Compounds with 3-electron bonds are most stable when the bound elements have comparable electronegativities, and they do not form easily with heavy elements where π-bonds are weaker. Probably NeF is the diatomic aerogen radical that will be formed in greatest concentration, and species like NeH, KrCl, XeF, etc., will be much harder to detect.

Proton Transfer Compounds

If a gaseous molecule is a Brönsted acid, it can transfer a proton to a basic species to form ions that condense to a crystalline solid similar to the electron transfer reaction forming $Xe^+PtF_6^-$. A familiar example is the reaction of the gaseous molecules HCl and NH_3 to form crystalline $NH_4^+Cl^-$.

Since the aerogens have significant proton affinities, they might in principle react

$$HX(g) + E(g) \longrightarrow EH^+X^-(c) \qquad (7)$$

If it is assumed that the lattice energy of crystalline EHX is the same as that of the isoelectronic alkali salt, it is possible to calculate the proton affinity of E necessary to make reaction 7 thermoneutral. Since considerable entropy is lost in this reaction, larger proton affinities are necessary if the reaction is to take place. These minimum proton affinities for formation of a halide salt are presented in Table II. The value quoted for He is that necessary to get HeHF. The values for the other elements are for iodide formation; values for bromide and chloride formation are the same within about 3 kcal./mole, and values for fluoride formation are somewhat higher.

TABLE II

MINIMUM PROTON AFFINITIES (IN KCAL./MOLE) FOR IONIC LATTICE FORMATION WITH A HYDROGEN HALIDE

He	Ne	Ar	Kr	Xe
122	144	159	164	170

The proton affinity of helium is calculable with moderate confidence from quantum mechanics. Evett[15] assigns a value between 40 and 47 kcal./mole. It is clear that helium will not react with a hydrogen halide to form a crystalline salt.

The proton affinities of the other aerogens are not known. Stevenson and Schissler[16] obsreved that neon, argon, and krypton positive ions all reacted without activation energy by the process

$$E^+ + H_2 \longrightarrow EH^+ + H \qquad (8)$$

Since the process

$$H_2 \longrightarrow H + H^+ + e^- \qquad (9)$$

requires 419 kcal./mole, this observation and the data in Table I indicate that the proton affinity of krypton is at least 96 kcal./mole and may of course be much more. Apparently the proton affinities of the heavier aerogens are greater than that of helium, and xenon and radon are the most apt to undergo reactions like eq. 7.

Even if hydrogen halides do not react to form stable solids, heavy aerogens may react with stronger Brönsted acids. The proton affinity of gaseous perchlorate ion is undoubtedly less than that of any halide, but perchlorate lattice energies are also less than those of halides.

Since ionic lattice energies can be calculated with moderate confidence, measurements of gaseous proton affinities would permit very reliable predictions as to which systems, if any, could produce crystalline EHX compounds. The data presently available are not very encouraging for their preparation.

Acknowledgment.—The preparation of this manuscript was supported in part by the Army Research Office under Grant DA-ARO(D)-31-124-G333. I am indebted to Professor Nancy P. Helmbold of the University of Oregon for suggesting the term aerogen.

(15) A. A. Evett, *J. Chem. Phys.*, **24**, 150 (1956).

(16) D. P. Stevenson and D. O. Schissler, *ibid.*, **23**, 1353 (1955); **24**, 926 (1956).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

The Temperature Dependence of the Steady State Kinetic Parameters of the Fumarase Reaction[1]

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The steady state kinetics of the hydration of fumarate and dehydration of L-malate by pig heart fumarase have been investigated from pH 5.5 to 8.5 and from 5 to 37° in tris-(hydroxymethyl)-aminomethane–acetic acid buffers of 0.01 ionic strength. The pH dependences of the Michaelis constants and maximum initial velocities can be represented by the equations used earlier which contain ten pH-independent kinetic parameters. The temperature dependence has been determined for each of these parameters. The results are discussed in terms of a general mechanism. The apparent activation energies for the lower limits to the bimolecular rate constants for combination of enzyme and substrate are both approximately 6 kcal. mole^{-1}, which is in accord with the fact that these lower limits approach the theoretical values for diffusion-controlled reactions.

Introduction

The steady state kinetics of the reaction catalyzed by fumarase have been thoroughly studied in tris-(hydroxymethyl)-aminomethane–acetic acid buffers.[4,5] In these studies the pH dependences of the maximum initial velocities and Michaelis constants for fumarate and L-malate have been determined over the pH range from about 5.0 to 8.5 and for a series of ionic strengths. From the effect of hydrogen ion concentration on these four kinetic parameters under given conditions of temperature and ionic strength a total of ten experimental parameters may be evaluated. In the present research the temperature dependence of each of these ten experimental parameters has been determined. An earlier study of the temperature dependence of fumarase kinetics was carried out by Massey[6] using phosphate buffers. Analysis of the effects of pH and temperature on fumarase kinetics cannot be made in as great detail

(1) Taken in part from the Ph.D. Thesis of D. A. Brant, University of Wisconsin, 1962. Presented in part at the 142nd National Meeting of the American Chemical Society, September 9–14, 1962, Atlantic City, N. J.

(2) National Science Foundation Predoctoral Fellow, 1958–1962.

(3) National Science Foundation Postdoctoral Fellow, 1959–1960.

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

(5) C. Frieden, R. G. Wolfe, Jr., and R. A. Alberty, *J. Am. Chem. Soc.*, **79**, 1523 (1957).

