Kinetic Behavior of Soluble and Particulate Succinic Dehydrogenase. I. Comparison*

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ABSTRACT: Initial rate data at pH 7.8, 30°, for the ferricyanide oxidation of L-chlorosuccinate catalyzed by soluble and by particulate succinic dehydrogenase is presented as $1/v$ *vs.* 1/(S) and $1/v$ *vs.* 1/(ferricyanide) plots. The two preparations differ in that slopes of the $1/v$ *vs.* 1/(S) plots for soluble enzyme decrease with increase in ferricyanide concentration while slopes of the corresponding plots for the particulate preparation increase with increasing ferricyanide concentration. Another fundamental difference is observed in the $1/v$ *vs.* 1/(ferricyanide) plots, initial slopes being positive with soluble enzyme and negative with particulate enzyme. A mechanistic scheme accounting for the kinetic behavior is presented and steady-state equations derived from the scheme are consistent with the data obtained for both enzyme preparations.

Detailed comparison of the kinetic behavior of an enzyme in an isolated, soluble state with its behavior in an insoluble, organized particulate would seem to be of fundamental interest.[1] The availability of soluble enzymes, isolated from the respiratory chain (Singer, 1963), makes such comparisons possible, and it is the purpose of this paper to report on the kinetic behavior of soluble and particulate succinic dehydrogenase. The two preparations show fundamental differences in kinetic behavior and a mechanistic hypothesis is suggested to account for the behavior of the enzyme in the soluble and in the particulate state.

For this study, L-chlorosuccinate, a recognized substrate for succinic dehydrogenase (Gawron *et al.*, 1962; Dervartanian, 1965; Dervartanian and Veeger, 1964) was the substrate of choice since slopes of $1/v$ *vs.* 1/(S) plots are greater with this substrate than with succinate, obviating the need for a competitive inhibitor (Dervartanian, 1965; Slater and Bonner, 1952) and since the reaction product, chlorofumarate, is inert to further enzyme attack. Further, L-chlorosuccinate in contrast to succinate possesses only a pair of *trans*-removable hydrogens (Gawron *et al.*, 1962) and is, therefore, particularly well suited for a study of isotope effects and kinetic data and interpretations obtained herein may be used as a basis for such a study. The oxidant of choice in these studies was ferricyanide since it has been employed in a detailed study of the succinate-soluble succinic dehydrogenase system (Dervartanian, 1965) and provides reproducible results with ease of handling.

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[1] Slater (1955) suggested a kinetic comparison of soluble and particulate succinic dehydrogenase.

Experimental Section

Enzyme Preparations. The particulate preparation employed was a cytochrome *c* deficient Keilin–Hartree muscle preparation prepared from beef heart by the procedure of Tsou (1952) with overnight extraction by phosphate buffer. Two particulate preparations with specific activities at 30° of 0.30 and 0.28 μmole of succinate oxidized/min per mg of protein in the standard assay were used. The soluble enzyme was prepared from the particulate preparation by the method of Wang *et al.* (1956) with the modification of a nitrogen atmosphere (Dervartanian, 1965; Dervartanian and Veeger, 1964). The soluble dehydrogenase was carried through calcium phosphate gel adsorption and one ammonium sulfate precipitation. Three soluble preparations were used with specific activities at 30° ranging from 0.39 to 0.79 μmole of succinate oxidized/min per mg of protein.

Standard Assay. Initial rates at 30° were followed at 455 mμ in cuvets of 1-cm light path in the thermostatted compartment of a Beckmann DB spectrophotometer with attached recorder. The standard assay reaction mixture, 3.0-ml total volume, was that of Dervartanian (1965) and contained 0.1 M phosphate, pH 7.8, 0.001 M potassium cyanide, 3 mg of bovine serum albumin, 0.04 M succinate, 0.006 M ferricyanide, and enzyme.

Kinetic Runs. Initial rates were determined as above, utilizing wavelengths appropriate to the concentration of ferricyanide employed. Reaction mixtures were identical with those utilized in the standard assay except that substrate, L-chlorosuccinate, and ferricyanide concentrations were varied. For the particulate studies, the individual preparations were used in separate runs at protein concentrations of 0.49 (sp act. 0.30) and 0.33 mg/ml (sp act. 0.28) of reaction mixture and the results for the two runs were normalized to the preparation with specific activity of 0.28 and then

