[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Studies of the Enzyme Fumarase. III.[1] The Dependence of the Kinetic Constants at 25° upon the Concentration and pH of Phosphate Buffers

BY ROBERT A. ALBERTY, VINCENT MASSEY, CARL FRIEDEN AND ARMIN R. FUHLBRIGGE

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The catalysis of the hydration of fumarate and dehydration of *l*-malate by fumarase has been studied spectrophotometrically over a range of substrate concentrations of 0.1 to 10^{-4} *M* for *l*-malate and 0.1 to 3×10^{-5} *M* for fumarate. When fumarate is the substrate linear Lineweaver–Burk plots are obtained at low substrate concentrations, but at higher concentrations the velocities are greater than expected. This effect is interpreted in terms of the alteration of the catalytic properties of the enzyme by the binding of substrate at neighboring sites which affect the enzymatic sites. Michaelis constants and maximum initial velocities for both substrates have been determined at low substrate concentrations over a *p*H range of 5.5 to 8.5 at phosphate buffer concentrations of 5, 15, 50, 60 and 133 m*M*. Although the kinetic constants vary considerably with the *p*H and phosphate concentration this variation is such that the Haldane relation between the kinetic constants for the two substrates and the equilibrium constant for the over-all reaction is always obeyed. The maximum initial velocities (V) increase with phosphate concentration and approach a value independent of phosphate concentration. Plots of V *vs.* *p*H are quite symmetrical bell-shaped curves as would be expected for the case where there are two ionizable groups in fumarase which are essential for its activity. The fact that the Michaelis constants (K_m) for both substrates vary strongly with the *p*H and are nearly directly proportional to the concentration of phosphate buffer at a given *p*H shows that they may not be given the usual simple interpretation. The results are interpreted in terms of a mechanism which allows specifically for interaction between the enzyme and a component of the buffer.

The catalysis of the hydration of fumarate and dehydration of *l*-malate by fumarase may be conveniently studied over a wide range of substrate concentrations, *p*H and buffer concentration because of the sensitivity of the spectrophotometric method for studying this reaction. The activating effect of phosphate and other ions on both the forward and reverse reactions has been studied recently by Massey[2] who has reviewed the earlier literature on this topic. The fact that various salts exert such different effects indicates that more than an ionic strength effect is involved. The present investigation has shown that the Michaelis constants for fumarate and *l*-malate vary considerably with the concentration of phosphate buffer. Thus the Michaelis constants cannot be interpreted simply in terms of $(k_2 + k_3)/k_1$ as provided by the Michaelis–Menten[3]–Briggs–Haldane[4] theory. In order to provide a theoretical basis for the interpretation of buffer effects mechanisms involving buffer–enzyme interactions have been treated.[5] In addition to providing data on the effect of phosphate on the kinetic constants, an objective of this investigation has been to test the Haldane relation[6] between maximum initial velocities, Michaelis constants and the equilibrium constant for the over-all reaction. This relation has already been found to be obeyed by the fumarase reaction at *p*H 7.3 in 0.05 *M* sodium phosphate buffer at 25°.[7]

Experimental

Crystalline fumarase was isolated from pig heart muscle by procedures developed in this Laboratory[1] and by the method of Massey.[8] Identical kinetic results have been obtained with materials isolated by the two procedures.

The Beckman DUR spectrophotometer used in the kinetic measurements has been described earlier.[7] The measurements have been facilitated by the use of logarithmic chart papers on which optical density is read directly.[9]

The values of the extinction coefficients for disodium fumarate given in Table I were determined with fumarate produced enzymatically from *l*-malate and are felt to be more accurate than values given earlier[7] since this method has the advantage of eliminating errors from absorbing impurities in fumaric acid. The determinations were made by preparing a solution of *l*-malate in 0.005 *M* phosphate buffer of *p*H 7.3, adding enzyme, allowing the reaction to come to equilibrium and then measuring the optical density at a series of wave lengths employing suitable blanks. Since only ratios of extinction coefficients may be determined directly by this method, the values of ϵ_λ have been calculated using $\epsilon_{250} = 1.45$ m$M.^{-1}$ cm.$^{-1}$.[7]

TABLE I

MOLAR EXTINCTION COEFFICIENTS[a] FOR DISODIUM FUMARATE AT 25° IN DILUTE PHOSPHATE BUFFER

λ, mμ	ϵ_λ, (mM.$^{-1}$ cm.$^{-1}$)	$\epsilon_{250}/\epsilon_\lambda$
205	16.6	0.0876
210	15.1	.0964
220	9.30	.156
230	4.52	.321
240	2.44	.595
250	1.45	1.000
260	0.900	1.61
270	.535	2.71
280	.278	5.22
290	.118	12.3
300	.0433	33.5

[a] Defined by $\epsilon = D/lc$ where D is log (I_0/I), l is the length of the optical path through the cell in cm. and c is concentration in m*M*.

In making kinetic measurements it has been found advantageous to keep the stock solution of the enzyme in a dilute phosphate buffer at 0°. A small volume (usually 0.2 ml.) of the enzyme solution is pipetted rapidly into a solution of substrate and buffer in a 3-ml. cuvette with a 1 cm. optical path, and the contents mixed by capping the cuvette and inverting it rapidly several times before placing it into the spectrophotometer. In some experiments it was necessary to correct for loss in enzymatic activity during a series of

(1) The preceding article in this series is C. Frieden, R. M. Bock and R. A. Alberty, THIS JOURNAL, **76**, 2482 (1954).

(2) V. Massey, *Biochem. J.*, **53**, 67 (1953).

(3) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

(4) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(5) R. A. Alberty and R. M. Bock, *Proc. Natl. Acad. Sci.*, **39**, 895 (1953).

(6) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930, p. 81.

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

(8) V. Massey, *Biochem. J.*, **51**, 490 (1952).

(9) Two new types of optical density paper are available from the Minneapolis-Honeywell Co.: No. 6304-N for 0-∞ density and No. 6300-N for 0–0.0969. For the use of both of these charts it is necessary to set the zero of the recorder at 1% full scale. This has the advantage of allowing a small amount of overtravel for the dark current adjustment.

velocity measurements. This was accomplished by rerunning a certain substrate concentration at intervals, plotting these velocities *vs.* time and interpolating to obtain the correction factors for the other experiments. All of the experiments were at 25°.

As shown in the Appendix it is necessary to obtain the initial velocities within the first 1.7% reaction when *l*-malate is the substrate or the first 7.8% reaction when fumarate is the substrate if the reaction is a reversible first-order reaction and it is desired to keep the error in a zero-order calculation of the initial rate less than 5%. Larger extents of reaction may be utilized in the zero-order calculation of the rate at substrate concentrations which are of the order of the Michaelis constant or greater. Fortunately, the sensitivity of the analytical method is increased 380 times in going from a wave length of 300 to 205 mμ. However, the lowest wave length which may be used at a given concentration of fumarate is limited by its absorption. An indication of the wave lengths used at various substrate concentrations is given in the Appendix.