(6) V. Massey, *Biochem. J.*, **53**, 72 (1953).

in phosphate buffers as in TRIS[7]–acetate buffers because of the activation and inhibition due to the singly and doubly charged phosphate ions.[8]

Massey observed distinct curvature in Arrhenius plots of the maximum velocities and Michaelis constants for the fumarase reaction at several pH values. Curvature in Arrhenius plots of maximum velocities and Michaelis constants has been observed for many enzymes, and in some cases questionable interpretations of such curvature have been offered. A critical discussion of the interpretation of the temperature dependence of maximum velocities and Michaelis constants has been given by Kistiakowsky and Lumry,[9] and this field has been surveyed by Dixon and Webb.[10]

Under given conditions of pH, temperature, and ionic strength and at sufficiently low substrate concentrations so that activation or inhibition by substrate is avoided, the steady state velocity, v, for the fumarase reaction is given by

$$v = \frac{\frac{V_F}{K_F}(F) - \frac{V_M}{K_M}(M)}{1 + \frac{(F)}{K_F} + \frac{(M)}{K_M}} \quad (1)$$

where (F) and (M) represent the total concentrations of fumarate and L-malate ions. The maximum initial velocities of the forward and reverse reactions are represented by V_F and V_M while K_F and K_M are the Michaelis constants for fumarate and malate. In TRIS–acetate buffers in the pH range from 5.0 to 8.5 the pH dependences of the parameters of equation 1 are given by

$$V_F = \frac{V_F'}{1 + (H^+)/K_{aEF}' + K_{bEF}'/(H^+)} \quad (2)$$

$$V_M = \frac{V_M'}{1 + (H^+)/K_{aEM}' + K_{bEM}'/(H^+)} \quad (3)$$

$$K_F = \frac{K_F'[1 + (H^+)/K_{0a}' + K_{0b}'/(H^+)][1 + (H^+)/K_{HF}]}{1 + (H^+)/K_{aEF}' + K_{bEF}'/(H^+)} \quad (4)$$

$$K_M = \frac{K_M'[1 + (H^+)/K_{0a}' + K_{0b}'/(H^+)][1 + (H^+)/K_{HM}]}{1 + (H^+)/K_{aEM}' + K_{bEM}'/(H^+)} \quad (5)$$

The quantities V_F', V_M', K_F', and K_M' are the pH-independent maximum velocities and Michaelis constants. The pH dependences are determined by the parameters K_{0a}', K_{0b}', K_{aEF}', K_{bEF}', and K_{aEM}' and K_{bEM}' and by the second acid dissociation constants of fumaric acid, K_{HF}, and L-malic acid, K_{HM}. The factors $1 + (H^+)/K_{HF}$ and $1 + (H^+)/K_{HM}$ in eq. 4 and 5 arise[4] if $F^=$ and $M^=$ are substrates and HF^- and HM^- are not. These factors are required to give the equilibrium constant, written in terms of total concentrations, the correct pH dependence. As can be seen from eq. 2 and 3 both V_F and V_M exhibit bell-shaped dependences upon pH, but the maxima do not in general occur at the same pH. The quantities

$$V_F[1 + (H^+)/K_{HF}]/K_F \text{ and } V_M[1 + (H^+)/K_{HM}]/K_M$$

also exhibit bell-shaped dependences upon pH, and in this case the maxima do occur at the same pH, as can be seen from eq. 6 and 7.

$$\frac{V_F}{K_F}\left[1 + \frac{(H^+)}{K_{HF}}\right] = \frac{V_F'/K_F'}{1 + (H^+)/K_{0a}' + K_{0b}'/(H^+)} \quad (6)$$

$$\frac{V_M}{K_M}\left[1 + \frac{(H^+)}{K_{HM}}\right] = \frac{V_M'/K_M'}{1 + (H^+)/K_{0a}' + K_{0b}'/(H^+)} \quad (7)$$

Setting $v = 0$ in eq. 1 yields

$$K_{eq} = (M)_{eq}/(F)_{eq} = V_F K_M/V_M K_F$$

Insertion of eq. 2–5 yields

$$K_{eq} = \frac{V_F'K_M'[1 + (H^+)/K_{HM}]}{V_M'K_F'[1 + (H^+)/K_{HF}]} \quad (8)$$

which shows the correct pH dependence for the pH range investigated. Thus V_F', K_F', V_M', and K_M' are not independent, but are related through the equilibrium constant. Determination of V_F, V_M, K_F and K_M as functions of pH under given conditions of temperature and ionic strength serves to determine the ten parameters of eq. 2–5, and the temperature dependence of the ten parameters is obtained from similar experiments at a series of temperatures.

Experimental

Preparation of Enzyme.—Crystalline fumarase was prepared from extracts of pig hearts by the method developed in this Laboratory.[11] The enzyme was stored at 0° as a suspension of crystals in 50% saturated $(NH_4)_2SO_4$ solution containing 0.01 M phosphate of pH 7.3. The enzyme solution used in the kinetic experiments was prepared by washing centrifuged crystals with cold water and dissolving the washed crystals in 0.01 M TRIS–acetate buffer at pH 7.7 and 0°. The concentration of the enzyme solution was adjusted to produce reaction velocities slow enough to permit easy observation of initial velocities at all substrate concentrations. A standard assay, run at frequent intervals during the course of a kinetic experiment, was used to correct for loss of enzyme activity during the experiment and to compare the enzyme concentrations used in different experiments. In the standard assay 0.5 ml. of enzyme solution was added to 25 ml. of 2×10^{-4} M malate at pH 7.7 at 25° in 0.01 M TRIS–acetate buffer, and the initial velocity was measured.