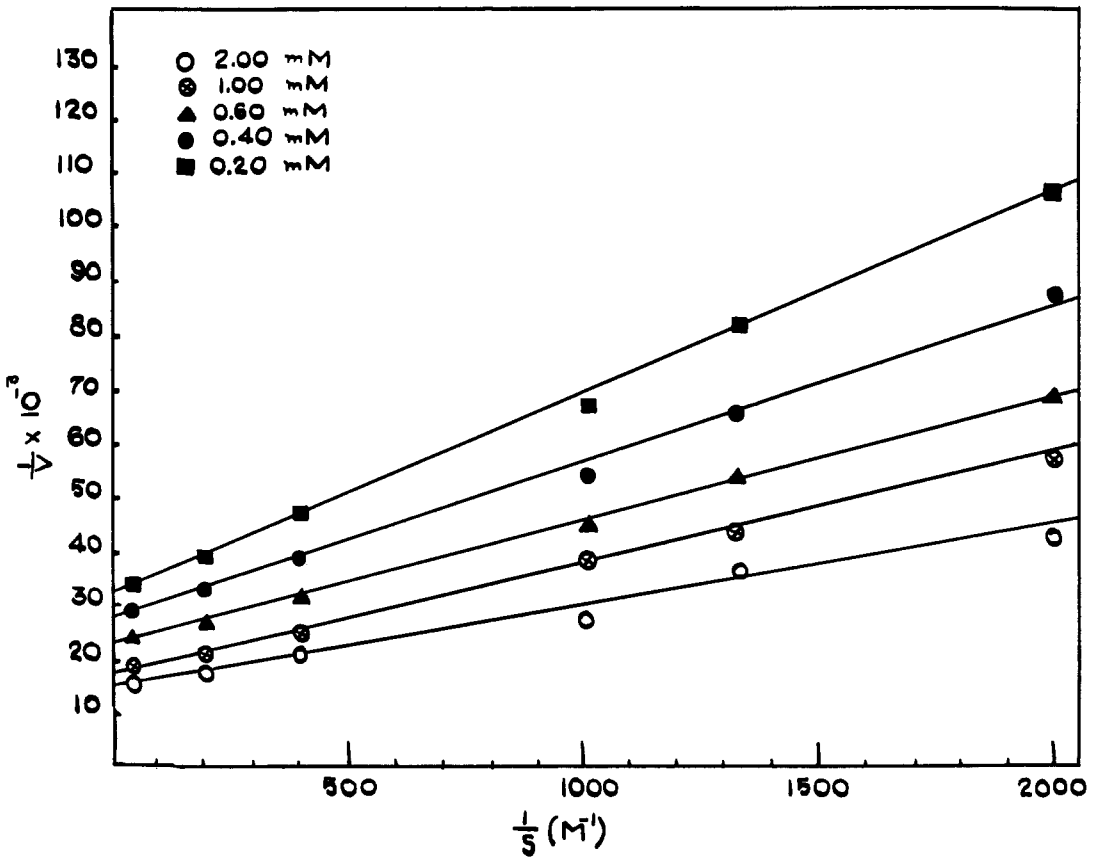

FIGURE 1: $1/v$ *vs.* 1/(S) plots for the oxidation of L-chlorosuccinate by the indicated concentrations of ferricyanide in the presence of soluble succinic dehydrogenase. Ordinate, reciprocal of initial rate, in moles^{-1} × liter × minutes. Average of three runs, 0.17–0.30 mg of protein/ml.

averaged. For the studies with soluble enzyme, three separate runs were conducted with 0.17–0.30 mg of protein/ml of reaction mixture and the results were normalized to a specific activity of 0.423. The unstable soluble enzyme was monitored during each run by the standard assay procedure.

L-*Chlorosuccinate.* L-Chlorosuccinic acid, mp 180–181°, $[\alpha]_D^{23}$ +26° (Gawron *et al.*, 1962), was used to prepare neutral stock solutions. These solutions were prepared fresh daily.

Results

Kinetic data relating initial velocity, v, to substrate concentration, (S), and to ferricyanide concentration, (A), is presented in the form of double-reciprocal plots. For the soluble enzyme $1/v$ *vs.* 1/(S) plots at several ferricyanide concentrations are presented in Figure 1 while the $1/v$ *vs.* 1/(A) plots at several substrate concentrations are presented in Figure 2. The corresponding plots for the particulate preparation are presented in Figures 3 and 4.

Comparing $1/v$ *vs.* 1/(S) plots (Figures 1 and 3) for the two preparations, it is immediately apparent that the preparations are similar in that both exhibit linear $1/v$ *vs.* 1/(S) plots but differ in effect of ferricyanide concentration on the slopes of these plots. Slopes of the $1/v$ *vs.* 1/(S) plots of Figure 1 for the soluble enzyme decrease with increasing ferricyanide concentration while slopes of the corresponding plots (Figure 3) for the particulate enzyme increase with increasing ferricyanide concentration. Comparison of $1/v$ *vs.* 1/(A) plots reveals further differences. With soluble enzyme, $1/v$ *vs.* 1/(A) plots (Figure 2) are linear at low 1/(A) values and fall off at high 1/(A) values while the corresponding plots (Figure 4) for particulate enzyme are linear at high 1/(A) values and show inhibition at low 1/(A) values.

The $1/v$ *vs.* 1/(A) curves of Figure 2 may be represented by eq 1 (Reiner, 1959)

$$\frac{1}{v} = \frac{\frac{a}{[A]^2} + \frac{b}{[A]} + c}{\frac{d}{[A]} + e} \qquad (1)$$

with intercept = c/e, initial slope = $(b/e - cd/e^2) > 0$, final slope = a/d, and extrapolated intercept = b/d. Equation 1 is also of the correct form to fit the $1/v$ *vs.*