In studying velocities over a wide range of phosphate and substrate concentrations there is the problem of whether to compare experiments at constant *p*H or constant ratio of the two ionic forms of phosphate since the *pK* for phosphate changes appreciably with concentration. Since the enzyme is a protein its activity varies markedly with *p*H, even in ranges where phosphate is predominately in either the monovalent or divalent form. Therefore, the kinetic constants reported were obtained from Lineweaver–Burk[10] plots at constant *p*H and constant total phosphate concentration.

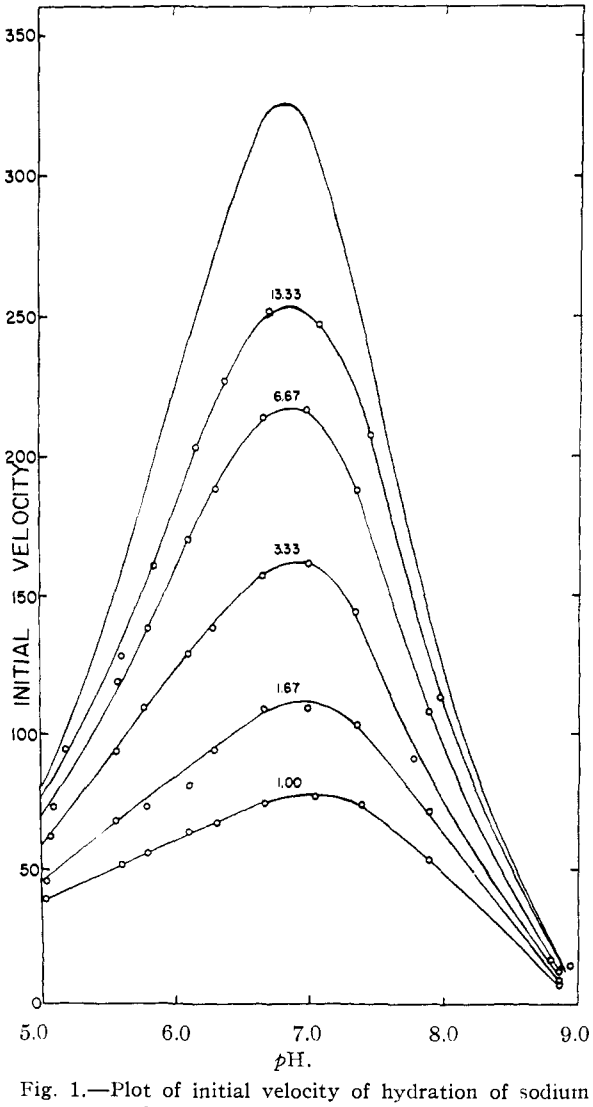

Fig. 1.—Plot of initial velocity of hydration of sodium fumarate at 25° *vs.* *p*H for 0.133 *M* sodium phosphate buffers and five fumarate concentrations indicated in m*M*. The top curve represents the maximum initial velocity obtained by extrapolation of Lineweaver–Burk plots.

In determining reaction velocities over a wide range of substrate concentrations it is a common problem that the *p*H values of the reaction mixtures in a series of experiments are not identical. This problem is especially serious at low buffer concentrations since the *p*H of the substrate solution may be somewhat different from that of the buffer. In order to avoid this problem and to obtain kinetic constants over a wide range of *p*H values, plots of initial velocities *vs.* *p*H were constructed for a series of five or more substrate concentrations. The *p*H values are those measured on the initial reaction mixtures shortly after the velocity determination. Such a plot is shown in Fig. 1. Velocities at chosen *p*H values are then interpolated from the graph and Lineweaver–Burk plots are constructed to obtain the Michaelis constant (K_m) and maximum initial velocity (V) at this *p*H and buffer concentration. A further type of experiment is required in order to compare the values of V for fumarate and *l*-malate at a series of phosphate concentrations. In this latter type of experiment velocities are determined at several phosphate concentrations in a narrow range of *p*H and a plot of V *vs.* phosphate concentration at constant *p*H is constructed. Having done this at one *p*H value the velocity curves at the various phosphate concentrations are normalized (that is, corrected to a common enzyme activity) and V *vs.* phosphate concentration plots obtained by interpolation.

Deviations from the Michaelis–Menten Relation.—Initial velocities of dehydration of *l*-malate at approximately *p*H 6.5 and four phosphate concentrations have been determined over a 1000-fold range of *l*-malate concentrations. The velocities were corrected to exactly *p*H 6.50 by use of plots of velocity *vs.* *p*H. The data for the range 0.33–10 m*M* are presented as a Lineweaver–Burk plot in Fig. 2. Data for the complete range of *l*-malate concentrations are given in Table II from which it may be seen that there is inhibition at 100 m*M*. If this substrate inhibition is ignored the data may be represented by the Michaelis–Menten[3] equation

$$v = \frac{V}{1 + K_m/(S)} \qquad (1)$$

where v is initial reaction velocity, V is the maximum initial velocity and K_m is the Michaelis constant. The calculated values of v in Table II have been obtained from equation 1 with $V = 7.7$ at all phosphate concentrations and $K_m = 0.27$, 0.38, 0.87 and 1.37 m*M* at 5, 15, 33.3 and 50 m*M* phosphate, respectively. Since the maximum initial velocity is independent of phosphate concentration above 5 m*M* at *p*H 6.5 the increase in the slopes of the Lineweaver–Burk plots with phosphate concentration

TABLE II

DEPENDENCE OF INITIAL RATE OF DEHYDRATION OF *l*-MALATE AT 25° AND *p*H 6.5 ON *l*-MALATE AND PHOSPHATE CONCENTRATIONS

(M), m*M*	Relative rates: 5 m*M* phos. Expt.	Eq. 1	15 m*M* phos. Expt.	Eq. 1	33.3 m*M* phos. Expt.	Eq. 1	50 m*M* phos. Expt.	Eq. 1
0.100	1.9	2.1	1.8	1.6	0.81	0.79	0.44	0.52
0.333	4.2	4.3	3.7	3.6			1.50	1.50
1.00	6.1	6.1	5.7	5.6	3.9	4.1		
3.33	6.5	7.1	6.7	6.9	6.2	6.1	5.3	5.4
10.0	7.2	7.5	7.4	7.4	6.7	7.1	6.7	6.8
33.3	7.4	7.6	8.0	7.6	7.8	7.5	7.7	7.4
100	6.9	7.7	6.3	7.7	5.8	7.6	5.3	7.6