Preparation of Buffers.—Glacial acetic acid was distilled using a 10-in. Vigreux column from a 2% solution by weight of $KMnO_4$ in du Pont reagent grade glacial acetic acid.[12] The main fraction, which boiled in the range 114.9–115.6°, was collected and used in all experiments with malate as substrate. In the experiments with fumarate as substrate du Pont reagent grade glacial acetic acid was used without distilling. Tris-(hydroxymethyl)-aminomethane, supplied by Sigma Chemical Co. under the name Sigma 121, was used without further purification. All experiments with malate as substrate were done with material from lot number 41-B-181, and all fumarate experiments were done with material from lot number 70-B-180. The water used in making all solutions was obtained by distillation from a solution of alkaline permanganate and had a specific conductance of 10^{-5} ohm^{-1} cm.$^{-1}$ or less. All experiments were performed in TRIS–acetate buffers in which the acetate ion concentration (and consequently the ionic strength) was maintained at 0.01 M at all pH values.

Preparation of Substrates.—A grade L-malic acid from California Corporation for Biochemical Research was recrystallized.[5] The recrystallized material was dried in a vacuum oven after which it melted at 102.4–103.6°. Fumaric acid supplied by the National Aniline Division of Allied Chemical and Dye Corporation was recrystallized from water and dried to constant weight at 100° in the vacuum oven.

Initial Velocity Measurement.—The initial velocity was obtained using a Cary model 14 recording spectrophotometer equipped with a 0.0–0.2 absorbancy expanded scale slide wire. The substrate solution (25 ml.) and enzyme solution (0.5 ml.) were mixed in a 10-cm. cylindrical spectrophotometer cell, and changes in absorbancy were determined. The initial slopes of the spectrophotometer traces were used to obtain initial velocities. In order to reduce the effect of random errors in the initial velocity due to spectrophotometer instability and other sources, the initial velocity was measured in quadruplicate at all substrate concentrations. In each determination of the maximum velocity and Michaelis constant under given conditions of pH, temperature, and ionic strength the initial velocity was measured with eight substrate concentrations. Whenever possible the range of substrate concentrations used for a given determination of V and K was from $0.2K$ to $5K$. It was not always possible to reach $0.2K$, since 1×10^{-6} M was about the lowest substrate concentration at which accurate values of the initial velocity could be obtained. In addition, since it was desired to avoid substrate activation and inhibition at high substrate concentrations, it was not possible to use substrate concentrations as high as $5K$ under some conditions of pH and temperature.

(7) The abbreviation, TRIS, will be used for the tris-(hydroxymethyl)-aminomethane cation.

(8) R. A. Alberty, *J. Cell. Comp. Physiol.*, **47**, Suppl. 1, 245 (1956).

(9) G. B. Kistiakowsky and R. Lumry, *J. Am. Chem. Soc.*, **71**, 2006 (1949).

(10) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, p. 150.

(11) C. Frieden, R. M. Bock and R. A. Alberty, *J. Am. Chem. Soc.*, **76**, 2482 (1954).

(12) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath and Co., Boston, Mass., 1955, p. 281.

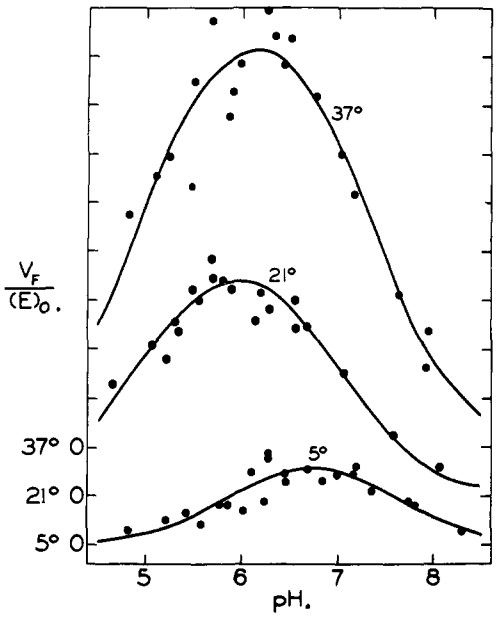

Fig. 1.—Molecular activity, $V_F/(E)_0$, with fumarate as substrate as a function of pH at three temperatures. The smooth curves are least square lines using eq. 2. The ordinate zero point used for each temperature is indicated along the ordinate. Each ordinate unit corresponds to 500 sec.$^{-1}$.

Temperature Control.—All substrate solutions used in the kinetic experiments were thermostated in a water-bath at the temperature of the experiment. Before filling with substrate solution the empty 10-cm. cuvettes were thermostated at the temperature of the experiment for 3 min. in a cell jacket through which water from a thermostated water-bath was circulated. After filling with 25 ml. of substrate solution the cuvettes were thermostated for another 3 min. in the same jacket. At the end of this time 0.5 ml. of enzyme solution at 0° was added to the cuvette. The cuvette was immediately rocked to mix the solutions and transferred quickly to a second thermostated cell jacket designed for insertion into the cell compartment of the spectrophotometer. The temperature of the reaction mixture, as measured at the beginning and completion of several initial velocity determinations during the course of a run, was within 0.3° of the desired temperature.

