

present, is strongly in favor of the proposed mechanism involving methyl radicals, because the latter are known²⁴ to react very rapidly with oxygen. On the other hand, the increase in the yield of ethane in the presence of inert gases (Table IV) eludes an explanation. The experiments with oxygen were the last to be performed before the unavoidable termination of this research and the effect of oxygen in changing the ratio between cyclopropane and propylene at low but not at higher pressures (Table I) could not be adequately explored.

There are thus several loose ends to this investigation which need further exploration before the reaction mechanisms here discussed can be con-

sidered as conclusively proven. What is definitely established by these experiments is the complexity of even supposedly simple reactions of methylene. The contradiction between the present experiments and those of Doering, as well as the effect of inert gases in modifying the reactivity of methylene, has only one logically tenable explanation. It is that methylene formed photochemically has an excess of energy over the equilibrium thermal value and that it reacts, in absence of inert gases, before this energy is dissipated on collisions. This means that "hot" methylene reacts very fast. What the reactivity of methylene thermally equilibrated at room temperature is, still remains to be determined.

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Studies of the Enzyme Fumarase. VII.¹ Series Solutions of Integrated Rate Equations for Irreversible and Reversible Michaelis-Menten Mechanisms²

BY ROBERT A. ALBERTY AND BARBARA M. KOERBER

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The integrated rate equations for the Michaelis-Menten mechanism and for simple reversible mechanisms have been expanded as power series in time to determine the relations between the intercepts and initial slopes of plots of $(P)/t$ or $-(1/t) \ln [1 - (P)/(P)_{eq}]$ vs. t and the kinetic parameters. The magnitudes of the experimental initial slopes for the fumarase reaction are in agreement with theory. It is shown that the initial slopes of these plots, as well as the intercepts, are useful in determining the kinetic parameters for enzymatic reactions. By calculating the coefficient of the t^2 terms in the series it is shown that the onset of appreciable deviations from linearity occurs at the same time for the two types of plots.

Introduction

The kinetic parameters for enzymatic reactions are generally calculated from steady-state velocities extrapolated to zero time to eliminate the effect of accumulation of product on the rate. Since the steady-state velocity may decrease rather rapidly as the reaction proceeds, there may be some difficulty in making the extrapolation to zero time. We have therefore investigated the relation between the slopes of plots of $(P)/t$ or $-(1/t) \ln [1 - (P)/(P)_{eq}]$ vs. t during the steady state and the steady state kinetic parameters, where (P) is the concentration of product and $(P)_{eq}$ is the equilibrium concentration. Such plots may be represented by power series expansions

$$\begin{aligned} (P)/t &= a\{1 + bt[1 + ct + \dots]\} & (1) \\ -(1/t) \ln [1 - (P)/(P)_{eq}] &= \alpha\{1 + \beta t[1 + \gamma t + \dots]\} & (2) \end{aligned}$$

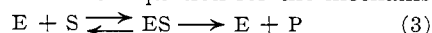
For a particular steady-state rate equation, a, b, c, \dots and $\alpha, \beta, \gamma, \dots$ may be obtained in terms of the rate parameters by means of series expansions of the integrated rate equation. These equations make it possible to calculate the length of the period during which a linear extrapolation to $t = 0$ may be made. They also permit the calculation of kinetic parameters from the initial slopes of such plots as well as from the intercepts or make possible a check on the consistency of the rate parameters determined from initial velocities with the decrease in velocity with time.

(1) The preceding article in this series is R. A. Alberty, W. G. Miller and H. F. Fisher, *THIS JOURNAL*, **79**, 3973 (1957).

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

Michaelis-Menten Mechanism

The integrated rate equation for the mechanism



was obtained by Walker and Schmidt³ on the assumptions that the time required to reach the steady state is negligible, that $d(ES)/dt = 0$ is a sufficiently good approximation,⁴ that the reaction goes to completion and the product is not inhibitory and that $(S)_0 \gg (E)_0$. The integrated rate equation may be written

$$V_{st} = (P) - K_s \ln [1 - (P)/(S)_0] \quad (4)$$

where V_s is the maximum steady-state velocity, and K_s is the Michaelis constant of the substrate and $(S)_0$ is the initial substrate concentration. Walker and Schmidt obtained V_s and K_s for the histidase reaction from a plot of $(1/t) \ln [1 - (P)/(S)_0]$ vs. $(P)/t$.