(10) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

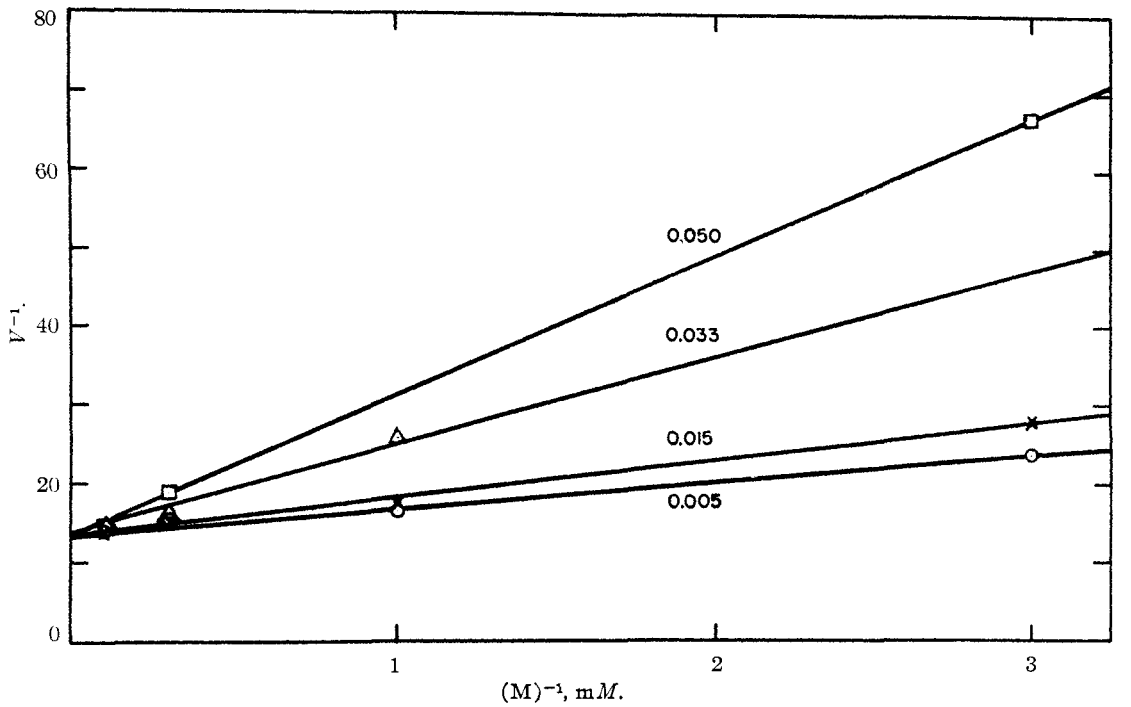

Fig. 2.—Plot of reciprocal initial velocity *vs.* reciprocal *l*-malate concentration at pH 6.5 and 25° for the indicated phosphate concentrations (mM).

suggests, according to the simple Michaelis–Menten theory, that phosphate is acting as a competitive inhibitor. However, an activating effect by phosphate may also be demonstrated by use of phosphate concentrations below 5 mM.[2]

Initial velocities of hydration of fumarate at pH 6.5 at the same four phosphate concentrations have been determined over a 3000-fold range of fumarate concentrations, and the data are given in Table III.

TABLE III

DEPENDENCE OF INITIAL RATE OF HYDRATION OF FUMARATE AT 25° AND pH 6.50 ON FUMARATE AND PHOSPHATE CONCENTRATIONS

(F), mM	Relative rates[a] 5 mM phos. Expt.	Eq. 1	Eq. 3	15 mM phos. Expt.	Eq. 1	Eq. 3	33.3 mM phos. Expt.	Eq. 1	Eq. 3	50 mM phos. Expt.	Eq. 1	Eq. 3
0.033							0.79	0.79	0.82	0.60	0.55	0.56
.100	4.4	4.2	4.2	2.7	2.7	2.8	2.20	2.16	2.25	1.48	1.57	1.60
.333	6.7	6.9	7.0	8.3	6.4	6.6	5.4	5.4	5.7	4.4	4.5	4.4
1.00	9.0	8.5	9.0	11.5	10.5	11.0	9.8	9.5	10.4	9.3	9.3	9.5
3.33	10.9	9.2	10.8	14.9	13.5	15.2	15.4	13.0	15.5	14.8	15.0	15.8
10.0	12.8	9.4	13.4	18.8	14.7	18.5	20.2	14.5	19.8	20.2	18.2	20.1
33.3	16.4	9.5	17.3	21.7	15.2	21.9	24.0	15.2	24.2	22.9	19.6	23.0
100	8.3	9.5	20.4	9.8	15.3	24.0	10.6	15.3	26.9	13.2	20.0	24.4

[a] The enzyme concentration used here is different than for Table II.

In the range below 1 mM fumarate the data may be represented quite well by equation 1 as shown in Table III. However, at higher fumarate concentrations the initial velocities are greater than expected from the kinetic constants obtained at low substrate concentrations. The curvature of the Lineweaver–Burk plots of the data for the range 1–33 mM fumarate is shown in Fig. 3. This activation is believed to result from the binding of substrate at sites on the enzyme molecule which have an effect upon the enzymatic sites. If the inhibition at 0.1 M fumarate is ignored, the simplest type of empirical equation which may be used to represent the variation of initial rate with substrate concentration is

$$v = \frac{V + a/(S)}{1 + b/(S) + c/(S)^2} \qquad (2)$$

where V, a, b and c are constants for a particular buffer, pH and temperature and V and a are directly proportional to enzyme concentration. In their investigations of the mechanism of action of urease, Kistiakowsky and Rosenberg[11] found deviations from the Michaelis–Menten equation which may also be represented by equation 2. They have pointed out that this type of rate equation results if there are two types of enzymatic sites which are either different and independent or which are identical but interact in pairs. In view of the activation of fumarase by various ions, including phosphate, activation by substrate itself appears to

(11) G. B. Kistiakowsky and A. J. Rosenberg, THIS JOURNAL, **74**, 5020 (1952).

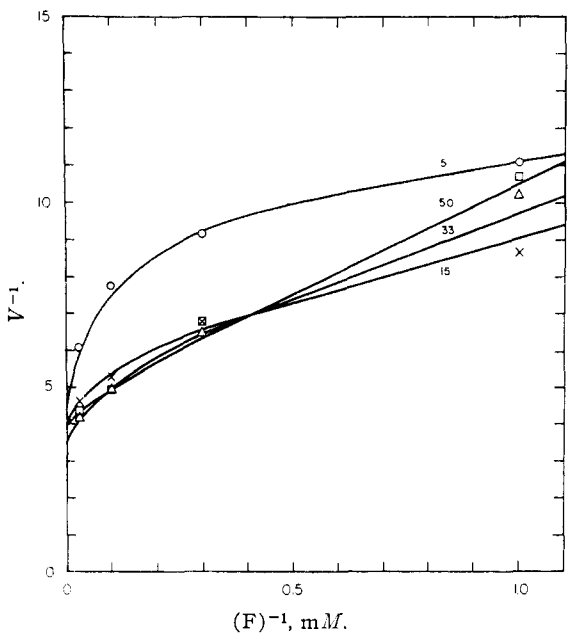

Fig. 3.—Plot of reciprocal initial velocity *vs.* reciprocal fumarate concentration (at *p*H 6.5 and 25° for the indicated phosphate concentrations (mM)). The curves have been calculated from equation 3 using the values of the kinetic constants given in Table IV.

be responsible for curvature in the present case. Mechanisms allowing for substrate activation[5] also yield rate equation 2.

