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Studies of the Enzyme Fumarase. IV.[1] The Dependence of the Kinetic Constants at 25° on Buffer Concentration, Composition and *p*H[2]

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The Michaelis constants and maximum steady-state velocities for the forward and reverse reactions catalyzed by fumarase depend upon the hydrogen ion concentration in a simple way in the range *p*H 5.5–8.5 when tris-(hydroxymethyl)-aminomethane acetate buffers are used. Altogether 10 kinetic parameters for the reaction at a particular buffer concentration may be calculated using the equations which have been derived earlier for the two Michaelis constants and two maximum steady-state velocities. The dependencies of these parameters on the concentration of tris-(hydroxymethyl)-aminomethane acetate and the effect of added sodium chloride have been determined. The "*p*H-independent" Michaelis constants are found to be a linear function of the acetate concentration.

It has been shown[4] that the Michaelis constants and maximum steady-state velocities for both the forward and reverse reactions catalyzed by fumarase vary in a simple way with *p*H in the range 5.5–8.5 in "tris"[5] acetate buffers of 0.01 ionic strength. The *pK* values and *p*H-independent Michaelis constants and maximum steady-state velocities are expected to depend upon the composition[6] and ionic strength[7,8] of the buffer. In order to explore these effects the various kinetic parameters for "tris" acetate buffers of 0.001, 0.005, 0.01, 0.02 and 0.05 ionic strength have been determined. They have also been determined for a 0.10 ionic strength buffer containing 0.09 *N* sodium chloride and 0.01 ionic strength "tris" acetate. Data of a similar nature were reported earlier for phosphate buffers,[1] but it was not possible to make a simple interpretation of the effect of *p*H, apparently because of the complications due to the change of the ratio of the concentrations of the mono- and divalent phosphate ions with *p*H. The "tris" acetate buffers have the very great advantage that the ionic composition of the buffer medium is independent of *p*H except for the changes in (H^+) and (OH^-).

Altogether 10 kinetic parameters may be evaluated at a given buffer concentration from the initial velocities of the forward and reverse reactions over a range of *p*H values. The interpretation of these parameters in terms of a mechanism which has been proposed[4] is discussed in detail in the following article.[9]

Experimental

Crystalline fumarase was isolated from pig heart muscle by the method described earlier.[10] The enzyme is quite stable when stored frozen in dilute buffer or in the crystalline form suspended in 50% saturated ammonium sulfate solution in a refrigerator. Small volumes of fumarase solution, containing about 0.1 mg. of enzyme per ml., were frozen and then thawed for use as needed.

Fumaric acid was crystallized twice from water and had a melting point of 287–292°. L-Malic acid (Pfanstiehl C.P.) was decolorized with activated charcoal and recrystallized twice from ethyl acetate–petroleum ether (boiling range 55–65°). The temperature of the solution was not allowed above 65° during the crystallization. The crystallized L-malic acid melted at 102.2–102.4° and had molar absorbancy indices at 210, 230 and 250 mμ of 124, 30 and 0.8 M^{-1} cm.$^{-1}$, respectively. These indices are identical with those of L-malic acid purified by chromatography.[11]

The Michaelis constants and maximum steady-state velocities at 25° were obtained as described earlier[1] using a

(1) The preceding article in this series is R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, THIS JOURNAL, **76**, 2485 (1954).

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

(3) Fellow of the National Foundation for Infantile Paralysis.

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

(5) The abbreviation "tris" will be used for tris-(hydroxymethyl)-aminomethane cation.

(6) R. A. Alberty, THIS JOURNAL, **76**, 2494 (1954).

(7) G. B. Kistiakowsky and W. H. R. Shaw, *ibid.*, **75**, 2751 (1953).

(8) G. B. Kistiakowsky and W. E. Thompson, *ibid.*, **78**, 4821 (1956).

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

(10) C. Frieden, R. M. Bock and R. A. Alberty, *ibid.*, **76**, 2482 (1954).

