nitrogen determination,[24] indicating that less than an estimated 3 to 4% of the amino groups reacted. No shift in the tyrosine light absorption curve was observed, indicating that less than an estimated 5% of the tyrosines were alkylated.[22] No decrease in the degree of coupling of diazotized sulfanilic acid was found, suggesting that there was also little alkylation of imidazole by DAA. Titration of free sulfhydryl[25] indicated that one of the two masked sulfhydryls had been alkylated.

Discussion

Previous reports,[6,13] showed that anti-A_p antibody appears to have a negative charge in the site which accommodates the positive charge of the antigen and that anti-X_p antibody formed against a negative charge has a positive charge in the site. The present work emphasizes the difference in the antibody combining sites of the anti-A_p and anti-X_p antibodies. When a mixture of these antibodies is treated with DAA, with resultant esterification of carboxylate, only the anti-A_p antibody is affected, although the anti-X_p was esterified simultaneously in the same solution. The extent of esterification must have been identical for both antibodies. This is proof that a carboxylate or at least a moiety capable of reacting with DAA is present in the anti-A_p site, while no such grouping is present in the anti-benzoate site. That it is the attack on the site itself and not the non-specific alteration of the anti-A_p molecule which is responsible for the effect is further emphasized by the fact that esterification of anti-A_p antibody in the presence of the specific hapten results in a retention of antibody activity. Specific protection would not be obtained unless the attack were on the antibody site. That esterification is responsible for the loss of activity is indicated by the fact that binding activity can be partially recovered by exposing the altered antibody to *p*H 11, which hydrolyzes the ester groups.

These findings provide clear-cut evidence, implied by previous work,[11-13] that the specific sites in antibodies against different antigens have different amino acid composition. This does not differentiate between involvement of different parts of the same polypeptide or the associated tertiary structure or to variations in the sequence of amino acids in the chain at the region of the sites.

From the rate of decrease of binding activity with esterification—70% loss of activity when 30% of the carboxyls are esterified—it would appear that the carboxyl in the site is more easily esterified than some of the other carboxyls. Part of this loss of binding activity may be due to a non-specific effect as shown by the fact that when 16% of the carboxyls are esterified there is a 35% decrease in the value of K_0'. This non-specific effect may be partially electrostatic due to the increased positive charge of the antibody as a whole, resulting in decreased attraction or increased repulsion of the positive hapten. Steric effects due to an ester group close to the antibody site and distortion of the molecule as a whole may also contribute to the decrease in binding constant.

(24) J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," The Williams and Wilkins Co., Baltimore, Md., 1932, pp. 386–399.

(25) Kindly performed by Dr. G. Markus by the method reported by G. Markus and F. Karush, THIS JOURNAL, **79**, 134 (1957).

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

The *p*H Dependence of the Competitive Inhibition of Fumarase[1,2]

BY PAUL W. WIGLER[3] AND ROBERT A. ALBERTY

RECEIVED APRIL 1, 1960

In order to obtain further information on the structure and function of the active site of fumarase, the inhibitor dissociation constants with four competitive inhibitors, succinate, D-tartrate, L-tartrate and *meso*-tartrate, were determined over a range of *p*H values between *p*H 5.3 and 8.3. The dissociation constants of the inhibitor–enzyme compounds were determined using plots of $(S)/v$ *versus* (I) at each *p*H. From these data, the two acid dissociation constants (K_{aEI} and K_{bEI}) for each of the inhibitor–enzyme compounds and the dissociation constants of the inhibitor–enzyme compounds of the three ionized forms of the enzymatic site were calculated. It was found that the K_{aEI} and K_{bEI} values for the D and L-tartrate compounds of fumarase are lower than the corresponding values for the succinate compound, while the values for the *meso*-tartrate compound are higher. The K_{aEI} and K_{bEI} values are related to the fact that the hydroxyl groups of D and L-tartrate are in the *cis* configuration while the hydroxyl groups of *meso*-tartrate are in the *trans* configuration when the carboxylate groups are *trans*. The results are interpreted in terms of a model for the active site based on a *trans* dehydration of L-malate by fumarase.

Introduction

The divalent anions of several dicarboxylic acids are competitive inhibitors of pig heart fumarase.[4,5] In order to further elucidate the relationships between the spatial configuration of the inhibitor and the enzymatic site, experiments with the three isomers of tartrate and with succinate were carried out over a range of *p*H.

It has been demonstrated that there are two acidic groups which have a total effect on the kinetics of fumarase.[6] The *p*H dependence of the inhibitor dissociation constant (K_I) may be interpreted in terms of this model of the enzymatic site.[7] The

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

(2) Presented in part before the 51st Annual Meeting of the American Society of Biological Chemists, Chicago, April 11–15, 1960.

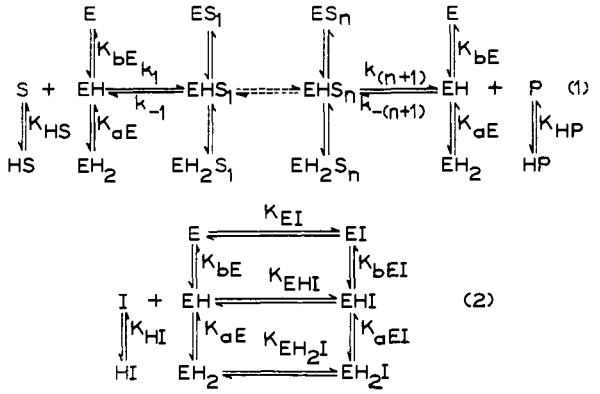
(3) This investigation was carried out during the tenure of a National Institutes of Health Postdoctoral Research Fellowship from the National Heart Institute.