It is characteristic of equation 2 that linear Lineweaver–Burk plots with different slopes are approached at very high and very low substrate concentrations. Thus the data may be characterized by four constants of the Michaelis type, V_1 and K_1 at low substrate concentrations and V_2 and K_2 at high substrate concentrations. Equation 2 may be expressed in terms of these constants by

$$v = \frac{V_2 + V_1K_2/(1 - V_1/V_2)(S)}{1 + K_2/(1 - V_1/V_2)(S) + K_1K_2/(1 - V_1/V_2)(S)^2} \quad (3)$$

As (S) → 0, equation 3 becomes $v = V_1/(1 + K_1/(S))$, while as (S) → ∞, it becomes $v = V_2/(1 + K_2/(S))$. The curves in Fig. 3 have been calculated from equation 3 using the values of the constants given in Table IV.

TABLE IV

KINETIC CONSTANTS FOR THE INITIAL RATE OF HYDRATION OF FUMARATE AT *p*H 6.5 AND 25°

Phos. concn., mM	5	15	33.3	50
V_1	9.5	15.4	15.4	20.3
K_1, mM	0.13	0.47	0.62	1.19
V_2	23	25	29	25
K_2, mM	14.0	6.6	7.3	3.9

Values of v calculated from equation 3 are also given in Table III to show that this equation satisfactorily represents the data if the substrate inhibition at 100 mM is ignored. It is interesting that V_2 is independent of phosphate concentration within the accuracy of these rather limited data. This is in accord with the idea of substrate activation since at (S) = ∞ buffer will be completely displaced from the activating sites, and so the rate would be expected to be independent of buffer concentration.

The constants V_1 and K_1 are considered to be characteristic of the enzyme which is in equilibrium with the buffer and does not have substrate bound at activating sites. The argument for this is simply based on the fact that no further deviations from linearity are encountered at the lowest substrate concentrations at which experiments are feasible with our present techniques. When the terms Michaelis constant and maximum initial velocity are used later in this article they apply to values obtained from the linear sections of Lineweaver–Burk plots at low substrate concentrations (K_1 and V_1).

Variation of Michaelis Constants and Maximum Initial Velocities with Phosphate Concentration.—The values of V_1 for fumarate increase with phosphate concentration to a maximum value as illustrated in Fig. 4. The experimental results may be represented by an empirical equation of the type

$$V = \frac{V_0K_B + V_B(B)}{K_B + (B)} \quad (4)$$

where V_0 is the maximum initial velocity in the absence of buffer and V_B is the value approached at high phosphate concentrations. Since K_B is equal to the buffer concentration at which $V = (V_0 + V_B)/2$, it will be referred to as the Michaelis constant for the buffer. The values of K_B for fumarate experiments as obtained from plots of $1/(V - V_0)$ *vs.* $1/(B)$ fall in the range 20–30 mM from *p*H 6–8.5.

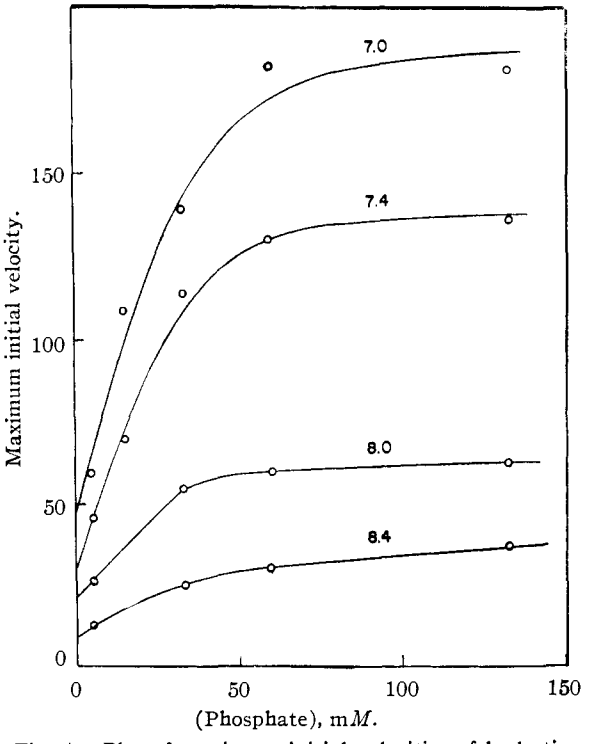

Fig. 4.—Plot of maximum initial velocities of hydration of fumarate *vs.* the concentration of sodium phosphate buffer for four *p*H values.

In the case of l-malate the maximum initial velocity (V_M) is independent of phosphate concentration above 5 mM at pH 6, 6.5 and 7. Thus at these pH values, $K_B < 5$ mM, as may be shown by use of lower phosphate concentrations. The fact that V_M is independent of phosphate concentration from 5 to 133 mM within our experimental error makes it unnecessary to consider ionic strength effects. Massey[2] has shown that the effects of various salts on the maximum initial velocity are quite specific since the pH optimum is shifted to different extents by different salts. It is important to note that the decrease in initial velocity at most finite substrate concentrations at high phosphate concentrations previously noted[2] and found throughout this study is not an ionic strength effect but results from the fact that the Michaelis constant continues to increase with increasing phosphate concentration beyond the concentration at which V_1 remains constant. As would be expected it is found that this inhibitory effect of phosphate increases as the substrate concentration is decreased; that is, besides being an activator phosphate also acts as a competitive inhibitor (*cf.* Figs. 2 and 3).

At pH values above 7–7.5, V_M increases with the phosphate concentration, and K_B is about 30 mM at pH 8 to 8.5. The fact that different values for K_B are obtained for the two substrates at lower pH values indicates that K_B cannot be interpreted as a simple dissociation constant for an enzyme–buffer complex. A theoretical basis for equation 4 is provided by the mechanism discussed later which shows why K_B may be expected to be different for the forward and reverse reactions.

The Michaelis constants for both substrates below pH 8 are directly proportional to the phosphate buffer concentration over the range 5 to 133 mM as illustrated in Fig. 5. Above this pH value there is some downward curvature. This is in marked contrast to the variation of V_1 with phosphate concentration. The fact that the plots of K_1 *vs.* phosphate concentration extrapolate to Michaelis constants in the range below 0.1 mM at zero phosphate concentration shows that the affinity of fumarate and l-malate is much greater than indicated by the values of Michaelis constants in phosphate buffers. Accurate values of K_1 in the absence of buffer cannot be obtained by extrapolation of values above 5 mM phosphate. This indication that the Michaelis constants in the absence of phosphate are so low is in agreement with the fact that the Michaelis constants in tris-(hydroxymethyl)-aminomethane chloride buffer of pH 7 are extremely low, too low to be measured accurately by the technique described in this paper.