Measurement of pH.—The pH of all solutions was determined with a Leeds and Northrop model 7664 pH meter. The standard buffer and unknown solutions were kept in tall beakers in the water-bath, and the instrument was standardized before each measurement with buffers at the same temperature and in the same pH range as the unknown solutions. Standard buffers (*ca.* pH 4, 7, and 9) supplied by Beckman were used. The pH values reported here are the result of measurements made on the reaction mixtures after the initial velocity had been measured. The reaction solutions from several initial velocity measurements were pooled in a tall beaker in the water-bath. When a sufficient amount of solution had accumulated for adequate immersion of the electrodes, the pH was measured. The reported pH values are the mean of a minimum of three such measurements. The results of the individual measurements generally agreed within 0.02 pH unit.

Results

In the kinetic experiments using malate as substrate a total of 82 separate determinations of V_M and K_M was made. The pH dependence of the malate kinetic parameters in 0.01 *M* TRIS–acetate buffers was determined over the pH range 5.5–8.5 at 5, 13, 21, 29, and 37°. In the fumarate experiments a total of 62 determinations of V_F and K_F was made, and the pH dependence of the fumarate kinetic parameters in 0.01 *M* TRIS–acetate buffers was determined in the pH range 4.7–8.2 at 5, 21 and 37°. The data from both series of experiments were treated identically. In order to reduce the effects of arbitrary weighting of particular experimental points by any one of the three possible linear plots of the Michaelis–Menten equation the data were plotted according to all three linear forms. Straight lines were drawn through the points by inspection, and the resulting values of V and K were averaged to give the reported results. The maximum velocities determined in this way were converted to molecular activities,[13] $V/(E)_0$, where $(E)_0$ is the molar concentration of the enzyme.

The results of the experiments to determine the molecular activity with fumarate as substrate, $V_F/(E)_0$, at a series of pH values are presented in Fig. 1 for each of three temperatures. The smooth curves drawn through the experimental points in Fig. 1 are the theoretical lines evaluated from equations of the form of eq. 2. The values of the parameters of eq. 2, $V_F'/(E)_0$, K_{aEF}', and K_{bEF}', which were used to calculate the theoretical lines, were obtained from a least squares fit to the equations using a digital computer[14] and are reported in Table I. The uncertainty limits given in Table I are estimates obtained by considering reasonable alternatives to the least squares curves. The molecular activity with malate as substrate, $V_M/(E)_0$, is presented as a function of pH at five temperatures in Fig. 2. The theoretical curves in Fig. 2 were calculated as just described using equations of the form of eq. 3, and the parameters are reported in Table I. Because substrate activation became a serious problem at the higher pH values where K_M is large, good data could not be obtained at pH values much above the pH optimum for $V_M/(E)_0$.

Plots of $V_M[1 + (H^+)/K_{HM}]/K_M(E)_0$ *vs.* pH are presented in Fig. 3 for five temperatures. The theoretical curves drawn through the data in Fig. 3 were calculated in the manner described above using eq. 7. The parameters of these curves are reported in Table I. Plots of $V_F[1 + (H^+)/K_{HF}]/K_F(E)_0$ *vs.* pH were not made, since it was difficult to obtain accurate values for the Michaelis constants of fumarate. The dependence of $V_F[1 + (H^+)/K_{HF}]/K_F(E)_0$ on pH must be identical with that of $V_M[1 + (H^+)/K_{HM}]/K_M(E)_0$ as is evident from eq 6 and 7. The values of $V_F'/K_F'(E)_0$ and K_F' given in Table I were calculated using eq. 8. Values of K_M', $(V_M' + V_F')/K_F'(E)_0$, and $(V_F' + V_M')/K_M'(E)_0$ were also calculated and are presented in Table I. The uncertainties in the calculated parameters in Table I were calculated from the estimated uncertainties in the directly determined parameters.

Discussion

The steady state kinetics of the fumarase reaction may be interpreted in terms of the mechanism[15]

$$\begin{array}{ccccccc}
F + E & \underset{k_{-1}'}{\overset{k_1'}{\rightleftarrows}} & X_1 & \cdots & X_i & \underset{k_{-(i+1)}'}{\overset{k_{(i+1)}'}{\rightleftarrows}} & \cdots X_n \underset{k_{-(n+1)}'}{\overset{k_{(n+1)}'}{\rightleftarrows}} E + M \\
 & \updownarrow K_{0b} & \updownarrow K_{1b} & & \updownarrow K_{ib} & & \updownarrow K_{nb} \quad \updownarrow K_{0b} \\
F + HE & \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} & HX_1 & \cdots & HX_i & \underset{k_{-(i+1)}}{\overset{k_{(i+1)}}{\rightleftarrows}} & \cdots HX_n \underset{k_{-(n+1)}}{\overset{k_{(n+1)}}{\rightleftarrows}} HE + M \qquad (9) \\
 & \updownarrow K_{0a} & \updownarrow K_{1a} & & \updownarrow K_{ia} & & \updownarrow K_{na} \quad \updownarrow K_{0a} \\
F + H_2E & \underset{k_{-1}''}{\overset{k_1''}{\rightleftarrows}} & H_2X_1 & \cdots & H_2X_i & \underset{k_{-(i+1)}''}{\overset{k_{(i+1)}''}{\rightleftarrows}} & \cdots H_2X_n \underset{k_{-(n+1)}''}{\overset{k_{(n+1)}''}{\rightleftarrows}} H_2E + M
\end{array}$$

In this mechanism F and M represent fumarate and L-malate. The substrate and product may combine

(13) Report of the Commission on Enzymes, Pergamon Press, New York, N. Y., 1961, p. 10.

