begin{vmatrix} a_{11} - \lambda & a_{12} & \cdots & a_{1,n+1} \\ a_{21} & a_{22}-\lambda & \cdots & a_{2,n+1} \\ \cdots & \cdots & \cdots & \cdots \\ a_{n+1,1} & a_{n+1,2} & \cdots & a_{n+1,n+1} - \lambda \end{vmatrix} = 0 \qquad (7)$$

The relaxation time is defined as $-1/\lambda_j$ and in principle equation 7 can be solved for the $n + 1$ relaxation times. The above procedure, of course, is analogous mathematically to the calculation of normal modes for vibration of a polyatomic molecule. In general, equation 7 cannot be solved exactly; however, solutions have been obtained for the important cases of one and two intermediates. Before presenting these results, a method will be given for obtaining a single relaxation time characteristic of steady state kinetics. Measurement of this relaxation time will give information similar to that obtained by conventional steady state rate measurements.

Steady State Relaxation Time.—The steady state rate equation for any mechanism of the type given by equation 1 can be written as

$$-\frac{ds}{dt} = \frac{(V_S/K_S)s - (V_P/K_P)p}{1 + s/K_S + p/K_P} \qquad (8)$$

when $s_0 >> e_0$. The V's and K's designate the usual maximum velocities and Michaelis constants. This equation can be simplified in a manner similar to that used previously: let $s = \bar{s} + \Delta s$, $p = \bar{p} - \Delta s$; neglect Δ^2 terms; and remember that $(V_S/K_S)\,\bar{s} - (V_P/K_P)\,\bar{p} = 0$. Utilizing these relationships, the equation obtained is

$$\frac{d\Delta s}{dt} = \frac{-\Delta s(V_P/K_S + V_P/K_P)}{1 + \bar{s}/K_S + \bar{p}/K_P + \Delta s(1/K_P - 1/K_S)} \qquad (9)$$

Since Δs is much smaller than $\bar{s}$ and $\bar{p}$, $\Delta s(1/K_P - 1/K_S)$ can be neglected compared to $1 + \bar{s}/K_S + \bar{p}/K_P$. Equation 9 then can be integrated easily, and the result can be written in the form

$$\Delta s = \Delta s_0 e^{-t/\tau_{ss}} \qquad (10)$$

where

$$\tau_{ss} = \frac{1 + \bar{s}/K_S + \bar{p}/K_P}{V_S/K_S + V_P/K_P} = \frac{1 + s_0(1 + V_S/V_P)/K_S(1 + K_{eq})}{V_S/K_S + V_P/K_P} \qquad (11)$$

The second form of equation 11 was obtained by using the relationships

$$K_{eq} = \frac{\bar{p}}{\bar{s}} = \frac{V_S K_P}{V_P K_S} \qquad (12a)$$

$$s + \bar{p} = s_0 \qquad (12b)$$

Looking at equation 11, one can see that τ_{ss} is *inversely proportional* to the total enzyme concentration since the maximum velocities are proportional to e and that it is a linear function of the total substrate concentration, s_0. Thus measuring τ_{ss} as a function of s_0 will give two independent parameters (a slope and an intercept). These two parameters, coupled with K_{eq}, give three independent relations between V_S, V_P, K_S and K_P. Therefore, if one of these four steady state kinetic parameters is known from some independent source, the other three can be calculated directly.

An alternative method of finding the steady state relaxation time is to set all of the $d\Delta x_i/dt$

equal to zero. Equations 4 will then consist of a set of n simultaneous equations linear in the Δx_i's and a differential equation of $d\,\Delta s/dt$. Eliminating the Δx_i's from the differential equation by solving the set of linear equations results in an equation of the form

$$\frac{d\Delta s}{dt} = -\frac{\Delta s}{\tau_{ss}} \tag{13}$$

and thus τ_{ss} can be evaluated directly. This procedure gives the same steady state relaxation time as the method described above.

Thus far no reason has been given for identifying the steady state relaxation time, τ_{ss}, with one of the relaxation times obtainable from equation 7 although such an identification seems to be physically reasonable. This relationship will be explicitly proved for the case of a mechanism involving one intermediate and then will be *assumed* valid for an n-intermediate mechanism.