Variation of Michaelis Constants with pH.—The pH variation of the kinetic constants may be attributed to ionization of the enzyme, enzyme–substrate complexes, enzyme–buffer complexes, substrate and buffer. In the pH range investigated the effect of ionization of the substrates is undoubtedly quite negligible since the pK values

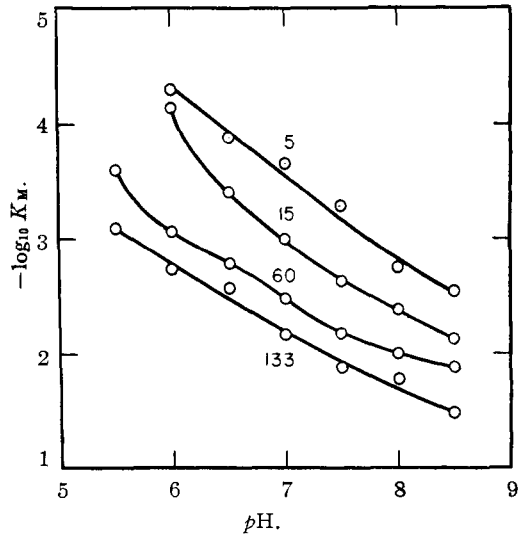

Fig. 6.—Plot of $-\log K_M$ *vs.* pH at 25° for four sodium phosphate concentrations (mM).

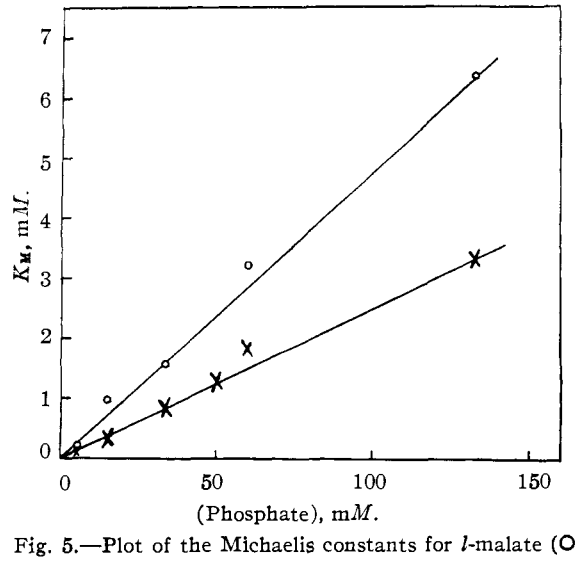

Fig. 5.—Plot of the Michaelis constants for l-malate (O) and fumarate (×) *vs.* the concentration of sodium phosphate buffer at pH 7.0.

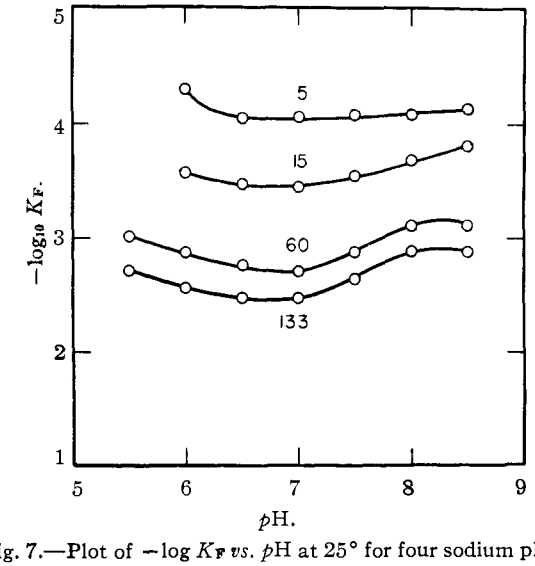

Fig. 7.—Plot of $-\log K_F$ *vs.* pH at 25° for four sodium phosphate buffer concentrations (mM).

for the second dissociations of fumarate and *l*-malate at 0.1 ionic strength and 25° are 4.18 and 4.73,[7] respectively. The variation of the Michaelis constants with *p*H is shown in Fig. 6 for *l*-malate and Fig. 7 for fumarate. Following the suggestion of Dixon[12] the data are presented as plots of $-\log K_1$ *vs.* *p*H. Dixon has discussed the use of such plots in the calculation of ionization constants of groups in the enzyme and enzyme–substrate complex. In Figs. 6 and 7 there are no regions in which the slopes are unity as would be expected from Dixon's theory if the pK's for ionizable groups were well separated. In the case of fumarase in phosphate buffer a more complicated theory is required since phosphate acts as an inhibitor, and the situation is further complicated by the fact that the ratio of the two forms of phosphate changes with *p*H. The fact that experiments with sodium, potassium and ammonium salts yield identical results indicate that it is the binding of $H_2PO_4^-$ and $HPO_4^=$ which affects the properties of the enzyme. In general, it must be expected that the effect of bound $H_2PO_4^-$ will be different from bound $HPO_4^=$ so that different ions may be responsible for the activating and inhibiting effects at high and low *p*H values. Thus a buffer utilizing tris-(hydroxymethyl)-aminomethane has the advantage over phosphate that the concentration of a single anion may be held constant over a wide range of *p*H.

In order to provide for the effect of buffer upon the variation of kinetic constants with *p*H, mechanisms involving both hydrogen ions and buffer ions have been considered and are discussed in the following paper.[13]

Variation of Maximum Initial Velocities with *p*H.—In the case of fumarase the interpretation of the *p*H variation of V_1 is simpler than the interpretation of K_1, since V_1 is independent of phosphate concentration at high concentrations. Plots of maximum initial velocity *vs.* *p*H are quite symmetrical for both substrates as illustrated in Fig. 8. Michaelis and Davidsohn[14] and Michaelis and Pechstein[15] suggested that such reversible loss of catalytic activity may result from ionization of acidic and basic groups of the enzyme. The mathematical formulation of their hypothesis leads[16] to the equation

$$V = \frac{V_{max}(1 + 2\sqrt{K_b/K_a})}{1 + (H^+)/K_a + K_b/(H^+)} \qquad (5)$$

where V_{max} is the maximum initial velocity at the optimum *p*H, and K_a and K_b are acid dissociation constants for groups in the enzyme molecule when it is saturated with substrate. The curves in Fig. 8 have been drawn according to equation 5 with values of K_a and K_b determined from the data by the method described earlier.[16]

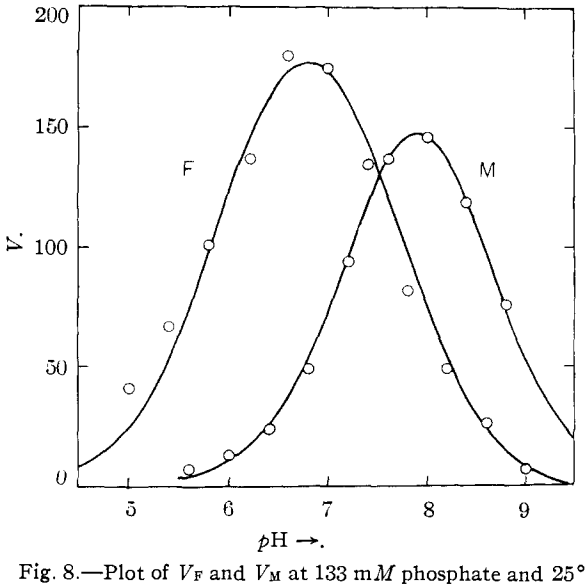

Fig. 8.—Plot of V_F and V_M at 133 mM phosphate and 25° *vs.* *p*H. The solid curves are calculated from equation 5 using pK_a 5.9 and pK_b 7.7 for fumarate and pK_a 7.3 and pK_b 8.5 for *l*-malate.