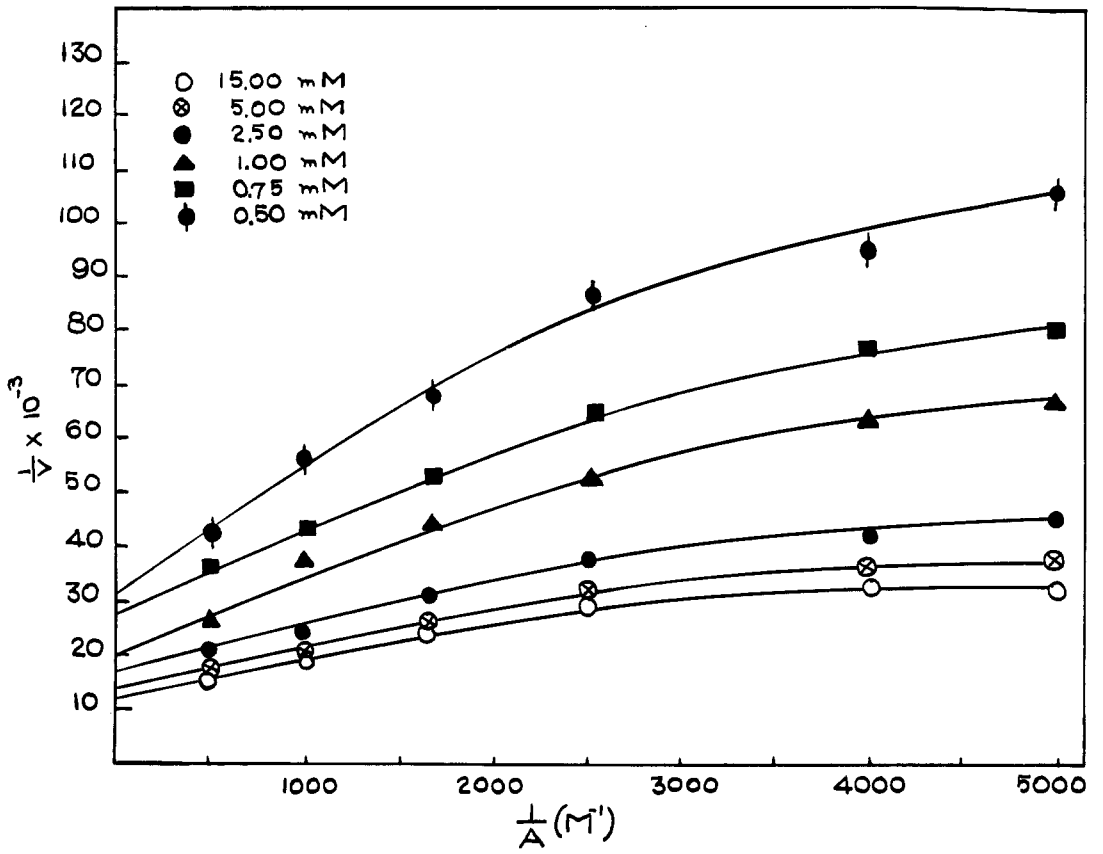

FIGURE 2: $1/v$ *vs.* 1/(A) plots (A = ferricyanide) for the oxidation of indicated concentrations of L-chlorosuccinate in the presence of soluble succinic dehydrogenase. Ordinate, reciprocal of initial rate, moles^{-1} × liter × minutes. Average of three runs, 0.17–0.30 mg of protein/ml.

1/(A) data (Figure 4) obtained with the particulate preparation. In this case, the initial slope is negative, *i.e.*, $b/e - cd/e^2 < 0$. The $1/v$ *vs.* 1/(A) plots of Figure 4 are also fitted by eq 2.

$$\frac{1}{v} = a + \frac{b}{[A]} + c[A] \qquad (2)$$

with $b = c(A)^2$ at the minimum; slope of final segment $= b$; extrapolated intercept of final segment $= a$.

The $1/v$ *vs.* 1/(A) curves of Figure 2 were fitted to the data by eye while the curves of Figure 4 were calculated from eq 2, the constants a and b being approximated graphically and c being calculated from the equation at each experimental point. Values of a and b were adjusted slightly until the average deviation of c values for a given curve seemed minimal.

Discussion

To rationalize the initial rate data and the kinetic relationships, reactions 3–7 are suggested as

$$E + S \rightleftharpoons ES \qquad (3)$$

$$ES \rightleftharpoons E''P \qquad (4)$$

$$E''P \rightleftharpoons E'' + P \qquad (5)$$

$$E + P \rightleftharpoons EP \qquad (6)$$

$$E'' + S \rightleftharpoons E''S \qquad (7)$$

descriptive of the interaction of oxidized enzyme, E, with reduced substrate, S, and in the presence of oxidant, A, reactions 8–10 are

$$E''P + A \rightleftharpoons EP + A_{red} \qquad (8)$$

$$E'' + A \rightleftharpoons E + A_{red} \qquad (9)$$

$$E''S + A \rightleftharpoons ES + A_{red} \qquad (10)$$

suggested for oxidation of the several, E'', E''P, E''S, reduced enzyme species. An essential feature of this scheme is the internal reaction of oxidized enzyme–substrate complex to give reduced enzyme–product complex, reaction 4, this reaction accounting