One-intermediate Enzyme Mechanism.—For the one-intermediate mechanism, a quadratic equation is obtained from 7 which can be solved easily. This results in terms involving a square root; the square root term can be expanded using the binomial theorem and it can be seen that usually only the first two terms need be retained. A *sufficient*, but *by no means necessary*, condition for this expansion is that $s_0 >> e_0$. The precise condition imposed is

$$[k_1(\bar{s}+\bar{e})+k_{-2}(\bar{p}+\bar{e})+k_{-1}+k_2]^2 >> 4\bar{e}[k_1k_{-2}(\bar{s}+\bar{p}+\bar{e})+k_1k_2+k_{-1}k_{-2}]$$

The two characteristic relaxation times obtained by the above procedure are

$$\tau_1 = [k_1(\bar{s}+\bar{e})+k_{-2}(\bar{p}+\bar{e})+k_{-1}+k_2]^{-1} \tag{14}$$

$$\tau_2 = \frac{k_1(\bar{s}+\bar{e})+k_{-2}(\bar{p}+\bar{e})+k_{-1}+k_2}{\bar{e}[k_1k_{-2}(\bar{e}+\bar{p}+\bar{s})+k_1k_2+k_{-1}k_{-2}]} \tag{15}$$

These relaxation times are functions of enzyme and substrate concentrations, so that by studying the relaxation times as a function of these parameters, aggregates of the rate constants may be obtained. If one assumes that $s_0 >> e_0$ and expresses τ_2 in terms of V_S, K_S, V_P and K_P by introducing

$$k_1k_2\bar{s} = k_{-1}k_{-2}\bar{p} \tag{16}$$

$$\bar{e} = \frac{e_0}{1+k_1\bar{s}/k_{-1}} \tag{17}$$

$$V_S = k_2e_0 \tag{18}$$

$$V_P = k_{-1}e_0 \tag{19}$$

$$K_S = \frac{k_{-1}+k_2}{k_1} \tag{20}$$

$$K_P = \frac{k_{-1}+k_2}{k_{-2}} \tag{21}$$

τ_2 can be shown to be equal to τ_{ss} as defined in equation 11.

Two-intermediate Enzyme Mechanism.—For the case of a two-intermediate mechanism, a cubic equation is obtained from equation 7 which is difficult to solve if one wishes to obtain usable results. However, a solution can be obtained for the case that $s_0 >> e_0$. The steady state relaxation time, τ_{ss}, for this will be assumed to be given by equation 11; in terms of rate constants this can be written as

$$\tau_{ss} = \frac{k_{-1}k_3+k_{-1}k_{-2}+k_2k_3+\bar{s}k_1(k_2+k_{-2}+k_3)+\bar{p}k_{-3}(k_{-1}+k_2+k_{-2})}{\bar{e}[k_1k_2k_3+k_{-1}k_{-2}k_{-3}+k_1k_{-3}(k_2+k_{-2})(\bar{s}+\bar{p})]} \tag{22}$$

This form of τ_{ss} was obtained using the relationships

$$\bar{e} = \frac{e_0}{1+\bar{s}k_1/k_{-1}+\bar{s}k_1k_{-1}/k_2k_{-2}} \tag{23}$$

$$k_1k_2k_3\bar{e} = k_{-1}k_{-2}k_{-3}\bar{p} \tag{24}$$

and by expressing the V's and K's in terms of rate constants.[8] Knowing τ_{ss} means that one of the roots of the cubic equation is known ($\tau_{ss} = -1/\lambda_{ss}$); therefore the cubic equation can be transformed to a quadratic by dividing the cubic by $(\lambda - \lambda_{ss})$. The remaining term is directly proportional to $\bar{e}$ and thus becomes negligible for sufficiently small $\bar{e}$. The resulting quadratic equation can be solved in the usual manner and the square root expanded in a two-term series. This expansion must be carefully scrutinized for individual cases since it will not always be a valid procedure. The results are