Equation 4 may be rearranged into the form of equation 1 by expanding the logarithmic term as a power series.

$$-\ln \left[1 - \frac{(P)}{(S)_0} \right] = \frac{(P)}{(S)_0} + \frac{(P)^2}{2(S)_0^2} + \frac{(P)^3}{3(S)_0^3} + \dots \quad (5)$$

Substituting into equation 4 and rearranging we obtain

$$\begin{aligned} \frac{(P)}{(S)_0} &= \frac{V_{st}}{(S)_0 + K_s} - \frac{K_s(P)^2}{2[(S)_0 + K_s](S)_0^2} - \\ &\quad \frac{K_s(P)^3}{3[(S)_0 + K_s](S)_0^3} - \dots \quad (6) \end{aligned}$$

For small extents of reaction, $(P)/(S)_0 \ll 1$ and the first term on the right-hand side of equation 6 may be substituted for $(P)/(S)_0$ in the second

(3) A. C. Walker and C. L. A. Schmidt, *Arch. Biochem.*, **5**, 445 (1944).

(4) M. F. Morales and D. E. Goldman, *THIS JOURNAL*, **77**, 6069 (1955).

term. Squaring this equation gives the second approximation to $(P)^2/(S)_0^2$. Substitution of this relation and $(P)^3/(S)_0^3 = [V_s t / ((S)_0 + K_s)]^3$ into equation 6 yields

$$\frac{(P)}{t} = v_0 \left\{ 1 - \frac{K_s v_0 t}{2V_s(S)_0^2} \left[1 - \frac{K_s v_0^2 (1 - 2(S)_0/K_s)t}{3V_s(S)_0^2} + \dots \right] \right\} \quad (7)$$

where $v_0 = V_s/[1 + K_s/(S)_0]$. Thus it can be seen that for sufficiently short times of reaction a linear extrapolation of $(P)/t$ vs. t back to $t = 0$ yields the initial velocity v_0 as given by the Michaelis-Menten rate equation. Determination of v_0 at two or more $(S)_0$ makes it possible to calculate K_s and V_s . However, if it is possible to determine the initial slope of the plot of $(P)/t$ vs. t , the values of K_s and V_s may be calculated from the intercept and initial slope of a single experiment by using equation 7. The coefficient of the t term in the innermost brackets indicates the time at which the plot will be appreciably curved. If $(S)_0 = K_s/2$, it can be seen that the coefficient of this term is equal to zero, and that the sign of this term changes at this substrate concentration.

Equation 4 may be rearranged into the form of equation 2 by solving equation 5 for (P) , and substituting into equation 4 to obtain

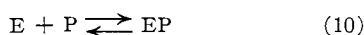
$$-(1/t) \ln [1 - (P)/(S)_0] = \frac{V_s}{(S)_0 + K_s} + \frac{(P)^2}{2[(S)_0 + K_s](S)_0^2} + \frac{(P)^3}{3[(S)_0 + K_s](S)_0^2 t} + \dots \quad (8)$$

By introducing the quantities derived earlier for $(P)^2/(S)_0^2$ and $(P)^3/(S)_0^3$ into the second and third terms on the right-hand side of equation 8, and rearranging, we obtain

$$-\left(\frac{1}{t}\right) \ln \left[1 - \frac{(P)}{(S)_0} \right] = \frac{v_0}{(S)_0} \left\{ 1 + \frac{v_0 t}{2V_s(S)_0} \left[1 - \frac{K_s v_0^2 (1 - 2(S)_0/K_s)t}{3V_s(S)_0^2} + \dots \right] \right\} \quad (9)$$

The initial steady-state velocity v_0 is therefore obtained by multiplying the intercept by $(S)_0$. If it is possible to determine the initial slope in addition, the values of V_s and K_s may be calculated from an experiment at a single substrate concentration. The coefficient of t in the innermost brackets is exactly the same as in equation 7, and so the onset of appreciable deviations from linearity in plots of $(P)/t$ vs. t or $-(1/t) \ln [1 - (P)/(S)_0]$ vs. t will occur at the same time.