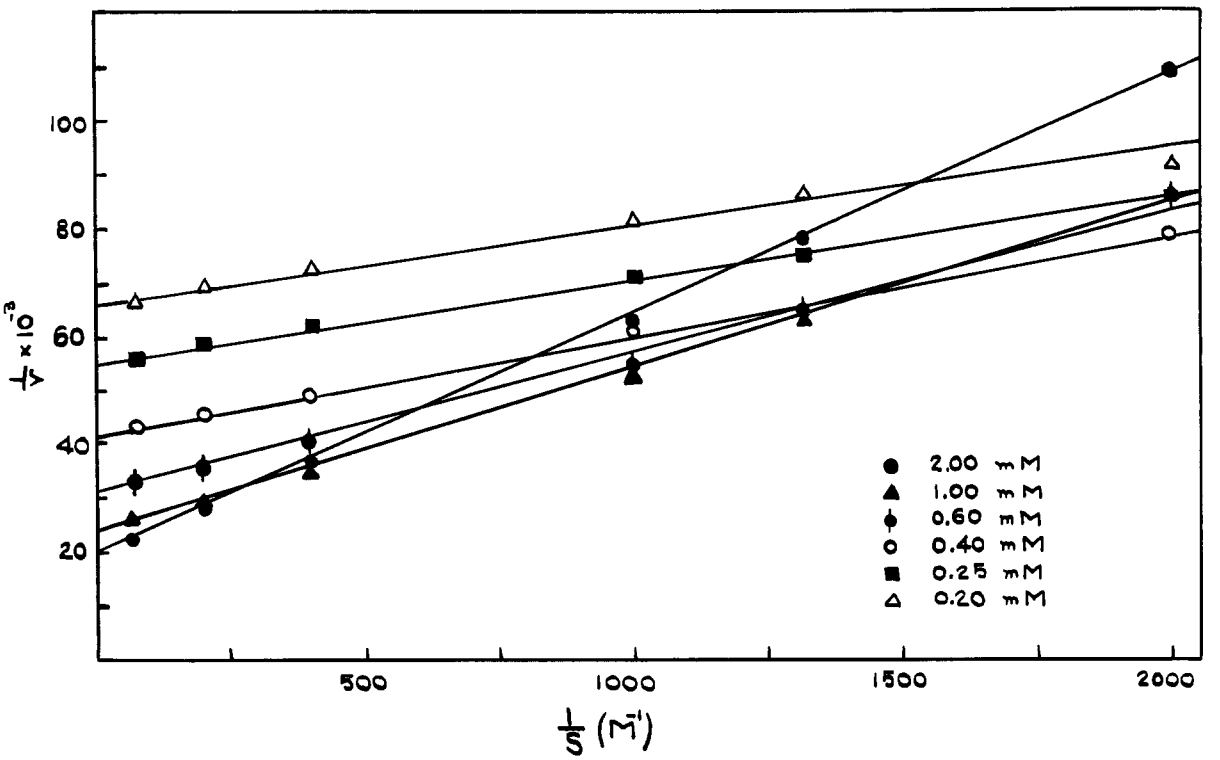

FIGURE 3: $1/v$ *vs.* 1/(S) plots for the oxidation of L-chlorosuccinate by the indicated concentration of ferricyanide in the presence of particulate succinic dehydrogenase, 0.33–0.49 mg of protein/ml. Ordinate, reciprocal of initial rate, moles^{-1} × liter × minutes.

for oxidation of substrate. As written, reaction 4 is a two-electron oxidation and E'' is the fully reduced form of enzyme. Of course, intermediate between ES and E''P there may be one-electron transfers from substrate to enzyme resulting in free-radical forms and it is also possible that fully reduced enzyme exists in a free-radical form. Oxidation of reduced enzyme by ferricyanide, independent of reduced enzyme species, is considered to take place *via* two one-electron transfers, the first being rate limiting. Further, dissociation of EP is not required to be rate limiting.

The scheme, as outlined above, is a departure from those proposed by Slater (1955) and Dervartanian (1965) in that oxidation of substrate by enzyme is considered to occur without intervention of oxidant and that dissociation of oxidized enzyme from oxidized enzyme–product complex, EP, is not considered rate limiting. Of further importance is consideration of reduced enzyme oxidation. While all three species of reduced enzyme are considered capable of being oxidized, oxidation of one or two of the three species may predominate under a given set of conditions. Indeed, as will be shown below, the initial rate data and kinetic relationships are accounted for by assuming that both reactions 8 and 9 must be taken into account with soluble enzyme while reaction 9 is predominant in the particulate preparation. The mechanistic scheme, as adopted here, is, of course, related to the formal mechanisms presented by Dalziel (1957) and Alberty (1953) with extension to the consideration of simultaneous oxidation of several reduced enzyme species. Further, reduced enzyme–reduced substrate complex, E''S, may be considered as an abortive complex (Bloomfield and Alberty, 1963).

The mechanistic scheme may be modified by omission of reactions 7 and 10 in order to provide a simpler scheme for derivation of an expression for initial steady state rates. Omission of reactions 7 and 10 is justified on the grounds that the $1/v$ *vs.* 1/(S) plots (Figures 1 and 3) for both soluble and particulate enzyme are linear over the concentration range studied. Were reactions 7 and 10 appreciable, it would be expected that $1/v$ *vs.* 1/(S) plots would depart from linearity either in the direction of inhibition or activation by substrate.