(4) V. Massey, *Biochem. J.*, **55**, 172 (1953).

(5) C. Frieden, Ph.D. Thesis, University of Wisconsin, 1955, p. 76.

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

kinetic equations are based on the following mechanism, where the dashed arrows indicate that there may be a number of additional intermediates. Rate constants are represented by lower case k's and equilibrium constants by capital K's.



The expression for the initial velocity (v) in the steady state is given in equation 3, where (S) and (I) are the total concentrations of substrate and competitive inhibitor, V_S is the maximal velocity and K_S is the Michaelis constant.

$$v = \frac{V_S}{1 + [K_S/(S)][1 + (I)/K_I]} \quad (3)$$

In equations 4–6, which may also be derived from the foregoing mechanism, K_{HS} and K_{HI} represent the second acid dissociation constants of the substrate and inhibitor. The total concentration of enzymatic sites is represented by $(E)_0$[8] and the pH-independent turnover number and Michaelis constant are represented by V'_S and K'_S. The derivation of the expressions for V'_S and K'_S for the multiple intermediate case is given by Peller and Alberty.[9] The inhibitor dissociation constant of the intermediate ionized form of the inhibitor–enzyme compound is K_{EHI}. In the following equations the quantities on the left side are determined by direct experimentation and the quantities on the right are to be calculated.

$$\frac{V_S\,[1 + (H^+)/K_{HS}]}{K_S(E)_0} = \frac{V'_S/K'_S}{1 + (H^+)/K_{aE} + K_{bE}/(H^+)} \quad (4)$$

$$V_S/(E)_0 = \frac{V'_S}{1 + (H^+)/K'_{aES} + K'_{bES}/(H^+)} \quad (5)$$

$$\Phi = \frac{K_1 V_S[1 + (H^+)/K_{HS}]}{K_S(E)_0[1 + (H^+)/K_{HI}]} = \frac{K_{EHI}V'_S/K'_S}{1 + (H^+)/K_{aEI} + K_{bEI}/(H^+)} \quad (6)$$

The acid dissociation constants of the free enzyme (K_{aE} and K_{bE}) and the inhibitor–enzyme compound (K_{aEI} and K_{bEI}) are macroscopic constants because there may be two intermediate ionized forms of the enzymatic site and of the inhibitor–enzyme compound. It should also be noted that the acid dissociation constants, K'_{aES} and K'_{bES} represent weighted averages of the acid dissociation constants for members of the series of intermediates.[9,10]

A plot of Φ *versus* pH may be used to determine K_{aEI} and K_{bEI} (see equation 6). The K_{EHI} value is obtained from the parameters of equations 4 and 6, and the inhibitor dissociation constant for the doubly-protonated form (K_{EH_2I}) and the non-protonated form (K_{EI}) of the inhibitor–enzyme compound are calculated with equations 7 and 8.

$$K_{EH_2I} = (K_{EHI})(K_{aEI})/(K_{aE}) \quad (7)$$

$$K_{EI} = (K_{EHI})(K_{bE})/(K_{bEI}) \quad (8)$$

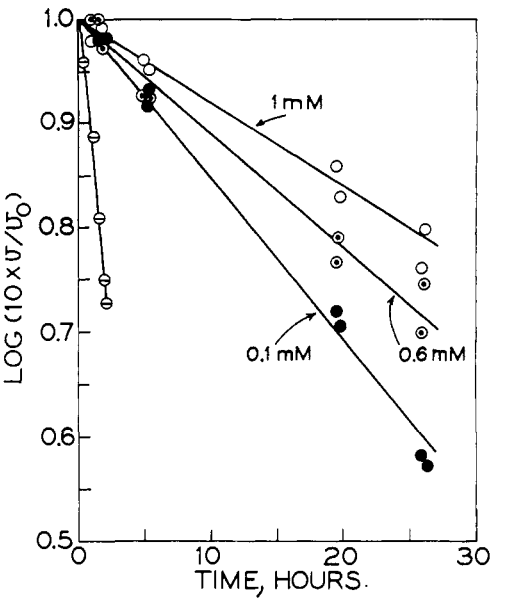


Fig. 1.—Decrease in activity of 6.0×10^{-10} M fumarase in "tris" acetate buffer of 0.01 ionic strength and pH 7.5 at 0° in the presence of varying concentrations of *meso*-tartrate: ⊖, no *meso*-tartrate; ● 0.1 mM *meso*-tartrate; ⊙, 0.6 mM *meso*-tartrate; O, 1 mM *meso*-tartrate. (v_0 is the steady state velocity at the beginning of the experiment.)

Experimental

The substrate, L-malic acid, was recrystallized as previously described.[11] Succinic acid (Mallinckrodt Reagent), D-tartaric acid (California Foundation, A.C.S. grade) and L-tartaric acid (California Foundation, CfP) were used without additional purification. *meso*-Tartaric acid (Aldrich Chemical Co.) was recrystallized from water, washed with cold methanol and dried for 7 hr. at 60° under 1 mm. pressure, m.p. 139–141°.