$$\lambda_1 = -B + \frac{C}{B+\lambda_{ss}} \tag{25}$$

$$\lambda_2 = -\frac{C+\lambda_{ss}(B+\lambda_{ss})}{B+\lambda_{ss}} \tag{26}$$

where B and C are coefficients in the cubic equation obtained from equation 7. Since the condition has

$$\lambda^3 + B\lambda^2 + C\lambda + D = 0 \tag{27}$$

been imposed that $\bar{e}$ be small, this means that λ_{ss} is small and can be neglected in comparison to B which is independent of $\bar{e}$. If the unimolecular rate constants are less than $k_1\bar{s}$ and $k_{-3}\bar{p}$ and λ_{ss} is made sufficiently small, $|B| >> |C/B| >> |\lambda_{ss}|$. Actually it is reasonable to expect these conditions to be satisfied; for example, the fumarase mechanism which is consistent with a two-intermediate mechanism, appears to obey these relationships exceedingly well.[9] The relaxation times then can be written as

$$\tau_1 = \frac{1}{B} = [k_1\bar{s}+k_{-3}\bar{p}+k_{-1}+k_2+k_{-2}+k_3]^{-1} \tag{28}$$

$$\tau_2 = \frac{B}{C} = \frac{k_1\bar{s}+k_{-3}\bar{p}+k_{-1}+k_2+k_{-2}+k_3}{k_1\bar{s}(k_2+k_{-2}+k_3)+k_{-3}\bar{p}(k_{-1}+k_2+k_{-2})+k_{-1}k_{-2}+k_{-1}k_3+k_2k_3} \tag{29}$$

Here $\bar{e}$ has been neglected in comparison to $\bar{s}$; also one term proportional to $\bar{e}$ has been neglected in the denominator of τ_2 since it not only will probably be small in comparison to the other terms but is also partially cancelled by a $B\lambda_{ss}$ term which has been neglected. Note that the transient state relaxation times are *independent* of enzyme concentration as was found in the one-intermediate case for $\bar{s}$ and $\bar{p}$ much greater than $\bar{e}$. If all three relaxation times could be studied as a function of substrate concentration, all six rate constants could be determined.

As is evident, calculating the relaxation times for a mechanism with more than two intermediates is

(8) L. Peller and R. A. Alberty, THIS JOURNAL, **81**, 5907 (1959).
(9) R. A. Alberty and W. H. Peirce, *ibid.*, **79**, 1526 (1957).

possible in principle but virtually impossible in practice.

Enzyme Inhibition.—The inclusion of enzyme inhibition in the n-intermediate mechanism and calculation of the resulting relaxation spectra is straightforward. Many types of enzyme inhibition are known but only the case of competitive inhibition will be treated in detail here since this will be sufficient to illustrate the general approach. Competitive inhibition can be included in the n-intermediate mechanism by adding reaction (30) (the concentration variables to be used are represented below the reaction as in equation 1).

$$\mathrm{E} + \mathrm{I} \underset{k'_{-1}}{\overset{k_1'}{\rightleftharpoons}} \mathrm{EI} \qquad (30)$$

At equilibrium	$\bar{e}$	$\bar{\imath}$	$\bar{x}'$
At any time t	e	i	x'
At any time t	$\bar{e} + \Delta e$	$\bar{\imath} + \Delta i$	$\bar{x}' + \Delta x'$

The enzyme conservation equation now becomes

$$e_0 = e + \sum_{j=1}^{n} x_j + x' \qquad (31)$$

and an additional conservation equation can be written down for the inhibitor, giving three conservation equations in all

$$i_0 = i + x' \qquad (32)$$

The relaxation spectrum now can be discussed exactly as before; however, an equation for $\mathrm{d}\Delta x'/\mathrm{d}t$ must now be included in equations 4. The determinant which now must be set equal to zero is of order $n + 2$. Thus, even in the simplest possible case of only one intermediate, a cubic equation is obtained.