The interpretation of the variation of maximum initial velocity with *p*H for fumarase in phosphate buffers has been discussed by Massey and Alberty.[17] The pK_a for enzyme saturated with *l*-malate is about 7.3 independent of phosphate concentration above 5 mM which is considerably higher than the corresponding value of about 5.8 for fumarate which is also independent of phosphate concentration. The pK_b for enzyme saturated with *l*-malate is about 8.5 independent of phosphate concentration above 5 mM, while the corresponding value for fumarate increases from 7.3 to 7.7 as the phosphate concentration is increased from 5 to 133 mM. Thus the two substrates have quite different effects upon the ionization of essential groups of the enzyme.

Test of the Haldane Relation.—The relationship between the two Michaelis constants, two maximum initial velocities and the over-all equilibrium constant for a simple reaction F $\rightleftarrows$ M which is catalyzed by an enzyme was derived by Haldane[6] and is given by equation 6.

$$K_{eq} = \frac{(M)_{eq}}{(F)_{eq}} = \frac{V_F K_M}{V_M K_F} \qquad (6)$$

This relation has been found to be obeyed by the fumarase-catalyzed reaction in 0.05 M phosphate buffer of *p*H 7.3 at 25°.[7] One of the possible corresponding relations for a reaction involving a coenzyme[18] has been tested by Kornberg[19] for the reaction: nicotinamide mononucleotide + ATP $\rightleftarrows$ DPN + inorganic pyrophosphate. In discussing the agreement between the calculated value of the equilibrium constant of 0.12 and the experimental values which range from 0.27 to 0.61, Kornberg pointed out that such a discrepancy may result from rather small errors in experimental values and that a simpler reaction such as that catalyzed by fumarase offers a more favorable opportunity for testing equation 6.

Since the derivation of equation 6 makes no provision for the variation of Michaelis constants and maximum initial velocities with buffer concentration or *p*H it is apparent that the derivation

(12) M. Dixon, *Biochem. J.*, **55**, 161 (1953).
(13) R. A. Alberty, THIS JOURNAL, **76**, 2494 (1954).
(14) L. Michaelis and H. Davidsohn, *Biochem. Z.*, **35**, 386 (1911).
(15) L. Michaelis and H. Pechstein, *ibid.*, **59**, 77 (1914).
(16) R. A. Alberty and V. Massey, *Biochem. Biophys. Acta*, in press.
(17) V. Massey and R. A. Alberty, *ibid.*, in press.
(18) R. A. Alberty, THIS JOURNAL, **75**, 1928 (1953).
(19) A. Kornberg, *J. Biol. Chem.*, **182**, 779 (1950).

TABLE V

RATIOS OF MAXIMUM INITIAL VELOCITIES (V_F/V_M), MICHAELIS CONSTANTS (K_M AND K_F IN mM) AND EQUILIBRIUM CONSTANTS CALCULATED WITH THE HALDANE RELATIONSHIP AT SIX CONCENTRATIONS OF SODIUM PHOSPHATE BUFFERS AT 25°

	5 mM				15 mM				33.3 mM			
pH	V_F/V_M	K_M	K_F	K_{eq}	V_F/V_M	K_M	K_F	K_{eq}	V_F/V_M	K_M	K_F	K_{eq}
5.5									10.0	0.21	0.21	10.0
6.0	4.65	0.05	0.050	4.7	8.3	0.072	0.27	2.3	7.85	.40	.53	6.0
6.5	3.10	.13	.090	4.5	4.1	0.40	.33	4.9	4.67	.77	.72	5.0
7.0	1.30	.22	.085	3.4	1.6	1.00	.35	4.6	2.28	1.59	.87	4.2
7.5	0.55	.52	.082	3.5	0.57	2.33	.28	4.8	1.05	3.03	.74	4.3
8.0	.21	1.8	.082	4.6	.266	4.1	.21	5.2	0.425	6.25	.54	4.9
8.5	.08	2.8	.072	3.1	.114	7.4	.15	5.6	0.16	12.5	.50	4.0
	50 mM				60 mM				133 mM			
5.5					16.6	0.25	0.98	4.3	12.7	1.24	1.90	8.3
6.0					9.75	0.87	1.35	6.3	9.2	1.82	2.70	6.2
6.5	4.7	0.94	1.10	4.8	5.65	1.64	1.70	5.4	5.8	2.67	3.33	4.7
7.0	2.7	1.50	1.21	3.4	2.72	3.33	1.96	4.6	2.38	6.7	3.33	4.8
7.5	0.95	2.82	0.89	3.0	0.95	6.7	1.32	4.8	0.92	11.8	2.22	4.9
8.0	0.52	4.96	0.72	3.6	.41	10	0.75	5.4	.425	16.7	1.28	5.5
8.5					.25	15	0.75	5.0	.285	33	1.28	7.3

represents a considerable oversimplification of the actual situation. It is, therefore, especially interesting to find experimentally that equation 6 is obeyed over a wide range of pH and buffer concentration provided the kinetic constants are calculated from the linear portions of the Lineweaver–Burk plots at low substrate concentrations. Values of the equilibrium constant for the over-all reaction calculated from the kinetic data using equation 6 are given in Table V. The average of all these values is $K_{eq} = 4.7$ with a standard deviation of 1.4. Since the data at pH 6.0 and 5.5 are more erratic the average also has been computed after excluding these data, and 4.4 ± 0.9 is obtained. Although the directly determined value for the equilibrium constant varies with the phosphate concentration the average value calculated from the kinetic data may be compared with the value at 50 mM phosphate which is $K_{eq} = 4.5$.[7] The large standard deviation for the values of K_{eq} calculated from the Haldane relation results from the accumulation of errors in the determination of the four kinetic constants.

Since there is evidently negligible activating effect by substrate at sufficiently low substrate concentrations so that the Lineweaver–Burk plots are linear, the maximum initial velocities and Michaelis constants obtained in this region are characteristic of the enzyme activated only by buffer. It is apparent from the discussion of mechanisms which follows why the Haldane relation is not obeyed by the kinetic constants determined at high substrate concentrations where there is substrate activation. At high substrate concentrations the enzyme is partially, or completely, activated by substrate and so the kinetic properties of the enzyme are different when the forward and reverse reactions are studied.