In testing equation 4 with chymotrypsin Huang and Niemann⁵ found that marked deviations were obtained after 40% hydrolysis of acetyl-L-tryptophan-amide as a result of inhibition by product. They showed that when product inhibition represented by



was added to mechanism 3, the integrated rate equation became

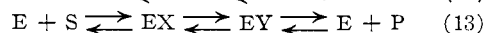
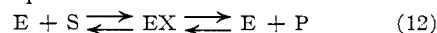
$$\frac{V_s}{K_s} t = \left(\frac{1}{K_s} - \frac{1}{K_P} \right) (P) + \left(1 + \frac{(S)_0}{K_P} \right) \ln \left[1 - \frac{(P)}{(S)_0} \right] \quad (11)$$

(5) H. T. Huang and C. Niemann, *THIS JOURNAL*, **73**, 1541 (1951).

where K_P is the competitive inhibition constant for the product P. The various ways in which this equation may be used have been discussed by Niemann and co-workers.⁶⁻⁹ Rather than consider product inhibition as an addition to mechanism 3, we prefer to treat it as shown in the following section.

Simple Reversible Mechanisms

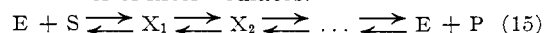
For the simple reversible mechanisms



Haldane¹⁰ showed that if $(S)_0 \gg (E)_0$ the steady-state rate equation may be written in the form

$$-\frac{d(S)}{dt} = \frac{d(P)}{dt} = \frac{V_s}{1 + \frac{(S)}{K_s} + \frac{(P)}{K_P}} \quad (14)$$

where $(S) + (P) = (S)_0$. This steady-state rate equation holds for mechanism (15) with an indefinite number of intermediates.



If the initial reactant is S the term containing (P) in the numerator will be negligible so long as the reaction is far from equilibrium. However, the second term in the denominator may become important at very small extents of reaction if K_P is small. This might make it extremely difficult to determine experimentally the initial steady-state velocity.

Assuming the time required to reach the steady state is negligible, equation 14 may be integrated¹¹ to obtain

$$\left[\frac{V_s}{K_s} + \frac{V_P}{K_P} \right] t = \left[\frac{1}{K_s} - \frac{1}{K_P} \right] (P) - \left\{ 1 + \frac{(S)_0}{K_P} + \frac{\left[\frac{1}{K_s} - \frac{1}{K_P} \right] V_P}{\left[\frac{V_s}{K_s} + \frac{V_P}{K_P} \right]} (S)_0 \right\} \times \ln \left[1 - \frac{(P)}{(P)_{eq}} \right] \quad (16)$$

As is evident from equation 14, the equilibrium concentrations of S and P are related to the kinetic parameters by

$$K_{eq} = \frac{(P)_{eq}}{(S)_{eq}} = \frac{(S)_0 - (S)_{eq}}{(S)_{eq}} = \frac{(P)_{eq}}{(S)_0 - (P)_{eq}} = \frac{V_s K_P}{V_P K_s} \quad (17)$$

Hence, only three of these kinetic parameters may have independent values.

Equation 16 may be used directly in determining¹² the kinetic parameters or it may be arranged in infinite series form.

Equation 16 may be arranged in the form of equation 1 by expanding the logarithmic term in a power series as illustrated in equation 5. Substituting into equation 16, eliminating V_P by use of

(6) R. J. Foster and C. Niemann, *Proc. Natl. Acad. Sci.*, **39**, 999 (1953).

(7) T. H. Applewhite and C. Niemann, *THIS JOURNAL*, **77**, 4923 (1955).

(8) R. R. Jennings and C. Niemann, *ibid.*, **77**, 5432 (1955).

(9) W. E. M. Lands and C. Niemann, *ibid.*, **77**, 6509 (1955).

(10) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930.

(11) R. A. Alberty, *Advances in Enzymology*, **17**, 42 (1956).