(14) Partial support for the services of the University of Wisconsin Numerical Analysis Laboratory was provided by the National Science Foundation and the Wisconsin Alumni Research Foundation through the Research Committee of the University of Wisconsin. All computer programs used in the present research are available upon request from Professor W. W. Cleland, Department of Biochemistry, University of Wisconsin.

(15) R. A. Alberty and V. A. Bloomfield, *J. Biol. Chem.*, in press.

TABLE I

VALUES AND ESTIMATED UNCERTAINTIES OF KINETIC PARAMETERS

T, °C.	37	29	21	13	5
$[V_F'/(E)_0] \times 10^{-8}$, sec.$^{-1}$	4.52 ± 0.49		2.52 ± 0.22		0.980 ± 0.055
pK_{aEF}'	4.92 ± .18		4.86 ± .14		5.87 ± .12
pK_{bEF}'	7.37 ± .13		7.04 ± .09		7.63 ± .07
$[V_M'/(E)_0] \times 10^{-8}$, sec.$^{-1}$	4.56 ± .41	2.27 ± 0.12	1.13 ± .10	0.593 ± 0.007	0.347 ± .009
pK_{aEM}'	6.57 ± .08	6.36 ± .06	6.36 ± .08	6.40 ± .03	6.55 ± .03
pK_{bEM}'	8.46 ± .15	8.88 ± .17	9.14 ± .23	9.17 ± .21	10.29 ± .62
$[V_M'/K_M'(E)_0] \times 10^{-9}$, sec.$^{-1}$ M^{-1}	0.571 ± .032	0.494 ± .041	0.299 ± .030	0.168 ± .008	0.0989 ± .0071
pK_{0a}'	5.97 ± .12	5.83 ± .14	5.75 ± .17	5.68 ± .11	5.87 ± .08
pK_{0b}'	6.88 ± .10	7.00 ± .10	7.10 ± .08	7.38 ± .07	7.46 ± .07
$[V_F'/K_F'(E)_0] \times 10^{-9}$, sec.$^{-1}$ M^{-1}	1.93 ± .16	1.99 ± .22	1.44 ± .18	0.976 ± .071	0.703 ± .068
$K_F' \times 10^6$, M	2.34 ± .44		1.74 ± .37		1.39 ± .21
$K_M' \times 10^6$, M	8.0 ± 1.2	4.59 ± .62	3.79 ± .62	3.54 ± .21	3.51 ± .34
$[(V_F' + V_M')/K_F'(E)_0] \times 10^{-9}$, sec.$^{-1}$ M^{-1}	3.9 ± 1.1		2.09 ± .63		0.95 ± .19
$[(V_F' + V_M')/K_M'(E)_0] \times 10^{-9}$, sec.$^{-1}$ M^{-1}	1.14 ± 0.28		0.96 ± .27		0.378 ± .055

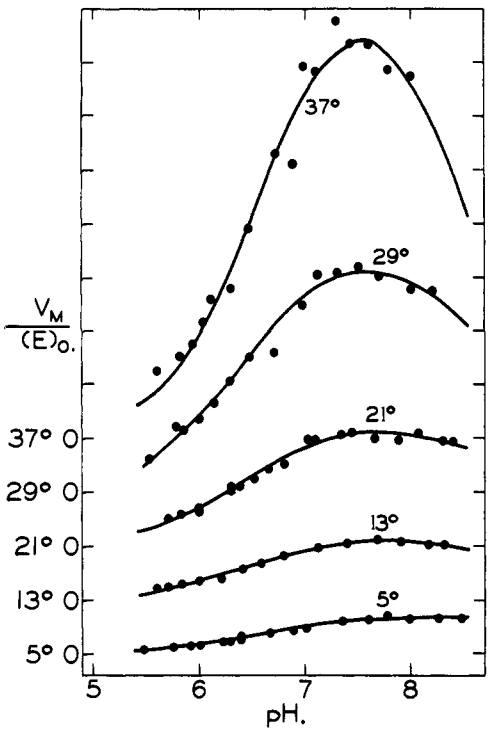

Fig. 2.—Molecular activity, $V_M/(E)_0$, with L-malate as substrate as a function of pH at five temperatures. The smooth curves are least square lines using eq. 3. The ordinate zero point used for each temperature is indicated along the ordinate. Each ordinate unit corresponds to 500 sec.$^{-1}$.

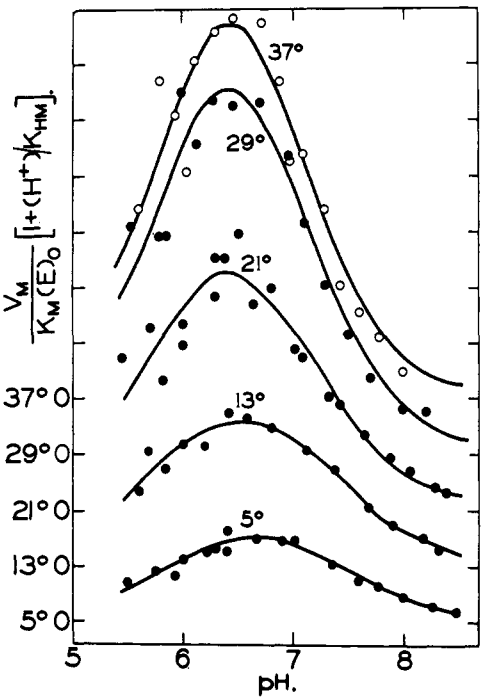

Fig. 3.—$V_M[1 + (H^+)/K_{HM}(E)_0$ *vs.* pH at five temperatures. The smooth curves are least square lines using eq. 7. The ordinate zero point used for each temperature is indicated along the ordinate. Each ordinate unit corresponds to 0.05 × 10^9 M^{-1} sec.$^{-1}$