Crystalline fumarase was prepared by the method of Frieden, Bock and Alberty.[12] The enzyme was found to be quite unstable in "tris"[13] acetate solutions of 0.01 ionic strength at 0°. The enzyme could be stabilized, however, by the addition of competitive inhibitor to the solution. The effect of *meso*-tartrate on the stability of 6.0×10^{-10} M fumarase in "tris" acetate of 0.01 ionic strength, pH 7.5 at 0° in polyethylene tubes, is shown in Fig. 1. In this stability experiment 0.5 ml. aliquots of enzyme–inhibitor solution were pipetted into 20 ml. of 75.3 μM L-malate in "tris" acetate of 0.01 ionic strength and pH 6.4,

(7) R. A. Alberty, in L. Pauling and H. A. Itano, eds., "Molecular Structure and Biological Specificity," Pub. No. 2, American Institute of Biol. Sciences, 1957, p. 155.

(8) The molar concentration of the enzyme is substituted for $(E)_0$ in the interpretation of the experimental data, because the number of enzymatic sites per molecule of fumarase is unknown. Acid titration data indicate that there are fewer than 6 sites per molecule. (See N. S. Shavit, R. G. Wolfe and R. A. Alberty, *J. Biol. Chem.*, **233**, 1382 (1958)).

(9) L. Peller and R. A. Alberty, THIS JOURNAL, **81**, 5907 (1959).

(10) R. A. Alberty and W. H. Pierce, *ibid.*, **79**, 1526 (1957).

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

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

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

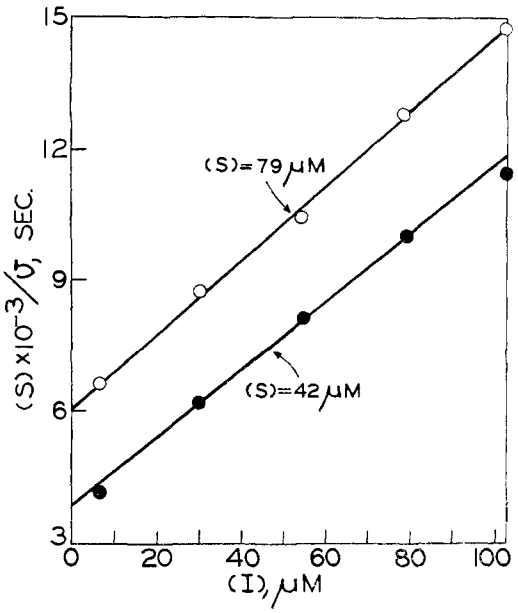


Fig. 2.—Two plots of $(S)/v$ *versus* (I) at 42 and 79 μM L-malate for *meso*-tartrate at 25°; the experiment was performed in "tris" acetate of 0.01 ionic strength and *p*H 6.1.

and the initial velocity was determined spectrophotometrically.

This procedure was adopted for use in the kinetic experiments: Enzyme crystals, stored under 50% saturated ammonium sulfate were centrifuged, washed with cold water and dissolved in "tris' acetate of 0.01 ionic strength and *p*H 7.5, containing one of the four inhibitors. A concentration of 10 mM succinate, 3 mM D-tartrate, 5 mM L-tartrate or 0.6 mM *meso*-tartrate was used to stabilize the enzyme. Losses in enzyme activity were small during an 8 hr. period when 75 ml. of enzyme–inhibitor solution were stored at 0° in a polyethylene bottle.

A 0.2 ml. aliquot of enzyme–inhibitor solution at a concentration which would produce an initial velocity less than 1.6×10^{-2} μM sec.$^{-1}$ was added to 20 ml. of *L*-malate and inhibitor in "tris" acetate of 0.01 ionic strength at 25°, adjusted to a predetermined *p*H. An Aupette (Clay-Adams Inc.) was used to deliver the enzyme–inhibitor solution into a cuvette of 100 mm. path length. The solution was rapidly mixed and the change in absorbancy at 2210 to 2260 Å. was determined with a Cary Model 14 Recording Spectrophotometer with a 0–0.1 absorbancy scale.

A standard assay was used throughout the course of the experiments in a single day and to compare the enzyme activity from day to day. For this assay a *p*H which gives half the maximal value for v was selected. For "tris" acetate solutions of 0.01 ionic strength at 25° the turnover number with L-malate at *p*H 6.3 is 700 sec.$^{-1}$, while at *p*H 7.5 it is 1,400 sec.$^{-1}$ (see ref. 9 and 12). The conditions for the standard assay were 75 μM L-malate in "tris" acetate of 0.01 ionic strength at *p*H 6.3 and 25°. (More concentrated solutions of the substrate and "tris" acetate were frozen and small aliquots were thawed when needed.)

Results

The method selected for treating inhibition data is based on the rearranged form of equation 3 in which the substrate concentration is divided by the initial steady state velocity.

$$(S)/v = [K_S + (S)]/V_S + (K_S/K_I V_S)(I) \quad (9)$$

From equation 9 it may be concluded that at a particular *p*H a plot of $(S)/v$ *versus* (I) will have a slope of $K_S/K_I V_S$. The reciprocal of this slope at several *p*H values and knowledge of the enzyme concentration are sufficient experimental data to determine pK_{aEI} and pK_{bEI} as may be seen from

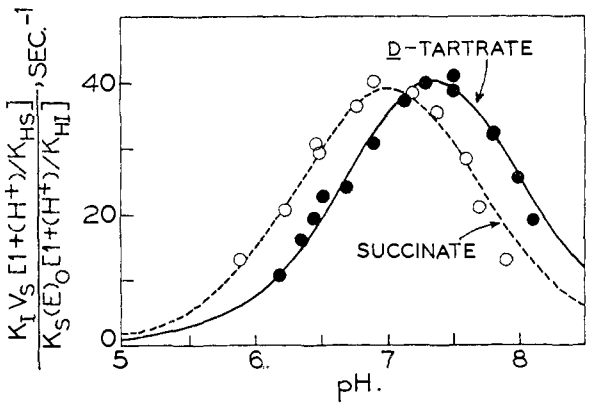


Fig. 3.—Plots of Φ *versus* *p*H for succinate and D-tartrate at 25°; the substrate was L-malate in "tris" acetate of 0.01 ionic strength.

equation 6. Plots of $(S)/v$ *versus* (I) were prepared for a range of *p*H values between 5.3 and 8.3 using 40 and 80 μM L-malate and five different concentrations of each inhibitor. The highest inhibitor concentrations were 2 mM succinate, 1.3 mM D-tartrate, 2 mM *L*-tartrate, and 0.1 mM *meso*-tartrate. The results of a series of experiments with *meso*-tartrate at *p*H 6.1 are given in Fig. 2. The lines through the points have been obtained by the method of least squares.