According to the derivation the Haldane relation yields the apparent equilibrium constant at the pH and salt concentration used. Although the variation of K_{eq} with phosphate concentration at constant pH is too small to be detected by $V_F K_M/V_M K_F$, the variation of K_{eq} with pH is sufficiently large so that it may be demonstrated.[7] Krebs[20] has recently discussed the calculation of the pH variation of the apparent equilibrium constant for the fumarase reaction. Table VI gives the kinetic constants at two low pH values in phosphate buffers. It is seen that the Haldane relation yields values of K_{eq} in agreement with the theoretical values at these pH values. The indicated range in the theoretical K_{eq} is due to the uncertainty (±0.05 in pK) in the second ionization constants of fumaric and l-malic acids.[7] The indicated uncertainty in the equilibrium constant calculated from the Haldane relation would result from a 5% error in each of the four kinetic constants.

TABLE VI

KINETIC CONSTANTS FOR FUMARASE IN 0.05 M SODIUM PHOSPHATE BUFFERS AT 25°

pH	V_F/V_M	K_F, mM	K_M, mM	K_{eq} Haldane	K_{eq} Theoret.
5.25	15.8	0.43	0.13	4.8 ± 1.0	5.1- 5.6
4.40	15.0	0.37	0.31	12.5 ± 2.7	7.4–10.2

Mechanism

If in addition to the basic Michaelis–Menten mechanism for the reaction of enzyme, E, and substrate, S, to yield product, P

$$E + S \underset{}{\overset{K_{ES}}{\rightleftharpoons}} ES \xrightarrow{k_1} E + P \qquad (I)$$

a component, B, of the buffer (other than hydrogen or hydroxyl ions) competes with substrate for the enzymatic site

$$E + B \overset{K_{EB}}{\rightleftharpoons} EB \qquad (II)$$

and is also bound at another site at which it affects the kinetic constants

$$E + B \overset{K_{BE}}{\rightleftharpoons} BE \qquad (III)$$

the following reactions may be important.

$$BE + S \overset{K_{BES}}{\rightleftharpoons} BES \xrightarrow{k_2} BE + P \qquad (IV)$$

$$BE + B \overset{K_{BEB}}{\rightleftharpoons} BEB \qquad (V)$$

The substance written at the right of E in a complex is bound at the enzymatic site while the substance

(20) H. A. Krebs, *Biochem. J.*, **54**, 78 (1953).

written to the left is bound at a neighboring site at which it has an effect on the enzymatic site. If the substrate competes with B for the non-enzymatic site the following steps also must be included.

$$E + S \overset{K_{SE}}{\rightleftharpoons} SE \qquad (VI)$$

$$SE + S \overset{K_{SES}}{\rightleftharpoons} SES \overset{k_3}{\longrightarrow} SE + P \qquad (VII)$$

$$SE + B \overset{K_{SEB}}{\rightleftharpoons} SEB \qquad (VIII)$$

It is unnecessary to include steps such as ES + B $\rightleftarrows$ BES since they are not independent of the above equilibria. The mechanism represented by reactions (I)–(VII) has previously been treated by Alberty and Bock[5] using the rapid-equilibrium method. The form of the rate equation obtained by the steady-state method is the same, but the constants (K) for complexes which dissociate to yield product are not equilibrium constants but of the type derived by Briggs and Haldane.[4] If S >> $(E)_0$ and (B) >> $(E)_0$, where (E_0) is the total molar concentration of enzymatic sites, the assumption that $d(ES)/dt = d(BES)/dt = d(SES)/dt = 0$ for the mechanism (I)–(VIII) leads to equation 3 for the initial velocity with

$$V_1 = \frac{k_1(E)_0}{1 + K_{ES}/K_{SE} + K_{ES}(B)(1/K_{BE}K_{BES} + 1/K_{SE}K_{SEB})} \qquad (7)$$

$$K_1 = K_{ES}\,\frac{1 + (B)(1/K_{BE} + 1/K_{EB}) + (B)^2/K_{BE}K_{BEB}}{1 + K_{ES}/K_{SE} + K_{ES}(B)(1/K_{BE}K_{BES} + 1/K_{SE}K_{SES})} \qquad (8)$$

$$V_2 = k_3(E)_0 \qquad (9)$$

$$K_2 = K_{SE}K_{SES}\{(1 - k_1/k_3)/K_{ES} + 1/K_{SE} + (B)[(1 - k_2/k_3)/K_{BE}K_{BES} + 1/K_{SE}K_{SEB}]\} \qquad (10)$$

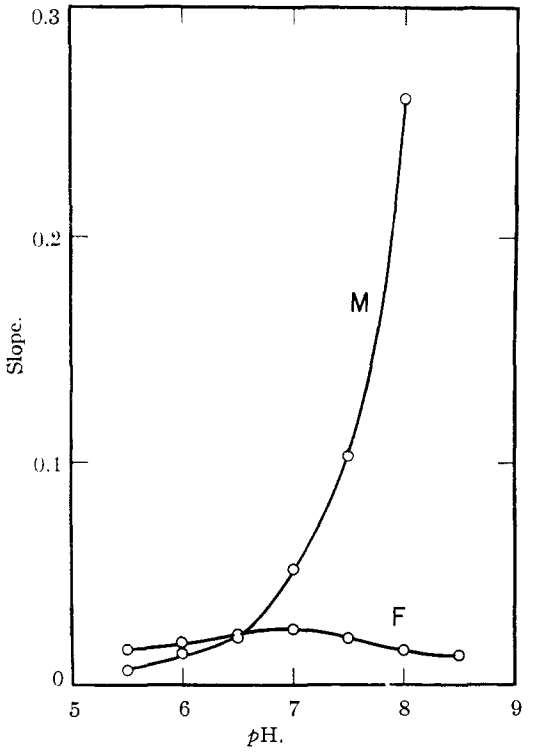

Fig. 9.—Initial slopes of plots of K_F and K_M *vs.* phosphate concentration at 25°.

where K_{EB}, K_{BE}, K_{BEB}, K_{SE} and K_{SEB} are dissociation constants for the equilibria II, III, V, VI, and VIII while K_{ES}, K_{BES} and K_{SES} are Michaelis constants of the Briggs–Haldane type. Thus this mechanism leads to a rate equation of the exact form found to represent the data for fumarase.

Since the Michaelis constants and maximum initial velocities reported in this paper have been computed from the linear regions of Lineweaver–Burk plots at low substrate concentrations, the variation of these kinetic constants with buffer concentration should be expressed by equations 7 and 8. Equation 7 has the same form as the empirical relation 4 found to represent V_1 as a function of phosphate concentration. The empirical constants of equation 4 are related to the equilibrium constants and rate constants of mechanism (I)–(VIII) by

$$V_0 = \frac{k_1(E)_0}{1 + K_{ES}/K_{SE}} \qquad (11)$$

$$V_B = \frac{k_2(E)_0}{1 + K_{BE}K_{BES}/K_{SE}K_{SEB}} \qquad (12)$$

$$K_B = \frac{1/K_{ES} + 1/K_{SE}}{1/K_{SE}K_{SEB} + 1/K_{BE}K_{BES}} \qquad (13)$$

Since the expression for K_B includes equilibrium constants involving substrate, it is not surprising that K_B has different values for fumarate and *l*-malate. According to mechanism (I)–(VIII), V_2 is independent of buffer concentration because at infinite substrate concentration B is displaced from both the enzymatic and neighboring sites by S, and this is borne out by the rather limited data on fumarate (Fig. 3).