(11) Personal communication of W. R. Miller.

modified Beckman DUR spectrophotometer. Cuvettes having light paths of 1, 5 and 10 cm. were employed as required to determine velocities during the first several per cent. reaction. Substrate activation[12] was avoided by using substrate concentrations less than about five times the Michaelis constant. The activities of the enzyme in different experiments were compared by use of a standard assay. Since the turnover number at 25° is 1.7×10^3 sec.$^{-1}$ for 0.05 *M* phosphate buffer of *p*H 7.3 containing 0.05 *M* L-malate, it is possible to express all maximum velocities as turnover numbers.[10] The stock solution of fumarase was held in an ice-bath during the experiment, and it was always allowed to stand at least one hour before starting the experiment. The rate of inactivation after the first hour was about 8–15% per hour and was determined at regular intervals by use of the standard assay.

Results

In the *p*H range of about 5.5 to 8.5 the maximum steady-state velocities for fumarate (F) and L-malate (M) may be represented by the equations

$$V_F = \frac{V'_F(E)_0}{1 + (H^+)/K'_{aEF} + K'_{bEF}/(H^+)} \quad (1)$$

$$V_M = \frac{V'_M(E)_0}{1 + (H^+)/K'_{aEM} + K'_{bEM}/(H^+)} \quad (2)$$

where the molar concentration of the enzyme is represented by $(E)_0$, the *p*H-independent turnover numbers are represented by V', and the apparent first and second acid dissociation constants by K'_a and K'_b. The acid dissociation constants were calculated from the *p*H-dependence of the maximum steady-state velocities using the hydrogen ion concentrations, $(H^+)_a$ and $(H^+)_b$, at which half the maximum velocity was obtained and the hydrogen ion concentration, $(H^+)_{max}$, at the maximum of the plot of V_F or V_M *versus* *p*H.[13]

$$K'_a = (H^+)_a + (H^+)_b - 4(H^+)_{max} \quad (3)$$

$$K'_b = (H^+)^2_{max}/K'_a \quad (4)$$

The *p*H-independent turnover numbers V'_F and V'_M were calculated with the equation

$$V' = V_{max}(1 + 2\sqrt{K'_b/K'_a}) \quad (5)$$

where V_{max} is the turnover number at the peak of the plot of maximum steady-state velocity *versus* *p*H.

In the *p*H range of about 5.5 to 8.5 the Michaelis constants at a given buffer concentration may be represented by the equations

$$K_F = K'_F \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K'_{aEF} + K'_{bEF}/(H^+)} \quad (6)$$

$$K_M = K'_M \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K'_{aEM} + K'_{bEM}/(H^+)} \quad (7)$$

where the *p*H–independent Michaelis constants are represented by K'. The values of K_{aE} and K_{bE} were calculated from plots of V_M/K_M and $V_F/4.4K_F$[14] *versus* *p*H. As required by the Haldane relation[15]

$$K_{eq} = \frac{(M)_{eq}}{(F)_{eq}} = \frac{V_F K_M}{V_M K_F} \quad (8)$$

these plots are superimposable.

Since according to equations 1–6 and 2–7

$$\frac{V}{K} = \frac{V'(E)_0/K'}{1 + (H^+)/K_{aE} + K_{bE}/(H^+)} \quad (9)$$

the first and second ionization constants of the enzymatic site in the free enzyme were evaluated by using equations 3 and 4. Similarly the *p*H-independent Michaelis constants were evaluated using equations similar to equation 5.

$$\frac{V'}{K'} = \left(\frac{V}{K}\right)_{max}\left(1 + 2\sqrt{K_{bE}/K_{aE}}\right) \quad (10)$$

Substitution of equations 1, 2, 6 and 7 into equation 8 shows that

$$K_{eq} = \frac{V'_F K'_M}{V'_M K'_F} \quad (11)$$

It is found that this relation is obeyed within the experimental error.