The maximal velocity (V_S) was calculated from the difference between two $(S)/v$ values at a particular (I) and two substrate concentrations. (V_S is independent of (I) for competitive inhibition.) The Michaelis constant was calculated from the intercepts of the $(S)/v$ *versus* (I) plots at two substrate concentrations and K_I was calculated from K_S, V_S and the slope of the $(S)/v$ *versus* (I) plot.

The V_S values were converted to turnover number $[V_S/(E)_0]$ with the V'_S value of 1,700 sec.$^{-1}$ given in references 9 and 12, and the experimental $K_S/K_I V_S$ values, K_{HS} and K_{HI} were used to determine Φ. The kinetic parameters are plotted as a function of *p*H in Figs. 3–5; the solid line curves were calculated by substituting the experimental values of K_{aEI}, K_{bEI}, K_{EHI}, V's and K's into equation 6. The K_{aEI} and K_{bEI} values were determined from the plots of Φ *versus* *p*H given in Figs. 3–5 with the following equations.[14]

$$K_{aEI} = (H^+)_a + (H^+)_b - 4(H^+)_{max} \quad (10)$$

$$K_{bEI} = (H^+)_{max}^2/K_{aEI} \quad (11)$$

In equations 10 and 11, $(H^+)_{max}$ represents the hydrogen ion concentration at the maximum, and $(H^+)_a$ and $(H^+)_b$ represent the hydrogen ion concentrations at the half maximum points on the acid side and the basic side of the bell-shaped plots of Φ *versus* *p*H.

The value of K_{EHI} is determined from the following equation where Φ_{max} represents the value of Φ at the maximum of the bell-shaped curves of Figs. 3–5.

$$K_{EHI} = K'_S \Phi_{max}[1 + 2(K_{bEI}/K_{aEI})^{1/2}]/V'_S \quad (12)$$

A K'_S of 8.5 μM was determined in these experiments (see equation 4). The acid dissociation con-

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

TABLE I

Dissociation constants of the free enzyme and the four inhibitor–fumarase compounds, in "tris" acetate of 0.01 ionic strength at 25°. The substrate was L-malate in these experiments.

	pK_a	pK_b	K_{EHSI} (μM)	K_{EHI} (μM)	K_{EI} (μM)
Free enzyme	6.3	6.9	.....	.....	.....
Succinate-enzyme compound	6.5	7.5	1.7×10^2	3.2×10^2	1.2×10^3
D-Tartrate–enzyme compound	6.9	7.8	80	3.4×10^2	2.5×10^3
L-Tartrate–enzyme compound	7.4	7.5	72	1.3×10^3	4.1×10^3
meso-Tartrate–enzyme compound	5.7	7.1	11	2.9	4.6

stants and inhibitor dissociation constants of the inhibitor–fumarase compounds are summarized in Table I.

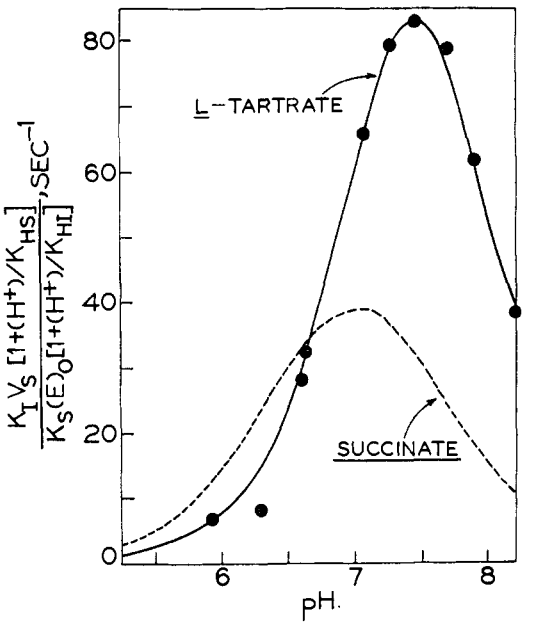


Fig. 4.—Plots of Φ *versus* pH for succinate and L-tartrate at 25°; the substrate was L-malate in "tris" acetate of 0.01 ionic strength.

Discussion

The observations that only one optical isomer of malate (the L form) is a substrate for fumarase and only one monodeutero malate is obtained from fumarate in D_2O solution show that the reaction is stereochemically specific at both the α and β carbons.[15] Furthermore, it has been shown[16] that the deuterium atom added to fumarate by fumarase in D_2O is *trans* to the hydroxyl group when the carboxylate groups are *trans*.