Once again, however, a steady state relaxation time can be calculated which should be valid regardless of the number of intermediates on the mechanism. Assuming that s_0 and $i_0 >> e_0$, equation 8 can now be written as

$$\frac{\mathrm{d}s}{\mathrm{d}t} = \frac{(V_S/K_S)s - (V_P/K_P)p}{1 + s/K_S + p/K_P + i/K_I} \qquad (33)$$

where

$$K_I = k_{-1}'/k_1' \qquad (34)$$

Proceeding exactly as before, the steady state relaxation time is found to be

$$\tau_{ss} = \frac{1 + \bar{s}/K_S + \bar{p}/K_P + i_0/K_I}{V_S/K_S + V_P/K_P} = \frac{1 + s_0(1 + V_S/V_P)/K_S(1 + K_{eq}) + i_0/K_I}{V_S/K_S + V_P/K_P} \qquad (35)$$

The steady state relaxation time, therefore, is a linear function of the concentration of inhibitor present at constant total substrate concentration. Proceeding exactly as in the two-intermediate case without inhibition, approximate values can be written down for τ_1 and τ_2 both of which are usually short relaxation times.

$$\tau_1 = \frac{1}{B} = [k_1\bar{s} + k_{-2}\bar{p} + k_{-1} + k_2 + k'_{-1} + k_1'\bar{\imath}]^{-1} \qquad (36)$$

$$\tau_2 = \frac{B}{C} = \frac{k_1\bar{s} + k_{-2}\bar{p} + k_{-1} + k_2 + k_{-1}' + k_1'\bar{\imath}}{[k_{-1}' + k_1'\bar{e}][k_1\bar{s} + k_{-2}\bar{p} + k_{-1} + k_2] + k_1'\bar{\imath}[\bar{e}(k_1 + k_{-2}) + k_{-1} + k_2]} \qquad (37)$$

In principle any reaction mechanism can be characterized by a relaxation spectrum in the manner described above; however, as the mechanism becomes increasingly complex, the characteristic equation becomes less amenable to simple solutions.

Application to the Fumarase Mechanism.—As previously indicated, the hydration of fumarate to L-malate catalyzed by fumarase can be represented by a two-intermediate mechanism at a constant pH.[10] From the lower limits of the rate constants,[8,9] the relaxation times can be calculated. The results indicate that an experimental situation is easily accessible whereby $\tau_1 \approx 10^{-6}$ sec., $\tau_2 \approx 10^{-3}$ sec. and $\tau_{ss} \approx 10^{2}$ sec. This means that the relaxation spectrum should be easily resolved. Unfortunately, τ_1 and τ_2 are so short that special techniques[4,11,12] would be necessary to measure such short relaxation times; however, τ_{ss} can be measured easily. The essential principle is that measurements must be made on systems close to equilibrium; for the measurement of τ_{ss} this can be accomplished easily by preparing a solution of both substrates having a concentration ratio slightly different from the equilibrium ratio. Enzyme then can be added and the rate of attainment of equilibrium can be measured. The fumarase system is well suited for this study since the equilibrium constant is about 4 at 25° and small changes in fumarate concentration easily can be measured.

Experimental

Measurements were made of the rate of approach to equilibrium using solutions of fumarate and L-malate at concentrations slightly different from their equilibrium values. In a given experiment, solutions of different total substrate concentrations were used in order to obtain the dependence of the relaxation time on this parameter. The experimental procedure consisted of pipetting 3 ml. of substrate solution into a 1 cm. cuvette, and then 0.5 ml. of enzyme solution from a syringe. The cuvette now was inserted into a Cary 14 recording spectrophotometer with a scale of 0–0.2 absorbancy unit; the wave length was adjusted to as low a value as possible; and a recording of absorbancy *versus* time was obtained. The equilibrium values of the absolute absorbancy also were measured, thus allowing determination of the equilibrium constant. (The absorbancy indices of fumarate have been previously published).[13]

The enzyme concentration was adjusted to give relaxation times falling in a reasonable time range (35–350 sec.): this concentration was roughly 1 mg./100 ml. Assays of the enzyme were made periodically during the course of an experiment by measuring the initial velocity at a convenient L-malate concentration. The enzyme activity was found to decrease slightly during the course of an experiment and the relaxation times were appropriately corrected. Determinations of K_M also were made at both pH's using conventional initial velocity techniques.[14]

A similar procedure to that outlined above was used for studying competitive inhibition by meso-tartrate, except that the total substrate concentration was kept constant during a given experiment, while the mesotartrate concentration was varied.