For initial rates, then, the reaction sequence becomes

$$E + S \underset{2}{\overset{1}{\rightleftharpoons}} ES \qquad (3)$$

$$ES \underset{4}{\overset{3}{\rightleftharpoons}} E''P \qquad (4)$$

$$E''P \xrightarrow{5} E'' + P \qquad (5)$$

$$E''P + A \xrightarrow{7} EP + A_{red} \qquad (8)$$

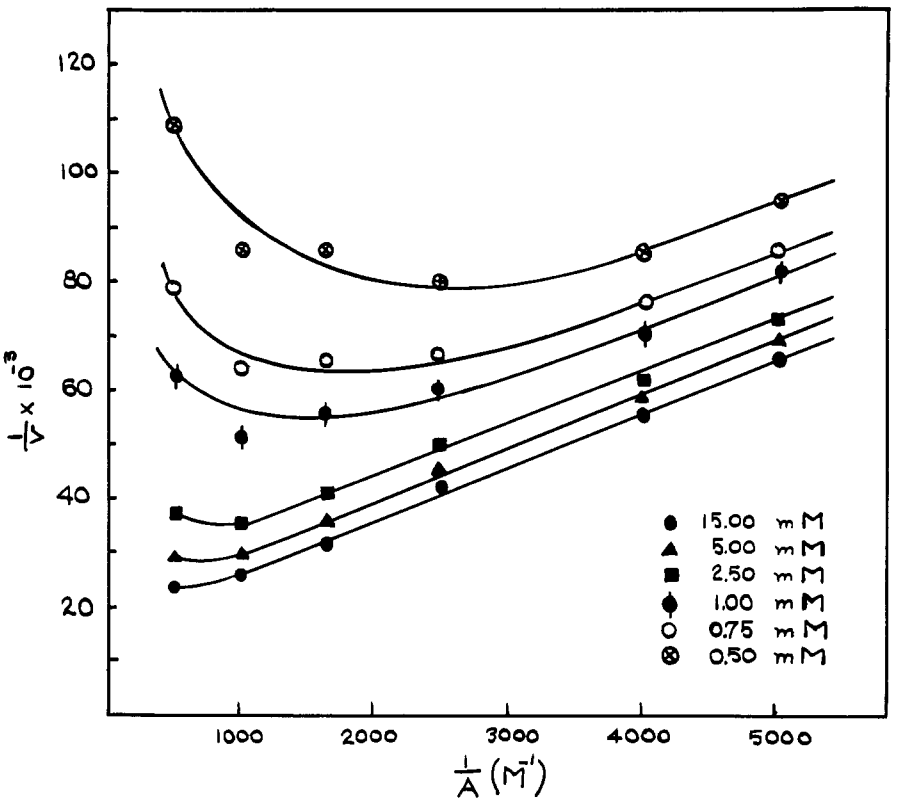

FIGURE 4: $1/v$ *vs.* 1/(A) plots (A = ferricyanide) for the oxidation of indicated concentrations of L-chlorosuccinate in the presence of particulate succinic dehydrogenase, 0.33–0.49 mg of protein/ml. Ordinate, reciprocal of initial rate, moles^{-1} × liter × minutes.

$$E'' + A \xrightarrow{9} E + A_{red} \qquad (9)$$

$$EP \xrightarrow{11} E + P \qquad (11)$$

Steady-state treatment gives for $v_7 = k_7(A)(E''P)$, eq 12, and for $v_9 = k_9(A)(E'')$, eq 13.

Addition of eq 12 and 13 gives for the total initial rate, eq 14, which in reciprocal form becomes eq 15.

Equation 15, linear in 1/(S) and of the same form as eq 1, is obviously qualitatively applicable to the $1/v$ *vs.* 1/(S) data of Figures 1 and 3 and to the $1/v$ *vs.* 1/(A) data of Figures 2 and 4.

The constants corresponding to eq 1 are

$$a = k_1k_3k_5k_{11}[S]$$

$$b = k_9k_{11}\{k_1[S](k_4 + k_5) + k_1k_3[S] + k_2(k_4 + k_5) + k_3k_5\}$$

$$v_7 = \frac{k_1k_3k_7k_9k_{11}E_0[S][A]^2}{k_1[S]\{k_9k_{11}[A](k_4 + k_5 + k_7[A]) + k_3k_9k_{11}[A] + k_3k_5k_{11} + k_3k_7k_9[A]^2\} + k_2k_9k_{11}[A](k_4 + k_5 + k_7[A]) + k_3k_9k_{11}[A](k_5 + k_7[A])} \qquad (12)$$

$$v_9 = \frac{k_1k_3k_5k_9k_{11}E_0[S][A]}{k_1[S]\{k_9k_{11}[A](k_4 + k_5 + k_7[A]) + k_3k_9k_{11}[A] + k_3k_5k_{11} + k_3k_7k_9[A]^2\} + k_2k_9k_{11}[A](k_4 + k_5 + k_7[A]) + k_3k_9k_{11}[A](k_5 + k_7[A])} \qquad (13)$$

$$v = \frac{k_1k_3k_7k_9k_{11}E_0[S][A]^2 + k_1k_3k_5k_9k_{11}E_0[S][A]}{k_1[S]\{k_9k_{11}[A](k_4 + k_5 + k_7[A]) + k_3k_9k_{11}[A] + k_3k_5k_{11} + k_3k_7k_9[A]^2\} + k_2k_9k_{11}[A](k_4 + k_5 + k_7[A]) + k_3k_9k_{11}[A](k_5 + k_7[A])} \qquad (14)$$

$$\frac{1}{v} = \frac{\frac{k_1k_3k_5k_{11}[S]}{[A]^2} + \frac{k_9k_{11}}{[A]}\{k_1[S](k_4 + k_5) + k_1k_3[S] + k_2(k_4 + k_5) + k_3k_5\} + k_7k_9\{k_1k_{11}[S] + k_1k_3[S] + k_2k_{11} + k_3k_{11}\}}{\frac{k_1k_3k_5k_9k_{11}E_0[S]}{[A]} + k_1k_3k_7k_9k_{11}E_0[S]} \qquad (15)$$