The fact that maleate is not a good competitive inhibitor of fumarase[5] suggests that two cationic groups of the active site interact with the carboxylate groups of the substrate in a *trans* position. These ideas are incorporated in the model shown in Fig. 6 where the carboxylate groups of L-malate are in the *trans* position, group R_L of the active site interacts with the hydroxyl group in the L configuration on the α carbon and group R_D interacts with a hydrogen atom in the D configuration on the β carbon. The groups R_L and R_D are the basic groups of the active site which have a total effect on the kinetics of fumarase; the acidic forms are represented by R_L–H and R_D–H. The macroscopic pK values of the site (considered as a dibasic acid) are represented by pK_{aE} and pK_{bE} for the free enzyme and pK_{aEI} and pK_{bEI} for the inhibitor–enzyme compounds.

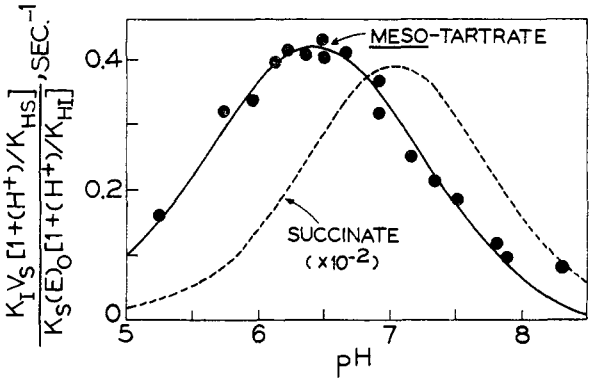


Fig. 5.—Plots of Φ *versus* pH for succinate and *meso*-tartrate at 25°; the substrate was L-malate in "tris" acetate of 0.01 ionic strength.

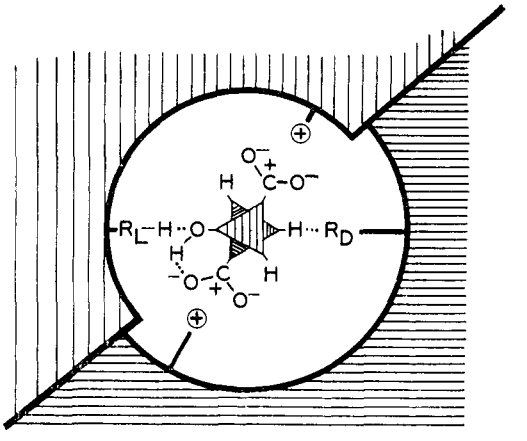


Fig. 6.—Model of the fumarase–L-malate compound based on a *trans*-mechanism for the dehydration of L-malate.

The interactions between the competitive inhibitors and groups R_L and R_D produce changes in pK_{aEI} and pK_{bEI} (see Table I). In particular the hydroxyl groups of D, L or *meso*-tartrate interact with R_L and R_D when the carboxylate groups of the inhibitor bind the cationic groups of the enzymatic site.

One interesting aspect of the data of Table I is the fact that the binding of D and L-tartrate increases the pK values of the site in the inhibitor–enzyme compound (in comparison with the pK values of the succinate–enzyme compound) while the opposite effect is observed with *meso*-tartrate.

(15) H. Fisher, C. Frieden, J. S. McKinley McKee and R. A. Alberty, THIS JOURNAL, **77**, 4436 (1955).

(16) O. Gawron and T. P. Fondy, *ibid.*, **81**, 6333 (1959); F. A. L. Anet, *ibid.*, **82**, 994 (1960).

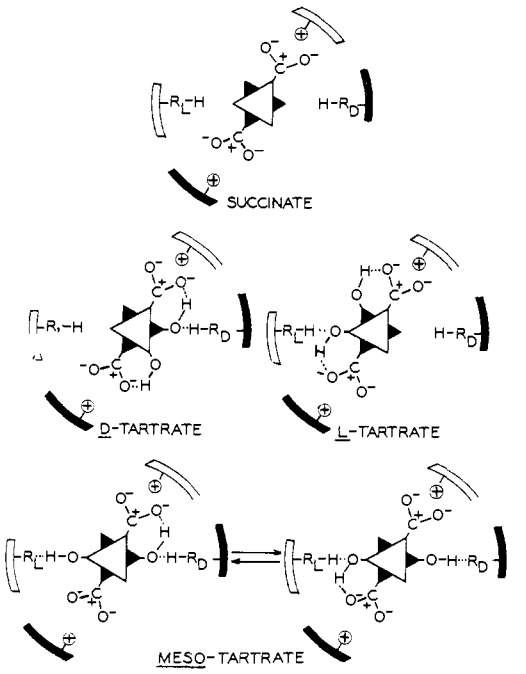


Fig. 7.--Models of the doubly protonated forms of the active site for the succinate, D-tartrate and L-tartrate compounds of fumarase. Models for the two intermediate ionized forms of the active site for the *meso*-tartrate compound of fumarase. (Shaded structures are in the background and C–H hydrogen atoms are omitted for simplicity.)

The hydroxyl group of an inhibitor may act as an electron donor in the formation of a hydrogen bond with R_L–H or R_D–H or as an electron acceptor in the formation of a hydrogen bond with R_L or R_D. These interactions are shown in the following equilibria, where the part of the inhibitor bearing a hydroxyl group is represented by HOI and the hydrogen bond dissociation constants of an R group of the enzymatic site in combination with HOI are represented by K_{I1} and K_{I2}

$$\begin{array}{ccc} R\text{–}H\cdots O\ [H]\ I & \overset{K_{aRI}}{\rightleftharpoons} & R\cdots HOI + H^+ \\ \downarrow\uparrow K_{I1} & & \downarrow\uparrow K_{I2} \\ R\text{–}H + HOI & \overset{K_{aR}}{\rightleftharpoons} & R + HOI + H^+ \end{array} \qquad (13)$$

The acid dissociation constant of the R group in combination with HOI, which is one of the microscopic acid dissociation constants for the D, L or *meso*-tartrate compound of fumarase, is represented by K_{aRI} and the corresponding constant for the free R group is K_{aR}. The interaction of the carboxylate groups of the inhibitors with the cationic groups of the enzymatic site affect the pK values of the R groups of the enzymatic site (see Table I). Thus, a K_{aRI} value for one of the tartrate–fumarase compounds should be compared with the K_{aR} of the succinate–fumarase compound rather than with the K_{aR} for the free enzyme.