(12) Forthcoming publication.

equation 17, and rearranging we obtain

$$\frac{(P)}{(P)_{eq}} = \frac{v_0 t}{(P)_{eq}} - \frac{\left\{1 + \frac{(S)_0}{(1 + K_{eq})} [K_{eq}/K_P + 1/K_S]\right\}}{1 + (S)_0/K_S} \times$$

$$\frac{(P)^2}{(P)_{eq}^2} - \frac{\left\{1 + \frac{(S)_0}{(1 + K_{eq})} [K_{eq}/K_P + 1/K_S]\right\}}{1 + (S)_0/K_S} \times$$

$$\frac{(P)^3}{(P)_{eq}^3} - \dots \quad (18)$$

After calculating $(P)^2/(P)_{eq}^2$ and $(P)^3/(P)_{eq}^3$ as described in connection with equation 6 and rearranging we obtain

$$\frac{(P)}{t} = v_0 \left\{1 - \frac{K_S v_0^2 R t}{2 V_S (S)_0^2} \left[1 - \frac{K_S v_0^2 [1 + 1/K_{eq} - (S)_0(2/K_S - 3/K_P - 1/K_S K_{eq})] t}{3 V_S (S)_0^2} + \dots \right]\right\} \quad (19)$$

where $v_0 = V_S/[1 + K_S/(S)_0]$ and

$$R = 1 + 1/K_{eq} + (S)_0(K_S/K_P + 1/K_{eq})/K_S \quad (20)$$

Thus for mechanisms 12, 13 and 15, the initial velocity v_0 obtained by extrapolation to $t = 0$ is related to the substrate concentration by the Michaelis-Menten equation. By determining the intercepts at two substrate concentrations, V_S and K_S may be calculated. From either initial slope, K_P may also be calculated if the equilibrium constant for the reaction is known. However, if $K_P \ll (S)_0$, the initial slope will be great and the extrapolation to $t = 0$ may be quite difficult. The coefficient of t in the innermost brackets will be equal to zero if

$$(S)_0 = \frac{1 + 1/K_{eq}}{2/K_S - 3/K_P - 1/K_{eq}K_S} \quad (21)$$

For some combinations of K_S , K_P and K_{eq} , there is no possible value of $(S)_0$ which satisfies this condition.

Equation 15 may be arranged in the form of equation 2 as described in connection with equations 8 and 9.

$$-\left(\frac{1}{t}\right) \ln \left[1 - \frac{(P)}{(P)_{eq}}\right] = \frac{v_0}{(P)_{eq}} \left\{1 + \frac{(1 - K_S/K_P)v_0^2 t}{2 V_S (S)_0} \left[1 - \frac{K_S v_0^2 [1 + 1/K_{eq} - (S)_0(2/K_S - 3/K_P - 1/K_S K_{eq})] t}{3 V_S (S)_0^2} + \dots \right]\right\} \quad (22)$$

It is seen that in this case the initial steady-state velocity v_0 is obtained by multiplying the intercept by the equilibrium concentration of the product. In contrast with the other plots which have been discussed a plot of $-(1/t) \ln [1 - (P)/(P)_{eq}]$ vs. t for a reversible reaction may have either a positive or negative slope depending upon whether $K_S < K_P$ or $K_S > K_P$. If $K_S = K_P$ the reaction will be pseudo first order as may also be seen from equation 16. Since V_S and K_S may be calculated from the intercepts for experiments at two substrate concentrations, the value of K_P may then be calculated from either initial slope.

The coefficient of t in the innermost brackets is exactly the same as in equation 19, and so the same remarks apply. Thus the onset of appreciable deviations from linearity in plots of $(P)/t$ and $-(1/t) \ln [1 - (P)/(P)_{eq}]$ vs. t occurs at the same time. If $K_{eq} = \infty$ and $K_P = \infty$, equation 22 reduces to equation 9, as it must.

Application to the Fumarase Reaction

The fumarase reaction follows rate equation 14 provided the substrate concentrations are low enough that substrate inhibition and activation are avoided.^{13,14} Recent calculations¹⁵ indicate that the transient phase of the reaction has a half-life of the order of 10^{-4} second and the time required to reach the steady state may be neglected for present purposes.

Several kinetic experiments were carried out at pH 7.8 and 8.5 using 15 mM phosphate buffers at a fumarase concentration such that 25–40% approach to equilibrium was attained in 25 minutes. Plots of $(P)/t$ and $-(1/t) \ln [1 - (P)/(P)_{eq}]$ vs. t were prepared, and four of these plots are shown in Figs. 1–4. For Figs. 1 and 2, fumarate is the sub-

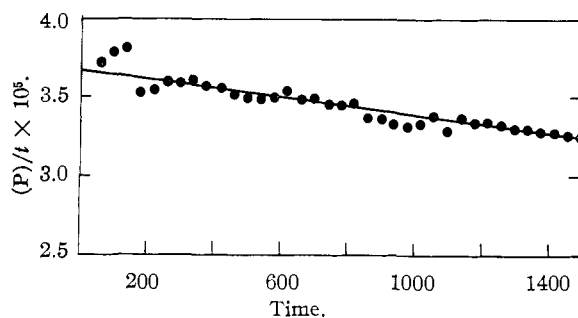


Fig. 1.—Plot of $(M)/t$ vs. t in seconds for $(F)_0 = 0.198$ mM in 15 mM sodium phosphate buffer of pH 8.5 at 25°. The L-malate concentrations are in mM.