with any one of the three acidic ionization forms of the enzymatic site E, HE, and H_2E. Corresponding to each of the three forms of the free enzymatic site are three forms of the n-intermediate species, X_i, HX_i, and H_2X_i. An arbitrary number of such intermediates is included, since steady state measurements on the rates of change of substrate or product concentration give no information concerning the number of intermediates; *i.e.*, the steady state rate law has the same form regardless of the number of intermediates. In deriving the steady state rate law for mechanism 9 it is assumed that the proton transfer steps remain in equilibrium. In the presence of buffers the protons are transferred to and from basic and acidic components of the buffer. The K's in the mechanism are the macroscopic acid dissociation constants for dissociation of protons from the various ionization forms of the free enzymatic site and the intermediates considered as dibasic acids. The k's are the rate constants for the several steps.

The steady state rate law for mechanism 9 is given by eq. 1. The general expressions for the maximum initial velocities and Michaelis constants[15] show a more complicated dependence on hydrogen ion concentration than that given in eq. 2 to 5 but include these equations as special cases. The accuracy of the experimental data is not always great enough to permit definite establishment of the exact form of the decline of V and V/K at high and low pH. A minimal requirement for V and V/K to go to zero at high and low pH would simply be that some pair of rate constants in the upper path, $k_{(i+1)}'$ and $k_{-(i+1)}'$, and some pair of rate constants in the lower path, $k_{(i+1)}''$ and $k_{-(i+1)}''$, be zero. However, since these quantities may not actually decline to zero as very high and very low pH values are approached, it is of interest to consider the lower limits to the weighted average rate constants as given in terms of the complete general treatment. Lower limits to weighted average rate constants for this mechanism may be calculated from the pH-dependent maximum initial velocities and Michaelis constants.[15] For example, the lower limit to the weighted

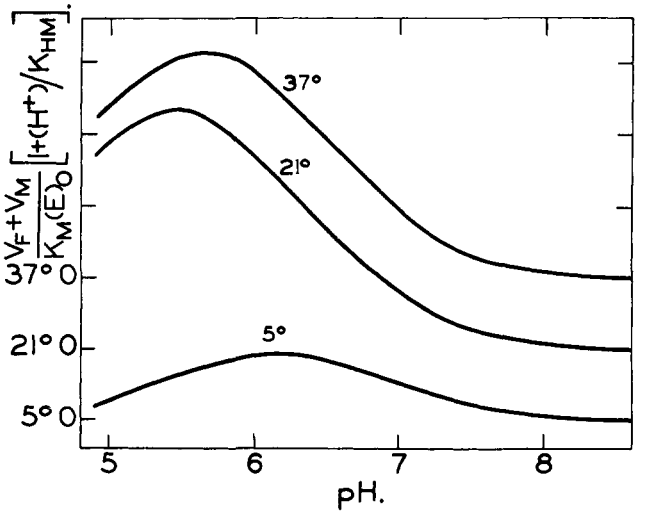

Fig. 4.—$(V_F + V_M)[1 + (H^+)/K_{HF}]/K_F(E)_0$ *vs.* pH at three temperatures. The curves were calculated using the parameters of eq. 2, 3, and 4 given in Table I. The ordinate zero point used for each temperature is indicated along the ordinate. Each ordinate unit corresponds to $0.5 \times 10^9\ M^{-1}$ sec.$^{-1}$.

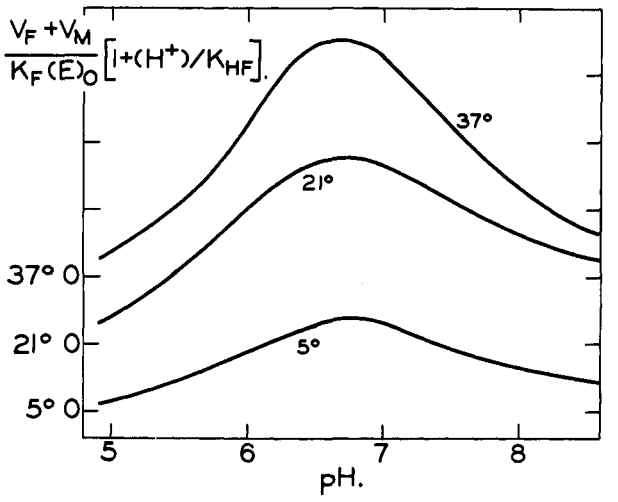

Fig. 5.—$(V_F + V_M)[1 + (H^+)/K_{HM}]/K_M(E)_0$ *vs.* pH at three temperatures. The curves were calculated using the parameters of eq. 2, 3, and 5 given in Table I. The ordinate zero point used for each temperature is indicated along the ordinate. Each ordinate unit corresponds to $0.5 \times 10^9\ M^{-1}$ sec.$^{-1}$.

average rate constant for combination of enzyme and fumarate is given by

$$\bar{k}_1 \equiv \frac{k_1 + k_1''(H^+)/K_{0a} + k_1'K_{0b}/(H^+)}{1 + (H^+)/K_{0a} + K_{0b}/(H^+)} \geqslant \frac{V_F + V_M}{K_F(E)_0} \quad (10)$$

where the equality holds only if $n = 1$. The lower limit to the weighted average rate constant for combination of enzyme and malate is given by

$$\bar{k}_{-(n+1)} \equiv \frac{k_{-(n+1)} + k_{-(n+1)}''(H^+)/K_{0a} + k_{-(n+1)}'K_{0b}/(H^+)}{1 + (H^+)/K_{0a} + K_{0b}/(H^+)} \geqslant \frac{V_F + V_M}{K_M(E)_0} \quad (11)$$