The equation which expresses the interdependence of the four equilibrium constants in (13) is $K_{aRI} = K_{aR}(K_{I1}/K_{I2})$. This equation shows that if $K_{I2} >> K_{I1}$ for an inhibitor-enzyme compound, K_{aRI} will be smaller than K_{aR}. The mathematical relationships between the microscopic acid dissociation constants and K_{aEI} and K_{bEI} will be discussed again; at this point it is sufficient to observe that a decrease in one of the four microscopic constants will cause a decrease in K_{aEI} and K_{bEI}. Since K_{aEI} and K_{bEI} for the D and L-tartrate compounds are smaller than for the succinate compound, the above mechanism indicates that the hydroxyl groups of the inhibitor act as electron donors in the formation of hydrogen bonds with R_L–H and R_D–H.

In the case where $K_{I1} >> K_{I2}$ for an inhibitor-enzyme compound, K_{aRI} will be larger than K_{aR} and the K_{aEI} and K_{bEI} values will be larger than the corresponding values of the succinate-fumarase compound. This shows that the acid strengthening effect of *meso*-tartrate may be due to the formation of a hydrogen bond between a hydroxyl group and the basic forms of R_L and R_D.[17]

It is interesting to speculate that groups R_D and R_L may be chemically identical and that the active site of fumarase is symmetrical. The data obtained with D-tartrate and L-tartrate would support the idea that R_D and R_L are chemically identical if the shift in pK_{aEI} and pK_{bEI} due to the interaction of the D-hydroxyl group with R_D were the same as the shift due to the interaction of the L-hydroxyl group with R_L. The data of Table I show that the pK values of the D-tartrate–fumarase compound are different from the pK values of the L-tartrate–fumarase compound. When the inhibition data are considered in terms of the structure given in Fig. 6, therefore, the hypothesis of a symmetrical site is not supported.

The detection of a group in the enzymatic site which interacts with a hydroxyl group of the competitive inhibitor in the D-configuration is in agreement with the *trans* nature of the hydration of fumarate by fumarase.[16] If the enzymatic hydration of fumarate were *cis*, the hydroxyl groups of D-tartrate could not interact with either proton dissociating group of the active site. For a *cis* model, therefore, the pK_{aEI} and pK_{bEI} values with D-tartrate should be very nearly the same as those observed with succinate. With the *trans* model, on the other hand, R_D interacts with one of the hydroxyl groups of D-tartrate leading to different pK_{aEI} and pK_{bEI} values from those obtained with succinate (see Table I).

The *trans* model of the fumarase active site is utilized in the structures for the inhibitor–fumarase compounds given in Fig. 7. The inhibitor–enzyme compound is shown in the doubly protonated form except with *meso*-tartrate, where the two intermediate ionized forms are shown. In Fig. 7, D and L-tartrate are shown with two hydrogen bonds between the carboxylate and the hydroxyl groups, and *meso*-tartrate is shown with one hydrogen bond of this type. The hydrogen-bonded forms are based on the work of Jones and Soper,[18] who proposed that the acid dissociation constants of the

(17) H. C. Brown, D. H. McDaniel and O. Häfliger, in E. A. Braude and F. C. Nachod, eds., "Determination of Organic Structures by Physical Methods," Academic Press, Inc., New York, N. Y., 1955, p. 628.

(18) I. Jones and F. G. Soper, *J. Chem. Soc.*, 1836 (1934).

tartaric acids indicate the presence of a double $-COO^- \cdots HO-$ bonded form for D (and L) tartrate and the absence of this double hydrogen bonded form for *meso*-tartrate.

The microscopic acid dissociation constants of the inhibitor–enzyme compounds would be more useful than the macroscopic constants. Physical methods for distinguishing between the two intermediate ionized forms of the enzymatic site would be necessary to determine the microscopic constants, but there is little chance of obtaining this information for a high molecular weight enzyme like fumarase. All the microscopic dissociations of R_L and R_D in the D-tartrate-fumarase compound are represented in Fig. 8. The macroscopic constants (K_{aEI}) and (K_{bEI}) are related to the microscopic constants (K_1, K_2, K_3 and K_4) by these relationships[19,20]

$$K_{aEI} = K_1 + K_2$$
$$1/K_{bEI} = 1/K_3 + 1/K_4 \qquad (14)$$
$$(K_{aEI})(K_{bEI}) = K_1K_3 = K_2K_4$$

The equilibrium constant for conversion of one intermediate ionized form of the site to the other is K_Z.

$$K_Z = K_1/K_2 = K_4/K_3 \qquad (15)$$

From the foregoing relationships it is readily shown that when two identical proton dissociating groups do not interact, $K_1 = K_2 = K_3 = K_4$ and $K_{aEI} = 4K_{bEI}$ (pK_{aEI} is 0.6 *p*H units lower than pK_{bEI}). It should be noted that this separation of pK values is actually found for the free enzyme at 0.01 ionic strength (see Table I), but the pK values for the fumarase compounds with succinate, D-tartrate and *meso*-tartrate are separated by more than 0.6 *p*H units.