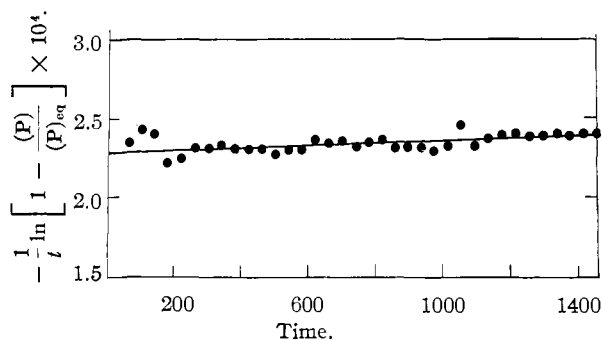


Fig. 2.—Plot of $-(1/t) \ln [1 - (M)/(M)_{eq}]$ vs. t in seconds for the same experiment as Fig. 1.

strate. In each case, the solid line is predicted for the extent of reaction covered both from the series expansion with terms through the t^2 term (equations 19 and 22) and from the total integrated equation (eq. 16). For Figs. 3 and 4, L-malate is the substrate, and the higher terms in the series expansion are of sufficient importance that the first two terms do not give a good representation of the data for a very large extent of reaction. Inclusion of the t^2 term does not produce a fit over a very great extent of reaction. In these figures, the solid line indicates the limiting slope obtained from the series expansion, the dotted line is calculated with the series expansion including both the t and t^2 terms, and the dashed line is calculated using the

(13) R. M. Bock and R. A. Alberty, *THIS JOURNAL*, **75**, 1921 (1953).

(14) R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, *ibid.*, **76**, 2485 (1954).

(15) R. A. Alberty and W. H. Peirce, *ibid.*, **79**, 1526 (1957).

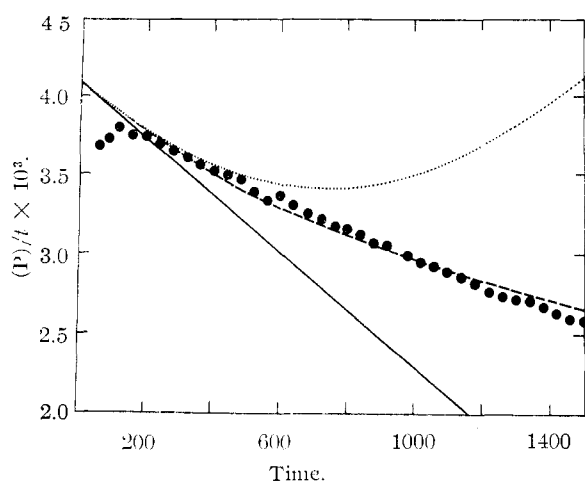


Fig. 3.—Plot of $(F)/t$ vs. t in seconds for $(M)_0 = 4.95$ mM in 15 mM sodium phosphate buffer of pH 8.5 at 25°. The fumarate concentrations are in units of absorbancy at 250 μ for a 10 cm. cuvette.

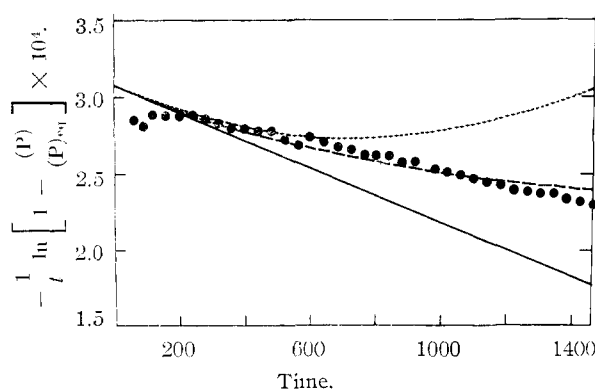


Fig. 4.—Plot of $-(1/t) \ln [1 - (F)/(F)_{eq}]$ vs. t in seconds for the same experiment as Fig. 3.