Measurements were made in 0.05 M phosphate buffer at pH's 6.75 ($K_M \approx 2K_F$) and 7.70 ($K_M \approx 7K_F$). Inhibition studies were made at pH 7.70. Phosphate buffer was selected because of the fact that fumarase is relatively stable

(10) C. Frieden and R. A. Alberty, *J. Biol. Chem.*, **212**, 859 (1955).

(11) M. Eigen and J. Schoen, *Zeit. für Elektrochem.*, **59**, 483 (1955); M. Eigen and L. de Maeyer, *ibid.*, **59**, 986 (1955).

(12) G. Kurtze and K. Tamm, *Acoustica*, **3**, 33 (1953).

(13) R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, THIS JOURNAL, **76**, 2485 (1954).

(14) R. M. Bock and R. A. Alberty, *ibid.*, **75**, 1921 (1953).

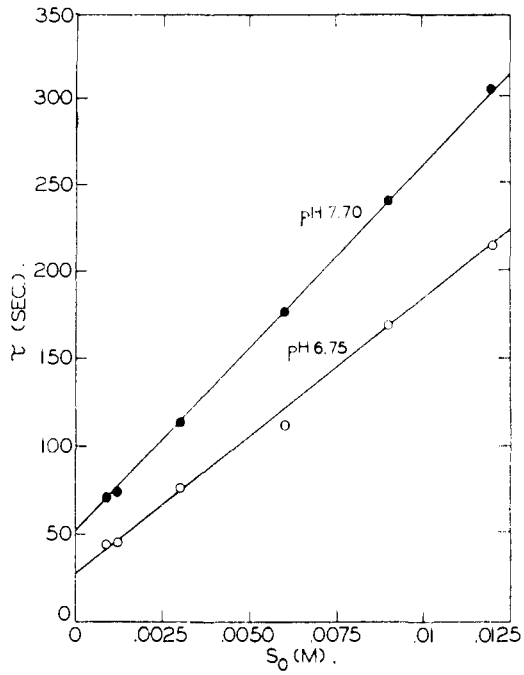

Fig. 1.—Plots of steady state relaxation time *versus* total substrate concentration at *p*H's 6.75 and 7.70.

in this medium. All solutions were thermostated at 25.0 ± 0.1°, as was the cell compartment of the Cary 14 spectrophotometer. The phosphate salts used were analytical grade; fumaric and meso-tartaric acids were recrystallized from water and most of the L-malic acid was recrystallized from ethyl acetate. Some of the malic acid was C.P. grade (California Biochemical Research) and was not recrystallized. No difference in initial velocities could be detected between the two types of L-malic acid. Crystalline fumarase was prepared by methods previously developed in this Laboratory.[15]

Results

All of the data were treated in a similar manner—a smooth line was drawn through the spectrophotometer tracing, and Guggenheim plots of $\ln(A_t - A_{t+\Delta t})$ or $\ln(A_{t+\Delta t} - A_t)$ *versus* time were used to obtain the relaxation times,[16] the relaxation time being equal to the reciprocal of the slope of such a plot. Here A_t represents the absorbancy at time t, and $A_{t+\Delta t}$ represents the absorbancy at a time $t + \Delta t$ later where Δt is a constant time interval.[17] The relative substrate concentration used in this analysis extended approximately over the range 5% from equilibrium to equilibrium. Most of the relaxation times were measured in duplicate for a given substrate concentration during the course of a single experiment; moreover the equilibrium was approached from both directions (*i.e.*, excess or deficient fumarate concentration) at both *p*H's. The relaxation times were generally reproducible within a few per cent. In accordance with equation 11, the relaxation times were plotted *versus* the total substrate concentration. Typical plots of this type at both *p*H's are shown in Fig. 1. A collection of typical data is given in Table I. The ratio of slope to intercept α_0 is independent of enzyme concentration; therefore this should be the same for all experiments in a given buffer. Also the ratio of slopes should equal the ratio of intercepts for a given pair of experiments since these quantities simply measure the relative enzyme activity. Included in this table are the experimentally determined equilibrium constants and the K_M obtained through initial velocity measurements. Assuming this value of K_M, values of K_F and V_F/V_M have been calculated from the experimentally determined α_0 and the equilibrium constant. The equations used were