The results with L-tartrate given in Table I are quite unusual since pK_{aEI} is approximately equal to pK_{bEI} and pK_1 and pK_2 are higher than pK_3 and pK_4. (See equations 14). This observation suggests that the addition of the first proton to the enzymatic site in the L-tartrate–fumarase compound slightly increases the affinity of the site for the second proton. A K_{aEI}/K_{bEI} ratio of unity, within the experimental error, is also found for the free enzyme at 0.1 ionic strength.[11] These observations are reminiscent of the oxygenation of haemoglobin; as the four haem groups are oxygenated, the successive oxygen dissociation constants have lower values.[21]

The K_{EH_2I}, K_{EHI} and K_{EI} values of Table I are equilibrium constants which illustrate the effect of the proton binding of the active site on the affinity for different inhibitors. It may be seen from Table I that the K_{EHI} value of the *meso*-tartrate–fumarase compound is lower than the K_{EH_2I}. This result shows that the intermediate ionized (and enzymatically active) form of the enzymatic site has the greatest affinity for *meso*-tartrate; the addition of a second proton to the site weakens the binding.

(19) E. Q. Adams, THIS JOURNAL, **38**, 1503 (1916).

(20) J. T. Edsall, in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943. p. 75.

(21) J. Wyman, in M. L. Anson and J. T. Edsall, "Advances in Protein Chemistry," Vol. IV, Academic Press, Inc., New York, N. Y. 1948, p. 407.

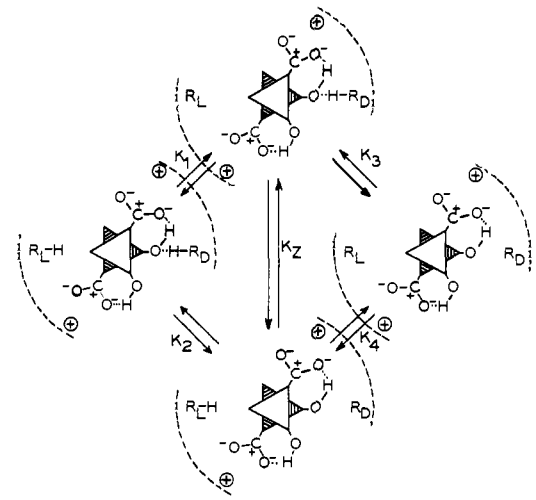


Fig. 8.—The microscopic acid dissociations of the fumarase active site for the D-tartrate–enzyme compound.

The ΔF^0 values for the displacement of succinate from the enzymatic site by D, L and *meso*-tartrate are given in Table II for the three ionized forms of the site. These values are considered to give approximately the free energies of the formation of hydrogen bonds between the tartrates and the enzymatic site.

TABLE II

THE ΔF^0 FOR THE DISPLACEMENT OF SUCCINATE FROM THE ACTIVE SITE BY D,L OR *meso*-TARTRATE TO FORM THE DOUBLY PROTONATED TARTRATE-FUMARASE COMPOUND (EH_2I), THE INTERMEDIATE IONIZED COMPOUND (EHI) AND THE COMPLETELY IONIZED COMPOUND (EI)

Fumarase compound formed	ΔF^0 (kcal. mole^{-1}) for displacement of succinate		
	EH_2I	EHI	EI
D-Tartrate	−0.5	0	+0.4
L-Tartrate	−0.5	+0.8	+0.7
meso-Tartrate	−1.6	−2.8	−3.3

Although differences in ΔF^0 values of less than 0.5 kcal. mole^{-1} may not be experimentally significant, weak hydrogen bond formation is indicated for the doubly protonated tartrate–fumarase compounds. In the case of the intermediate ionized form and the completely ionized form of the D and L-tartrate compounds of fumarase, however, the tartrates are less strongly bound than succinate and there is no evidence of hydrogen bond formation. The positive ΔF^0 values could be due to steric hindrance between the hydroxyl groups (which do not interact with R_D and R_L) and the enzymatic site.

The ΔF^0 values of −2.8 and −3.3 kcal. mole^{-1} in the case of the *meso*-tartrate–fumarase compound are suggestive of fairly strong hydrogen bonding. The ΔF^0 for the *meso*-tartrate compound is similar to that obtained for the association of hydrogen-bonded dimers in non-polar solvents. For example, a ΔF° of −3.6 kcal. mole^{-1} is obtained for the association of the δ-valerolactam dimer in CCl_4 at 25°.[22]

The temperature dependence of ΔF^0 could be used to determine the enthalpies and entropies of the inhibitor dissociation and proton dissociation of

(22) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," W. H. Freeman and Co., San Francisco, 1960, p. 218.

the fumarase active site. A comparison of these thermodynamic parameters with those for reactions of low molecular weight compounds should provide further information on the nature of the binding to the active site.

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Optical Rotatory Dispersion Studies. XXXVI.[1] α-Haloketones (Part 7).[2] Demonstration of Boat Form in the Bromination of 2α-Methylcholestan-3-one[3,4]

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Kinetically controlled bromination of the enol acetate of 2α-methylcholestan-3-one yields 2α-bromo-2β-methylcholestan-3-one (V) in which ring A exists in the boat conformation (XIV). This conclusion, first uncovered by rotatory dispersion measurements, was confirmed by hydrogen bromide-promoted equilibration which led to the thermodynamically more stable 2β-bromo-2α-methylcholestan-3-one (VI) accompanied by the rearrangement product 2α-methyl-4α-bromocholestan-3-one (VII). Bromination of the enol acetate of cholestan-3-one affords directly 2α-bromocholestan-3-one under conditions where 2β-bromocholestan-3-one is stable. These results appear to be inconsistent with Corey's generalizations on the stereochemical course of ketone bromination.