The *pK* values and *p*H-independent kinetic constants obtained at a series of ionic strength values are summarized in Table I. Because of

TABLE I

KINETIC PARAMETERS FOR THE FUMARASE REACTION IN "TRIS" ACETATE BUFFERS AT 25°

Ionic strength	0.001	0.005	0.01[a]	0.02	0.05	0.10[b]
pK_{aE}	6.5	6.3	6.2	6.3	..	7.4
pK'_{aEF}	6.5	5.8	5.3	5.6	5.7	6.9
pK'_{aEM}	7.1	7.1	6.6	6.8	6.9	7.7
pK_{bE}	6.9	6.8	6.8	6.9	..	7.4
pK'_{bEF}	7.1	6.9	7.3	7.3	7.4	7.8
pK'_{bEM}	8.5	8.3	8.5	8.7	8.4	9.0
$V'_F \times 10^3$ (sec.$^{-1}$)	1.2	2.0	2.3	3.2	3.8	2.2
$V'_M \times 10^3$ (sec.$^{-1}$)	1.2	1.8	1.7	1.8	2.3	1.8
$K'_F \times 10^6$ (*M*)	1.8	1.9	2.6	4.5	..	40
$K'_M \times 10^6$ (*M*)	7.9	8.1	9.0	11.1	..	145

[a] The values in ref. 5 have been corrected. [b] The buffer contained 0.09 *M* NaCl and 0.01 ionic strength "tris" acetate.

difficulties in determining V_F at low *p*H values at 0.10 ionic strength the position of the V_F *versus* *p*H curve in this region was calculated from the other three kinetic parameters using equation 8 with $K_{eq} = 4.4$.

At *p*H values above about 8.5 the Michaelis constants deviate from equations 6 and 7 by considerably more than the experimental error. In making the present calculations these deviations were ignored since it is not yet certain whether these deviations are due to an additional dissociation or to electrostatic repulsion of the substrate ions by the fumarase molecule which has a fairly large negative charge at such high *p*H values.[16] The magnitude and significance of these deviations will be discussed in a future publication.

In order to permit a comparison of the kinetic parameters for "tris" acetate buffers with those for phosphate buffers[1] the kinetic constants for 0.005 *M* sodium phosphate and "tris" acetate buffers at *p*H 7.0 and 25° are given in Table II. It is evident that the maximum steady-state velocities are affected to a very much smaller extent than the Michaelis constants by the change of buffer. The fact that the Michaelis constants are 20–30 fold

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

(13) R. A. Alberty and V. Massey, *Biochim. Biophys. Acta*, **13**, 347 (1954).

(14) The equilibrium constant for the over-all reaction at 25° and for low ionic strength values is *K*eq = 4.4. The ratios of maximum steady-state velocities to Michaelis constants may be further corrected by multiplying by factors providing for the secondary ionizations of the substrates.[4] However, these corrections are negligible at the lowest *p*H values used here.

(15) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930, pp. 80–82.

(16) R. A. Alberty, *J. Cell. Comp. Physiol.*, **47**, 245 (1956).

TABLE II

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES FOR 0.005 *M* SODIUM PHOSPHATE AND "TRIS" ACETATE BUFFERS AT *p*H 7.0 AND 25°

	K_F (μM)	$[V_F/(E)_0]$ $\times 10^{-3}$ (sec.$^{-1}$)	K_M (μM)	$[V_{M'}(E)_0]$ $\times 10^{-3}$ (sec.$^{-1}$)	K_{eq}[a]
Phosphate	72	1.9	190	1.1	4.6
Acetate	2.4	0.75	10	0.81	4.1

[a] Calculated using equation 8.

larger for phosphate buffer suggests that the phosphate ions are much better competitive inhibitors of fumarase than acetate ions.

Discussion

The *p*H-independent maximum velocities for fumarate and L-malate increase somewhat with increasing "tris" acetate concentration. The activating effect may be a consequence of the binding of acetate ions at binding sites neighboring the enzymatic site. It would not be expected that the experimental data should conform exactly with equations derived for such a mechanism[6] since the ionic strength was not held constant in the present experiments. However, the effect is very similar to that which would be expected.

The *p*H-independent Michaelis constants for fumarate and L-malate increase linearly with increasing "tris" acetate concentration as shown in Fig. 1. According to these plots the *p*H-independent Michaelis constants at zero buffer concentration would be 1 μM for fumarate and 7.5 μM for L-malate. While the change of the *p*H-independent Michaelis constants with "tris" acetate concentration is not inconsistent with equations[6] derived on the assumption of simple activation and inhibition the fact that the ionic strength varies precludes any simple interpretation of the present data.