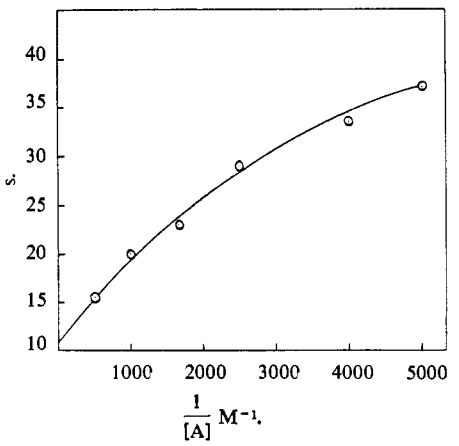

FIGURE 5: Slopes, s, of the $1/v$ *vs.* 1/(S) plots of Figure 1, soluble enzyme, plotted against the reciprocal of the ferricyanide concentration. The curve is calculated from eq 18, using visually estimated intercepts and slope and the experimental data for solution. Constants were adjusted until a close fit was obtained. The points are experimental.

$$c = k_7k_9\{k_1k_{11}[S] + k_1k_3[S] + k_2k_{11} + k_3k_{11}\}$$

$$d = k_1k_3k_5k_9k_{11}E_0[S]$$

$$e = k_1k_3k_7k_9k_{11}E_0[S]$$

Further, considering eq 15 with respect to the $1/v$ *vs.* 1/(A) data for both preparations, it may be noted that the initial slope, s', $b/e - cd/e^2$, of $1/v$ *vs.* 1/(A) plots according to eq 15 is represented by eq 16.

$$s' = \frac{k_1k_4[S] + k_1k_3[S]\left\{1 - \frac{k_5}{k_{11}}\right\} + k_2k_4}{k_1k_3k_7E_0[S]} \quad (16)$$

Equation 16 predicts dependence of the sign of initial slopes on the ratio of k_5/k_{11}. A positive initial slope is predicted for $k_5/k_{11} < 1$ and for $k_5/k_{11} > 1$ provided $k_1k_3(S)[1 - (k_5/k_{11})]$ is numerically less than the other terms in the numerator. This is the case for the $1/v$ *vs.* 1/(A) plots (Figure 2) obtained with soluble enzyme. A negative initial slope is predicted only for $k_5/k_{11} > 1$, provided $k_1k_3(S)[1 - (k_5/k_{11})]$ is numerically greater than the other forms in the numerator. Since a negative initial slope is observed for the $1/v$ *vs.* 1/(A) plots (Figure 4) obtained with particulate enzyme, k_5 must be greater than k_{11} for this preparation. Equation 15, therefore, may be used to also account for the differences in initial slopes of the $1/v$ *vs.* 1/(A) plots for the two preparations.

Considering slopes, s, of $1/v$ *vs.* 1/(S) plots, eq 15 predicts

$$s = \frac{k_7[A](k_2 + k_3) + k_2(k_4 + k_5) + k_3k_5}{k_1k_3k_7E_0[A] + k_1k_3k_5E_0} \quad (17)$$

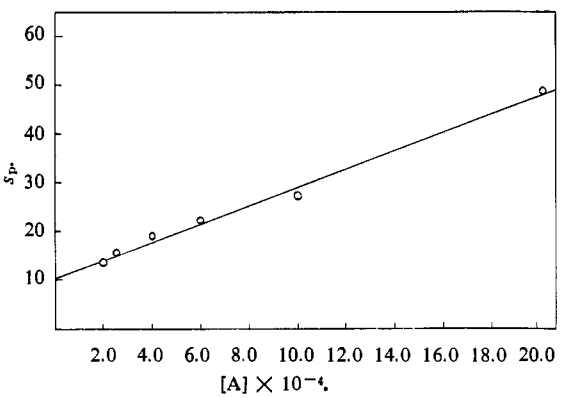

FIGURE 6: Slopes, s_p, of the $1/v$ *vs.* 1/(S) plots of Figure 3 particulate enzyme, plotted against ferricyanide concentration.

Equation 17 on dividing numerator and denominator by (A) is of the same form as

$$s = \frac{\frac{a}{[A]} + b}{\frac{c}{[A]} + d} \quad (18)$$

with initial slope = $(a/d) - (bc/d^2) > 0$; intercept = b/d; and limiting value of $s = a/c$. Equation 17 fits (Reiner, 1959) the nonlinear plot (Figure 5) of slopes, s, *vs.* 1/(A) where the slopes, s, are those taken from the primary $1/v$ *vs.* 1/(S) plots (Figure 1) for the soluble enzyme. The slopes, s_p, of $1/v$ *vs.* 1/(S) plots (Figure 3) for the particulate enzyme, on the other hand, are linear with (A) (Figure 6). This difference in the dependence of slopes of $1/v$ *vs.* 1/(S) plots constitutes, as already noted, a major difference in the kinetic behavior of the two preparations. The linear plot of s_p *vs.* (A) (Figure 6) may also be rationalized on the basis of eq 17 by assuming $k_5 \gg k_7(A)$ for the particulate preparation. On this basis eq 17 becomes

$$s_p = \frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1k_3k_5E_0} + \frac{k_7(k_2 + k_3)[A]}{k_1k_3k_5E_0} \quad (19)$$

and predicts the observed linear relationship between s_p and (A).