The fact that K_1 is found to increase with phosphate concentration over the whole *p*H range investigated is in agreement with equation 8. If it were not for the $(B)^2$ term in the numerator, resulting from step V in the mechanism, the variation of K_1 with phosphate concentration would closely resemble the variation of V_1. It is found that the values of K_1 for fumarate and *l*-malate are linear functions of phosphate concentration, except in the region *p*H 8–8.5 where the plots are concave downward for both substrates. The initial slopes of the plots of K_F and K_M *vs.* phosphate concentration are given in Fig. 9 as a function of *p*H to show the different behavior of the two substrates.

According to equation 10 the value of K_2 is expected to be a linear function of buffer concentration, and the fact that K_2 for fumarate decreases with increasing phosphate concentration at *p*H 6.5 indicates that k_2 is enough larger than k_3 to make the coefficient of (B) negative.

Derivation of the Haldane Relation for the Case that Buffer Affects the Kinetics.—In order to allow for the reverse reaction, steps I and IV of the mechanism are rewritten

$$E + F \overset{K_{EF}}{\rightleftharpoons} EF \underset{k_{1M}}{\overset{k_{1F}}{\rightleftharpoons}} EM \overset{K_{EM}}{\rightleftharpoons} E + M \qquad (I')$$

$$BE + F \overset{K_{BEF}}{\rightleftharpoons} BEF \underset{k_{2M}}{\overset{k_{2F}}{\rightleftharpoons}} BEM \overset{K_{BEM}}{\rightleftharpoons} BE + M \qquad (IV')$$

If the expressions for V_1 and K_1 (with F or M sub-

stituted for S) are substituted for V_F, V_M, K_F and K_M in equation 6

$$K_{eq} = \frac{V_F K_M}{V_M K_F} = \frac{k_{1F}K_{EM}(1 + k_{2F}K_{EF}(B)/k_{1F}K_{BE}K_{BEF})}{k_{1M}K_{EM}(1 + k_{2M}K_{EM}(B)/k_{1M}K_{BE}K_{BEM})} \quad (14)$$

It is obvious that equation 14 is equal to the ratio of the equilibrium concentrations of M and F for the cases that (B) = 0 and (B) = ∞ since

$$K_{eq} = \frac{(M)_{eq}}{(F)_{eq}} = \frac{k_{1F}K_{EM}}{k_{1M}K_{EF}} = \frac{k_{2F}K_{BEM}}{k_{2M}K_{BEF}} \quad (15)$$

but it may also be seen that equation 14 should give the equilibrium constant at *any* value of buffer concentration since the coefficients of the (B) terms in the numerator and denominator of 14 are identical according to 15.

On the other hand, it is not possible to relate the kinetic constants at high substrate concentration (K_2 and V_2) with the equilibrium constant for the over-all reaction. If there is substrate activation the kinetic constants obtained with one substrate will be characteristic of enzyme activated by that substrate, while the kinetic constants for the reverse reaction will be characteristic of the enzyme activated by the other substrate. When this is the case there will be no simple connection between the kinetic constants for the forward and reverse reactions.

Appendix

In determining Michaelis constants it is important to obtain truly *initial* rates since the derivations of rate equations are all based upon this assumption. The following derivation shows the conditions under which this may be accomplished in the case of a reversible reaction like that catalyzed by fumarase. The greatest difficulty is encountered at the lowest substrate concentrations where the reaction is nearly first order. Under these conditions the over-all reaction may be represented by the reversible first-order reaction

$$F \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M \quad (16)$$

For this case the differential equation is

$$dx/dt = k_1(c_0 - x) - k_2x = k_1[c_0 - (1 + K_{eq}^{-1})x] \quad (17)$$

where c_0 is the initial concentration of F, x is the concentration of M at any time and $K_{eq} = k_1/k_2$. Integration yields the concentration of M.

$$x = c_0[1 - e^{-k_1t(1 + K_{eq}^{-1})}][1 + K^{-1}{}_{eq}]^{-1} \quad (18)$$

For low extents of reaction the initial velocity may be calculated from x/t, whereas the true initial velocity is $dx/dt = k_1c_0$. In order that the initial velocity calculated from x/t be within 5% of the true initial velocity, it is necessary for the ratio x/k_1c_0t be not less than 0.95. Introduction of equation 18 yields

$$0.95 < \frac{1 - e^{-k_1t(1 + K_{eq}^{-1})}}{k_1t(1 + K_{eq}^{-1})} \quad (19)$$

The value of k_1t for which the ratio is 0.95 is 0.10 when fumarate is the substrate (*i.e.*, $K_{eq} = 4.5$). Substitution of $k_1t = 0.10$ into equation 18 leads to the conclusion that the extent of reaction must not be allowed to exceed 7.8% if it is desired to obtain the initial velocity with an error of less than 5% by a zero-order calculation. When *l*-malate is the substrate (*i.e.*, $K_{eq} = 1/4.5$) a similar calculation shows that only the first 1.7% of the reaction may be used because of the unfavorable equilibrium.

TABLE VII

INITIAL CONCENTRATIONS OF FUMARATE AND *l*-MALATE FOR WHICH 7.8% AND 1.7% REACTION, RESPECTIVELY, CORRESPOND TO $\Delta D_\lambda = 0.1$

λ, mμ	(F), mM	(M), mM	mμ	(F), mM	(M), mM
205	0.077	0.35	260	1.4	6.5
210	.085	.39	270	2.4	11
220	.14	.63	280	4.6	21
230	.28	1.3	290	11	50
240	.53	2.4	300	30	136
250	.88	4.1	-		

The initial concentrations of fumarate and *l*-malate for which 7.8% and 1.7% reaction, respectively, correspond to changes in optical density of 0.1 at various wave lengths are given in Table VII. The full width of the 80–100% scale of the recorder represents an optical density of 0.097. The optical density for the concentration of fumarate at each wave length is 1.3, and so it may be seen that the fumarate concentrations given cannot be greatly exceeded because of the large light absorption. Since it is necessary to obtain a change of $\Delta D \times 10^3$ of about 20 in 20 seconds in order to obtain a satisfactory rate measurement with the present apparatus, and about 10 seconds are required for mixing, it may be seen that it is not possible to obtain satisfactory rate measurements at fumarate concentrations below about 0.02 mM or *l*-malate concentrations below 0.1 mM without employing longer cells.

The values given in Table VII apply with exactness only at such low substrate concentrations that the reaction is first order and larger extents of reaction may generally be used at concentrations in the neighborhood of the Michaelis constant or greater. However, since effects such as inhibition by product may cause the rate to drop off faster than it otherwise would,[21] it is desirable to use as small extent reaction as possible in determining the initial velocity.

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MADISON, WISCONSIN

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