The apparent pK values calculated using equations 1, 2 and 9 are nearly independent of "tris" acetate concentration but are markedly different for the 0.10 ionic strength buffer containing 0.09 *M* sodium chloride. Since the probable errors in the apparent pK values are somewhat larger[13] than the experimental errors in the determination of the *p*H values at which half the maximum velocity is obtained, changes in pK values of 0.3 are not considered significant. In all cases $pK'_{aEF} \leqslant pK_{aE}$. The pK values are consistently higher at 0.10 ionic strength as shown by the differences in Table III. This is probably due to the acid-weakening

TABLE III

DIFFERENCE IN pK' VALUES AT 0.1[a] AND 0.01 IONIC STRENGTH AT 25°

	E	EF	EM
pK_a	1.2	1.6	1.1
pK_b	0.6	0.6	0.6

[a] The ionic strength was increased by adding 0.09 *M* NaCl.

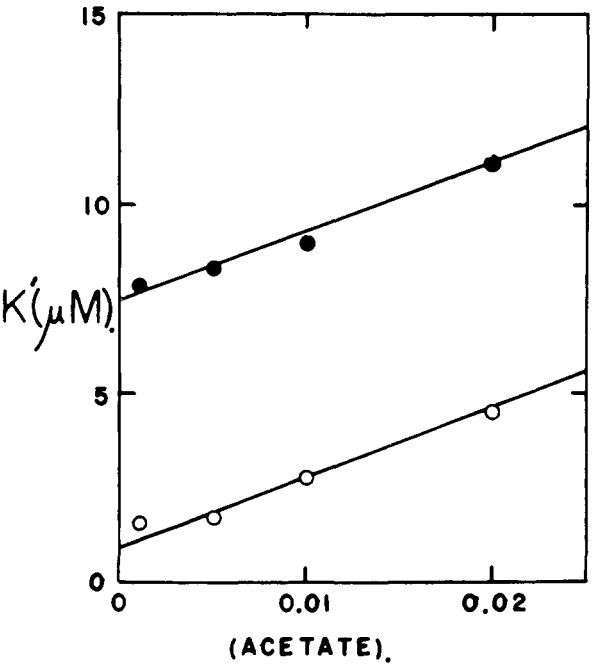

Fig. 1.—Dependence of *p*H-independent Michaelis constants for fumarate (○) and L-malate (●) on "tris" acetate concentration (moles of salt per liter).

effect of the greater negative charge on the fumarase molecule at 0.10 ionic strength due to a greater extent of anion binding. The fact that the pK_a values are shifted about twice as much as the pK_b values when 0.09 *M* sodium chloride is added is something which will have to be explained by future theories of the enzyme structure.

The differences $pK'_{aES} - pK_{aE}$ and $pK'_{bES} - pK_{bE}$ do not vary greatly with ionic strength with the exception of $pK'_{aEF} - pK_{aE}$ although the pK values are considerably higher for the 0.10 ionic strength buffer. The shifts at 0.01 and 0.10 ionic strengths are summarized in Table IV. The con-

TABLE IV

DIFFERENCES IN pK' VALUES AT 25°

Ionic strength	0.001	0.005	0.01	0.02	0.10
$pK'_{aEF} - pK_{aE}$	0	−0.5	−0.9	−0.7	−0.5
$pK'_{aEM} - pK_{aE}$	0.6	0.8	0.4	0.5	0.3
$pK'_{bEF} - pK_{bE}$	0.2	0.1	0.5	0.4	0.4
$pK'_{bEM} - pK_{bE}$	1.6	1.5	1.7	1.8	1.6

centration of "tris" acetate was the same for the 0.01 and 0.1 ionic strength buffers. The fact that the binding of fumarate by the enzyme causes a lowering of pK_a indicates a more profound change upon formation of the enzyme–substrate complex than simple ion pair formation.

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