Rationalization of the particulate data by eq 15 with the proviso $k_5 \gg k_{11}$ and the assumption $k_5 \gg k_7$ (A), leads to the expectation that v_7 (eq 12) makes a negligible contribution to the total rate for the particulate preparation. Accordingly, the total rate for the particulate preparation should be represented by v_9 (eq 13). Equation 13 in reciprocal form is

$$\frac{1}{v_9} = \frac{(k_3 + k_4 + k_5)}{k_3k_5E_0} + \frac{1}{k_9E_0[A]} + \frac{k_7[A](k_3 + k_{11})}{k_3k_5k_{11}E_0} + \frac{k_2(k_4 + k_5 + k_7[A]) + k_3(k_5 + k_7[A])}{k_1k_3k_5E_0[S]} \quad (20)$$

and it is immediately apparent that eq 20 is linear in 1/(S), of the correct form (eq 2) to fit the $1/v$ *vs.* 1/(A) plots (Figure 4) for the particulate, and correctly predicts the dependence of s_p on (A) (Figure 6). The particulate rate data is thus equally well fitted by the equation for v_9, seemingly justifying the assumption that v_7 makes a negligible contribution to the total rate for the particulate preparation.

The above considerations suggest that of the several reduced enzyme species postulated, only E'', essentially, is oxidized in the particulate preparation while E'' and E''P are oxidized in the soluble enzyme and that this difference in species oxidized accounts for the differences in kinetic behavior of the two preparations. Assuming the correctness of this analysis, several explanations may be advanced. A hindered approach, as might be expected, of oxidant to the particulate would result in a diminished rate for v_7 (reaction 8) without alteration in the rate of dissociation of E''P (reaction 5). Accordingly, oxidation of reduced enzyme, E'', after dissociation from reduced enzyme–product complex would be favored. Alternatively, an unextracted respiratory chain component, coenzyme Q being a likely candidate, in the cytochrome *c* deficient particulate may be interacting with E''P to give another reduced enzyme–product species, E''_1P, which may rapidly dissociate into E''_1 and product. This interaction may be complex, perhaps requiring a protein component, albeit not changing the form of the kinetic equation. It should also be noted here that the inhibitory effect of relatively high ferricyanide concentrations on the particulate preparation (Figure 4) is due to, according to the developed scheme, the slow dissociation of product from EP, oxidized enzyme–product complex, the latter arising by oxidation of E''P (reaction 8).

As might be expected, the reaction scheme may be altered, by changing the nature of reaction 8, without a change in the form of the equations for v_7, v_9, and the total rate. Writing reaction 8 as

$$E''P + A \xrightarrow{7} E + P + A_{red}$$

and, thereby, omitting reaction 11 from the scheme gives equations of the same form albeit with fewer constants. When reaction 8 is written as

$$E''P + A \underset{8}{\overset{7}{\rightleftharpoons}} E''PA$$

and reaction 11 as

$$E''PA \xrightarrow{13} E + P + A_{red}$$

equations of the same form again result, albeit with a greater number of constants. However, when reaction 8 is written to indicate reversible formation of a ternary complex, E''PA, of reduced enzyme, product, and oxidant

$$E''P + A \underset{8}{\overset{7}{\rightleftharpoons}} E''PA$$

which does not react further, then steady-state treatment yields eq 21 for the reciprocal of v_9.

$$\frac{1}{v_9} = \frac{k_3 + k_4 + k_5}{k_3k_5E_0} + \frac{1}{k_9E_0[A]} + \frac{k_7[A]}{k_5k_8E_0} + \frac{1}{k_1E_0[S]}\left[\frac{k_2(k_4 + k_5) + k_3k_5}{k_3k_5}\right] \quad (21)$$

Equation 21 fits the particulate $1/v$ *vs.* 1/(A) data (Figure 4) but not the particulate $1/v$ *vs.* 1/(S) data (Figure 3) since slopes of $1/v$ *vs.* 1/(S) plots, according to eq 21, should be independent of (A) and obviously they are not.

The scheme, as presented, does not require separate "binding sites" for oxidant, ferricyanide in this case, as is often suggested as an explanation for $1/v$ *vs*

TABLE I: Secondary Plots from Primary $1/v$ *vs.* 1/(S) Plots.

Plot[a]	Intercept	Slope[b]	Other
s vs. 1/(A) (Figure 5)	$\frac{k_2 + k_3}{k_1k_3E_0}$	$\frac{k_2k_4}{k_1k_3k_7E_0}$	$\frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1k_3k_5E_0}$
s_p *vs.* (A) (Figure 6)	$\frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1k_3k_5E_0}$	$\frac{k_7(k_2 + k_3)}{k_1k_3k_5E_0}$	
i vs. 1/(A) (Figure 7)	$\frac{k_3 + k_{11}}{k_3k_{11}E_0}$	$\frac{k_4k_{11} + k_3[k_{11} - k_5]}{k_3k_7k_{11}E_0}$	$\frac{1}{k_9E_0}$
i_p *vs.* 1/(A) (Figure 8)	$\frac{k_3 + k_{11}}{k_3k_{11}E_0}$	$\frac{k_4k_{11} + k_3[k_{11} - k_5]}{k_3k_7k_{11}E_0}$	$\frac{1}{k_9E_0}$

[a] *s*, slopes of $1/v$ *vs.* 1/(S) plots of Figure 1, soluble enzyme; s_p slopes of $1/v$ *vs.* 1/(S) plots of Figure 3, particulate enzyme; *i*, intercepts of $1/v$ *vs.* 1/(S) plots of Figure 1; i_p, intercepts of $1/v$ *vs.* 1/(S) plots of Figure 3. [b] Initial slope for nonlinear plot. [c] Limiting value of *s* for Figure 5; final slope for Figures 7 and 8.

TABLE II: Secondary Plots from Primary $1/v$ *vs.* 1/(A) Plots.