$$V_F/V_M = \left[\alpha_0\left(\frac{1 + K_{eq}}{K_{eq}}\right)K_M - 1\right]^{-1} \quad (38)$$

$$K_F = V_F K_M / V_M K_{eq} \quad (39)$$

The results of these calculations are included in Table I.

The data obtained for competitive inhibition by meso-tartrate were basically handled in the same way as described above. However, since the total substrate concentration was kept constant during these experiments, the relaxation time was plotted *versus* meso-tartrate concentration (see eq. 35). A typical graph of this type is shown in Fig. 2. In order to calculate the inhibition constant, three quantities must be known: the ratio of slope to intercept α_I from a plot of τ_{ss} *versus* meso-tartrate concentration, the ratio of slope to intercept α_0 from a plot of τ_{ss} *versus* total substrate concentration in the same buffer and the total substrate concentration present when determining α_i. In this case, K_I can be written as

$$K_I = \frac{1}{\alpha_i(\alpha_0 s_0 + 1)} \quad (40)$$

The inhibition constants as calculated from equation 40 are tabulated in Table II. As is clear from Table II, inhibition constants can be obtained with good precision by relaxation methods.

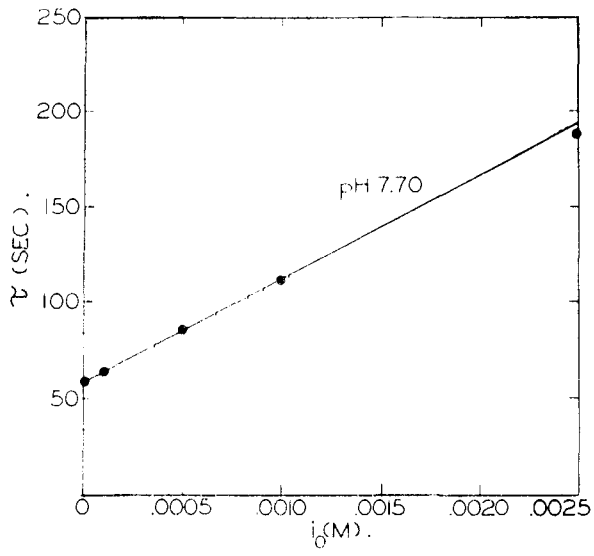

Fig. 2.—Plot of steady state relaxation time *versus* total meso-tartrate concentration at *p*H 7.70.

(15) C. Frieden, R. M. Bock and R. A. Alberty, THIS JOURNAL, **76**, 2482 (1954).

(16) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, N. Y., 1953, p. 48.

(17) The pertinent equations used can be obtained from equation 10 in the usual manner. For example, $\ln(s_t - s_{t+\Delta t}) = -t/\tau_{ss} + \ln \Delta s_0(1 - e^{-t/\tau_{ss}})$.

TABLE I

DETERMINATION OF STEADY STATE PARAMETERS FROM RELAXATION TIMES

$\left(\frac{\text{Malate}}{\text{Fumarate}}\right)_{t=0}$[a]	Intercept (sec.)	Slope $\times 10^{-4}$ (sec. M^{-1})	α_0 (M^{-1})	K_F (mM)	V_F/V_M
5[b]	51.5	2.09	406	0.751	0.652
5[b]	49.0	1.94	396	.783	.680
3.5[b]	68.0	2.60	382	.833	.723
3.5[b]	78.0	2.86	367	.893	.775
		Av.	388 ± 13	0.815 ± 0.048	0.708 ± 0.042
5[c]	27.5	1.56	567	1.32	2.91
5[c]	32.5	1.97	606	1.04	2.29
3.5[c]	43.5	2.79	641	0.872	1.93
3.5[c]	52.0	3.29	633	0.904	2.00
		Av.	612 ± 25	1.03 ± 0.15	2.28 ± 0.32

[a] This ratio is that of initial substrate solutions before enzyme is added. [b] pH = 7.70, K_M = 5.09 mM, K_{eq} = 4.42 ± 0.09. [c] pH = 6.75, K_M = 1.92 mM, K_{eq} = 4.25 ±0.1.