total integrated rate equation. These theoretical lines and those in Figs. 1 and 2 were calculated using values of K_M , K_F and V_F/V_M calculated from the intercepts and limiting slopes of plots of $(P)/t$ vs. t at pH 8.5 for 4.95 and 0.991 mM L-malate and at pH 7.8 for 4.95 and 0.793 mM L-malate. These kinetic parameters are summarized in Table I.

TABLE I

KINETIC PARAMETERS FOR THE FUMARASE REACTION AT 25° IN 15 mM SODIUM PHOSPHATE BUFFERS

	Series expansion method		Initial velocity method ¹⁴	
	pH 8.5	pH 7.8	pH 8.5	pH 7.8
K_M (mM)	6.57	2.97	7.4	3.25
K_F (mM)	0.27	0.37	0.15	0.24
V_F/V_M	0.181	0.548	0.114	0.339

The agreement with the kinetic parameters determined earlier¹⁴ is not as good as might be hoped. The most serious discrepancies involve the Michaelis constants for fumarate, and it is just these determinations which are most difficult for both methods because of the low value of this Michaelis constant.

The new parameters satisfy the equilibrium equation 17 because this is assumed in the treatment of the data while some of the old parameters are not quite consistent with that equation.

Discussion

In using the steady-state kinetic method for investigating the mechanism of any enzymatic reaction, the question always arises of whether the steady-state velocity obtained by using data from the first part of the reaction actually corresponds with the given initial substrate concentration. The present equations show that a linear extrapolation to v_0 may always be obtained at sufficiently short times for a simple reversible reaction, and they permit a calculation of the time during which such an extrapolation may be made for a given set of kinetic parameters. As is apparent from the data presented, the length of time that the first two terms of the infinite series are a good approximation to the whole integrated rate equation may vary over a wide range. Under conditions where $K_S \approx K_P$, the apparent first-order plot has been found to be linear for more than eight half-lives.¹¹ The smaller is K_P relative to K_S , the larger is the coefficient of the t^2 term. Since K_P may be calculated from the initial slope, all four kinetic parameters may be obtained by studying just the forward reaction provided the equilibrium constant for the over-all reaction is known.

Experimental

Crystalline fumarase was isolated from pig heart muscle by the procedure developed in this Laboratory.¹⁶ Fumaric and L-malic acids were purified by methods described earlier.¹⁷

A Beckman DU spectrophotometer operated in conjunction with a stopwatch was used to determine the extent of reaction at various times. Maximum sensitivity was attained by operating at the lowest possible wave length. Absorbancies were corrected to 250 μ and converted to concentration of fumarate by means of the known molar absorptivity indices.¹⁴ The temperature was maintained at $25 \pm 0.5^\circ$ by means of Beckman thermospacers.

Loss of enzymatic activity was found to be rapid immediately after the enzyme solution was introduced into a quartz cuvette. After about an hour, the rate of loss of activity became constant and so small that it was not necessary to take it into account during the rate measurements. In order to take advantage of this, 20 ml. of a solution of enzyme in the buffer was allowed to stand in a 10 cm. quartz cuvette for an hour or longer. Then 0.2 ml. of a substrate solution which would give the desired substrate concentration in the cuvette was introduced. At the same time the stopwatch was started. Absorbancy measurements were made every 40 seconds.

Data were taken in phosphate buffers with either L-malate or fumarate as substrate. The substrate concentration was always less than five times the Michaelis constant so that substrate activation might be avoided. The conditions of the experiments were those under which kinetic constants had been obtained previously by the initial velocity method.¹⁴

Acknowledgment.—The authors are indebted to Dr. Edward L. King and Dr. Manuel Morales for helpful discussions of rate laws of the type of equation 14 which led to the present research.

MADISON, WISCONSIN

(16) C. Frieden, R. M. Beck and R. A. Alberty, *THIS JOURNAL*, **76**, 2482 (1951).

(17) C. Frieden, R. G. Wolfe, Jr., and R. A. Alberty, *ibid.*, **79**, 1523 (1957).