Lower limits may also be determined for the weighted average unimolecular rate constants of mechanism 9. These lower limits are given by

$$\bar{k}_{(i+1)} \equiv \frac{k_{(i+1)} + k_{(i+1)}''(H^+)/K_{ia} + k_{(i+1)}'K_{ib}/(H^+)}{1 + (H^+)/K_{ia} + K_{ib}/(H^+)} \geqslant \frac{V_F}{(E)_0} \quad (12)$$

$$\bar{k}_{-i} \equiv \frac{k_{-i} + k_{-i}''(H^+)/K_{ia} + k_{-i}'K_{ib}/(H^+)}{1 + (H^+)/K_{ia} + K_{ib}/(H^+)} \geqslant \frac{V_M}{(E)_0} \quad (13)$$

where the equality signs hold only for $n = 1$.

Plots of $(V_F + V_M)[1 + (H^+)/K_{HF}]/K_F(E)_0$ and $(V_F + V_M)[1 + (H^+)/K_{HM}]/K_M(E)_0$ *vs.* pH at three temperatures are shown in Fig. 4 and 5, and as indicated in eq. 10 and 11 these plots give lower limits for $\bar{k}_1 + \bar{k}_{-(n+1)}$. Since these lower limits closely approach the maximum values permitted by the theory of diffusion controlled reactions,[16,17] the lower limits may be considered to be approximately equal to second-order rate constants. Activation energies for these lower limits are given in Table II. The lower limits for $\bar{k}_{(i+1)}$ and $\bar{k}_{-i}$ given by eq. 12 and 13 are presented in Fig. 1 and 2.

If the combination of the enzymatic site with its substrates is independent of the degree of ionization of the site then $k_1 = k_1' = k_1''$ and $k_{-(n+1)} = k_{-(n+1)}' = k_{-(n+1)}''$ so that eq. 10 and 11 become

$$k_1 \geqslant (V_F + V_M)/K_F(E)_0 \quad (14a)$$

$$k_{-(n+1)} \geqslant (V_F + V_M)/K_M(E)_0 \quad (14b)$$

Thus k_1 at a given temperature would be greater than or equal to the greatest value attained by the appropriate plot in Fig. 4, and $k_{-(n+1)}$ would be greater than the greatest value attained by the appropriate plot in Fig. 5.

The above discussion has not taken cognizance of the fact that the pH dependences for the kinetic parameters are given by eq. 2–5. While there are several conditions under which the expressions for the maximum initial velocities and Michaelis constants for mechanism 9 reduce to these forms, the simplest special case is that which was treated earlier[18] in which all the primed and double primed rate constants are set equal to zero. In terms of this more restrictive mechanism lower limits to the unprimed rate constants may be expressed in terms of the pH-independent maximum velocities and Michaelis constants, and the quantities K_{0a}' and K_{0b}' are interpreted as the first and second acid dissociation constants of the free enzymatic site, *i.e.*, $K_{0a}' = K_{0a}$ and $K_{0b}' = K_{0b}$. The expressions for the lower limits to the two bimolecular rate constants are $k_1 \geqslant (V_F' + V_M')/K_F'(E)_0$ and $k_{-(n+1)} \geqslant (V_F' + V_M')/K_M'(E)_0$, while the lower limits to the unimolecular rate constants of the forward and reverse path are given by $k_{(i+1)} \geqslant V_F'/(E)_0$ and $k_{-i} \geqslant V_M'/(E)_0$, respectively. The equality signs in these expressions hold only for $n = 1$. The activation energies for these various lower limits are given in Table II. The Arrhenius plots are given in Fig. 6. The type of pH dependence represented by eq. 2–5 would also be obtained from mechanism 9 if a "bottleneck step" ($k_{(s+1)}' = k_{(s+1)}'' = k_{-(s+1)}' = k_{-(s+1)}'' = 0$), or a series of such steps, were slow compared with other steps in the mechanism.[16] In this case plots of $V/K(E)_0$ *vs.* pH would also yield the correct acid dissociation constants for the enzymatic site. Plots of pK_{0a}' and pK_{0b}' *vs.* $1/T$ are given in Fig. 7 and the apparent activation energies obtained from these plots by the method of least squares are -1.7 kcal. mole^{-1} and 7.7 kcal. mole^{-1}, respectively.

For both of the special cases of mechanism 9 discussed immediately above, K_{0a}' and K_{0b}' are to be interpreted as acid dissociation constants of groups in the enzymatic site. If such an interpretation may be made, it is of interest that the standard enthalpies of dissociation, -1.7 kcal. mole^{-1} and 7.7 kcal. mole^{-1}, are of the magnitude to be expected for carboxyl and imidazole groups, respectively. However, the kinetic parameters