Kinetically controlled bromination in the presence of pyridine[5] of the enol acetate IV of 2α-methylcholestan-3-one (III) affords[6] a monobromo derivative, which can also be obtained[6] by direct bromination of 2α-methylcholestan-3-one (III) itself. The location of the bromine atom at C-2 was established[6] by dehydrobromination to 2-methyl-Δ[1]-cholesten-3-one (VIII) and since infrared[7] and ultraviolet[8] measurements[9] indicated an axial orientation for the bromine atom, the product was assigned[6] the 2β-bromo-2α-methylcholestan-3-one (VI = XV) structure. Such an assumption seemed reasonable on the basis of Corey's generalization[10] that kinetically controlled bromination of a cyclohexanone always affords the axially oriented bromo ketone.

According to the axial haloketone rule[11,12] it would be predicted that a steroidal 2β-bromo-2α-methyl-3-ketone of the 5α-series (*e.g.*, VI) should exhibit a rotatory dispersion curve characterized by a strongly positive Cotton effect. Through the courtesy of Drs. Y. Mazur and F. Sondheimer,[6] a sample of their presumed 2β-bromo-2α-methylcholestan-3-one (VI) was obtained and its rotatory dispersion curve measured. In contrast to the anticipated strongly positive Cotton effect curve, a negative one was observed (see Fig. 1 in ref. 4) and this unexpected rotatory dispersion behavior prompted the presently recorded[4] re-examination of the bromination of 2α-methylcholestan-3-one (III). The results embodied in this article and the two succeeding ones[13,14] demonstrate that the stereochemical course of such brominations is considerably more complicated than envisaged originally[10] and that some modification is required in the currently accepted stereochemical picture[10] of the halogenation of cyclohexanones.

In our hands, the earlier reported[6] synthesis of 2α-methylcholestan-3-one (III) was not as convenient as the hydrogenolysis[15] of 2-hydroxymethylenecholestan-3-one (II). The formation of the enol acetate IV and its kinetically controlled[16] bromination were carried out as described earlier[6] and we were able to confirm the axial orientation of the bromine atom by infrared and ultraviolet spectral measurements (see Experimental). As noted above, the bromo ketone exhibited a negative Cotton effect (Fig. 1 in ref. 4), which according to the axial haloketone rule[11,12] is incompatible with the proposed[6] 2β-bromo-2α-methyl-3-ketone formulation VI. Nevertheless, the rotatory disper-

(1) Paper XXXV, J. Fishman and C. Djerassi, *Experientia*, **16**, 138 (1960).

(2) Part 6, C. Djerassi, E. J. Warawa, R. E. Wolff and E. J. Eisenbraun, *J. Org. Chem.*, **25**, 917 (1960).

(3) Part of the experimental work was performed in the Departments of Chemistry of Wayne State University (Detroit, Mich.) and of Birkbeck College (London). Carl Djerassi and Neville Finch are indebted to the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, for financial assistance (grants CY-2919 and CY-4818).

(4) For preliminary communication see C. Djerassi, N. Finch and R. Mauli, THIS JOURNAL, **81**, 4997 (1959).

(5) E. R. H. Jones and D. J. Wluka, *J. Chem. Soc.*, 911 (1959).

(6) Y. Mazur and F. Sondheimer, THIS JOURNAL, **80**, 5220 (1958).

(7) R. N. Jones, D. A. Ramsay, F. Herling and K. Dobriner, *ibid.*, **74**, 2828 (1952).

(8) R. C. Cookson, *J. Chem. Soc.*, 282 (1954).

(9) The ultraviolet spectral properties are recorded in the Experimental section of the present paper.

(10) (a) E. J. Corey, *Experientia*, **9**, 329 (1953); (b) THIS JOURNAL, **76**, 175 (1954). A critical discussion of this paper will be found in ref. 14.

(11) (a) C. Djerassi and W. Klyne, *ibid.*, **79**, 1506 (1957); (b) C. Djerassi, J. Osiecki, R. Riniker and B. Riniker, *ibid.*, **80**, 1216 (1958).

(12) C. Djerassi, "Optical Rotatory Dispersion: Applications to Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1960, Chapter 9.

(13) R. Mauli, H. J. Ringold and C. Djerassi, THIS JOURNAL, **82**, 5494 (1960).

(14) R. Villotti, H. J. Ringold and C. Djerassi, *ibid.*, **82**, Nov. 5 (1960).

(15) For pertinent references see Y. Abe, T. Harukawa, H. Ishikawa, T. Miki, M. Sumi and T. Toga, *ibid.*, **75**, 2567 (1953); M. Yanagita and R. Futaki, *J. Org. Chem.*, **21**, 949 (1956); H. J. Ringold, E. Batres, O. Halpern and E. Necoechea, THIS JOURNAL, **81**, 427 (1959).

(16) The experimental conditions used by Mazur and Sondheimer (ref. 6) in the bromination of 2α-methylcholestan-3-one (III) itself (in contrast to those employed for its enol acetate IV) do not necessarily imply kinetic control since no particular precaution was taken to remove the hydrogen bromide formed in the bromination of the ketone. However, we have shown in the accompanying two papers (ref. 13, 14) that kinetically controlled bromination of a 2α-methyl-3-keto-5α steroid or its enol acetate leads to similar products.