TABLE II

DETERMINATION OF THE INHIBITION CONSTANT OF MESOTARTRATE FROM RELAXATION TIMES AT pH 7.70

$\left(\frac{\text{Malate}}{\text{fumarate}}\right)_{t=0}$	α_1(sec. M^{-1})	s_0(mM)	K_I(mM)
5	915	1.2	0.746
5	951	1.2	.719
3.5	1050	0.9	.704
		Av.	0.723 ± 0.015

Discussion

As can be seen from Tables I and II, steady state parameters can be obtained from relaxation experiments with fair precision. All of the results are consistent with earlier data obtained through initial velocity measurements.[18] Under all conditions studied, the ratio of slope to intercept, α_0 or α_1, obtained from plots of relaxation time *versus* total substrate concentration or mesotartrate concentration were determined with an average deviation of less than 5%. Moreover, the results were the same, within experimental error, regardless of the direction (excess or deficient fumarate) from which equilibrium was approached. The quantity α_0 is not devoid of physical significance since $\alpha_0(1 + K_{eq})$ and $\alpha_0(1 + 1/K_{eq})$ are equal to the dissociation constants for the formation of enzyme–substrate complex for the forward and reverse reactions respectively *if* there is only a single intermediate in the mechanism.

$$\alpha_0(1 + K_{eq}) = k_1/k_{-1} \quad (41)$$

$$\alpha_0(1 + 1/K_{eq}) = k_{-2}/k_2 \quad (42)$$

At pH 6.75, the Michaelis constants are approximately equal ($K_M \approx 2\ K_F$) and K_F and V_F/V_M cannot be determined with good precision because the algebraic solution for these parameters involves subtracting numbers of comparable magnitude. However, at pH 7.70 where the Michaelis constants are quite different ($K_M = 7K_F$), all of the steady state constants in Table I have an average deviation of less than 6% The data obtained at pH 7.70 are also more precise than those at pH 6.75 because the decay of enzyme activity during an experiment was less at the higher pH—correcting relaxation times for this phenomenon is difficult because the decay does not appear to follow any simple relationship. The ionic strength was not constant during a series of relaxation time measurements since substrate concentrations approached buffer concentrations at the highest subtrate concentrations used. This was unavoidable because substrate concentration should be the same order of magnitude as the Michaelis constants. The ionic strength variation was negligible at pH 7.70 but amounted to about 36% at pH 6.75 for the highest substrate concentration used. Obviously, if desired the individual maximum velocities can be obtained from the data providing the enzyme concentration is known.

One point should be made clearly: measurements of steady state relaxation times *will not* yield any information not obtainable through initial steady state velocity measurements. A more detailed discussion of what information can be obtained from steady state enzyme kinetics recently has been published.[8] The shortcomings of steady state relaxation experiments are obvious: only three independent relationships are obtained for the four independent quantities characterizing the steady state. Even if one of these parameters is known, the other three cannot be obtained very precisely if the Michaelis constants are approximately equal. Also for practical purposes the equilibrium constant of the enzyme reaction should be of the order of magnitude of unity if this method is to be employed. The determination of competitive inhibition constants by this method appears particularly promising since good precision can be obtained, and the minimum data for determining an inhibition constant can be found in two relaxation experiments.

Mention should be made of the fact that the temperature must be controlled closely if significant results are to be obtained. This is because the equilibrium constant changes with temperature (unless $\Delta H = 0$); consequently poor temperature control would cause concentration fluctuations to be superimposed on the concentration change being studied.

The next point of interest appears to be experimental determination of short relaxation times, which coupled with steady state data will give information about specific rate constants not available at present.

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(18) Carl Frieden, Ph.D. Thesis, University of Wisconsin, 1956.

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