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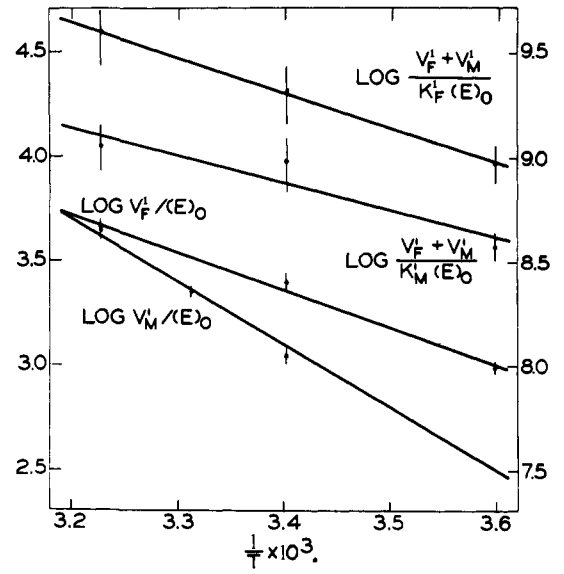

Fig. 6.—Arrhenius plots of $(V_F' + V_M')/K_F'(E)_0$, $(V_F' + V_M')/K_M'(E)_0$, $V_F'/(E)_0$, and $V_M'/(E)_0$. Least square lines are drawn through the experimental points. The ordinate scale used for each plot is indicated along the ordinate.

TABLE II

VALUES AND ESTIMATED UNCERTAINTIES OF ACTIVATION ENERGIES AND APPARENT STANDARD ENTHALPY CHANGES

Paramcter	Activation energy or apparent standard enthalpy change
$V_F'/(E)_0$	8.2 ± 0.9
$V_M'/(E)_0$	13.9 ± 0.8
$V_F'/K_F'(E)_0$	5.9 ± 1.1
$V_M'/K_M'(E)_0$	9.9 ± 1.0
K_F'	2.8 ± 1.8
K_M'	4.0 ± 1.4
$(V_F' + V_M')/K_F'(E)_0$	7.5 ± 2.6
$(V_F' + V_M')/K_M'(E)_0$	6.0 ± 2.2
pK_{0a}'	−1.7 ± 1.8
pK_{0b}'	7.7 ± 1.5
$(V_F + V_M)[1 + (H)^+/K_{HF}]/K_F(E)_0$ at pH	
6.0	5.1
6.5	5.4
7.0	4.8
7.5	4.8
$(V_F + V_M)[1 + (H^+)/K_{HM}]/K_M(E)_0$ at pH	
6.0	6.3
6.5	4.6
7.0	2.9
7.5	2.1
8.9	1.0
8.5	1.7

K_{0a}' and K_{0b}' may not be equilibrium constants. It has been shown[15] that equations of the form of eq. 6 and 7 will be obtained if the first series of several intermediates formed by the combination of F with E, HE, and H_2E have identical pK values and the primed and unprimed rate constants for these steps have the same values and there is one or more "bottle-neck steps." In this case $V/K(E)_0$ will show the simple dependence upon pH given by eq. 6 and 7, but the pK_{0a}' will be lower than pK_{0a} or pK_{0b}' will be higher than pK_{0b}. A similar admonition is appropriate with regard to the apparent acid dissociation constants, K_{aEF}', K_{bEF}', K_{aEM}', and K_{bEM}'. In none of the cases discussed here

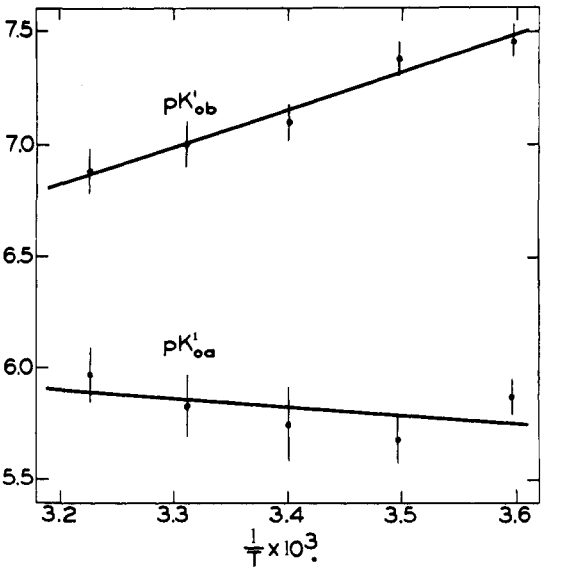

Fig. 7.—pK_{0a}' and pK_{0b}' *vs.* $1/T$. Least square lines are drawn through the experimental points.

can these four parameters be interpreted as acid dissociation constants.

The analysis of the effect of temperature on the steady state kinetic parameters of the fumarase reaction could in principle be carried one step further by studying the effect of buffer composition at constant ionic strength and the effect of ionic strength. The pH-independent maximum velocities and Michaelis constants are functions of buffer concentration and composition.[5] These effects are of the general type to be expected to result from the binding of buffer components at the enzymatic site and at neighboring sites where an effect is exerted on the catalytic site.[19] A detailed study of these effects would still only yield aggregates of rate constants, but would be expected to yield buffer-independent Michaelis constants with lower values than those reported here, and consequently still higher lower limits for bimolecular rate constants. In addition such a study would lead to the evaluation of buffer-binding constants and the temperature dependence of these constants. A preliminary study of the effect of temperature at 0.10 ionic strength with malate as substrate bears out these ideas.[20]

The differences between the experimentally determined values of the kinetic parameters reported here and values for these parameters reported earlier[5,21] are felt to reflect differences in the purity of reagents and buffer components used in the various investigations. Since the steady state kinetic parameters of this enzyme are sensitive to impurities in the buffer medium, the present work was done with carefully purified components. The uncertainties reported here reflect the precision of our results under conditions such that the sources of enzyme and all reagents and buffer components were constant for all experiments.

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