Plot[a]	Intercept	Slope
s' *vs.* 1/(S)	$\frac{k_4k_{11} + (k_3k_{11} - k_3k_5)}{k_3k_7k_{11}E_0}$	$\frac{k_2k_4}{k_1k_3k_7E_0}$
i' *vs.* 1/(S)	$\frac{k_3 + k_{11}}{k_3k_{11}E_0}$	$\frac{k_2 + k_3}{k_1k_3E_0}$
i'_e *vs.* 1/(S)	$\frac{k_3 + k_4 + k_5}{k_3k_5E_0}$	$\frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1k_3k_5k_9k_{11}E_0}$
i'_{pe} *vs.* 1/(S)	$\frac{k_3 + k_4 + k_5}{k_3k_5E_0}$	$\frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1k_3k_5E_0}$[b]

[a] s', initial slopes of $1/v$ *vs.* 1/(A) plots for both preparations; i', intercept of $1/v$ *vs.* 1/(A) plots for both preparations; i'_e, extrapolated intercepts of $1/v$ *vs.* 1/(A) plots of Figure 2; i'_{pe}, extrapolated intercepts of final segment $1/v$ *vs.* 1/(A) plots of Figure 4 particulate enzyme. [b] From equation 20, from equation 15 same as corresponding slope for Figure 5.

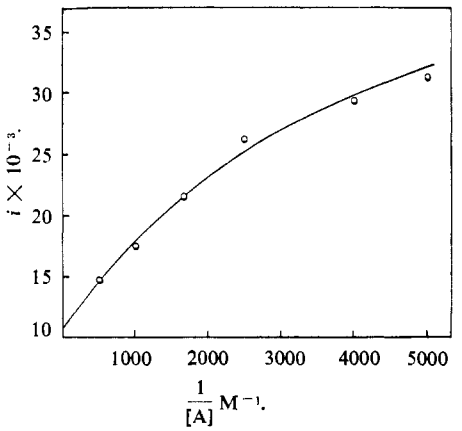

FIGURE 7: Intercepts, i, of $1/v$ *vs.* 1/(S) plots of Figure 1, soluble enzyme, plotted against the reciprocal of the ferricyanide concentration. The curve is calculated from eq 1, using visually estimated intercepts and slopes and the experimental data for solution. Constants were adjusted until a close fit was obtained. The points are experimental.

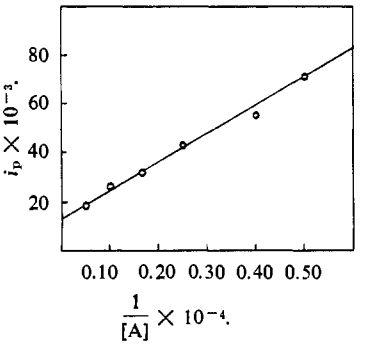

FIGURE 8: Intercepts, i_p, of $1/v$ *vs.* 1/(S) plots of Figure 3, particulate enzyme, plotted against the reciprocal of the ferricyanide concentration.

$$i = \frac{\frac{k_3k_5k_{11}}{[A]^2} + \frac{k_9k_{11}}{[A]}(k_4 + k_5 + k_3) + k_7k_9(k_{11} + k_3)}{\frac{k_3k_5k_9k_{11}E_0}{[A]} + k_3k_7k_9k_{11}E_0} \quad (22)$$

1/(oxidant) curves which are not linear and, apparently, are segmented. In the absence of physical binding data with pure protein, it is also difficult to conceive of a given binding site capable of binding the diverse oxidants that are usually employed with flavoproteins.

Tables I and II list secondary plots which may be derived from the primary $1/v$ *vs.* 1/(S) and $1/v$ *vs.* 1/(A) plots, respectively. Several of these plots are depicted in Figures 5–8. Figures 5 and 6, representing plots of s *vs.* 1/(A) and s_p *vs.* (A), respectively, have already been discussed in connection with the kinetic differences between the two preparations. Figures 7 and 8 present plots of i *vs.* 1/(A) and i_p *vs.* 1/(A), respectively. The curve of Figure 7 is fitted by eq 22 eq 22 being of the same form as eq 1 and being derived from eq 15. Equation 22 is also to be applied to Figure 8 albeit a straight-line plot results from the data. In theory, the curve of Figure 8 should resemble the curves of the $1/v$ *vs.* 1/(A) plots (Figure 4) obtained with the particulate enzyme and, in particular, the $1/v$ *vs.* 1/(A) plots as substrate concentration is increased. Accordingly, the expected initial negative slope would occur at very high concentrations of A, concentrations not obtained in this study. Accordingly, the straight line is the line for the final segment of the curve with slope equal to $1/k_9E_0$ and intercept (extrapolated) equal to $(k_4 + k_5 + k_3)/k_3k_5E_0$. Similar consideration of eq 20 for $1/v_9$ leads to the same conclusions. Further com-

parison of the two preparations is made difficult by the fact that no absolute measure of E_0 is available and because composite constants obtained by dividing slope by intercept, for example, are complex.

Essentially, the over-all reaction scheme, with its several oxidized enzyme species and its several reduced enzyme species is one which permits choice of reaction combinations to meet the demands of a given experimental situation and may well be applicable to, at least, other flavoprotein enzymes. The over-all reaction scheme may, of course, be written according to the notation of Cleland (1963) and the steady-state equations with rate constants, derived herein, may be transformed into equations containing kinetic constants (Cleland, 1963).[2]

The kinetic relationships obtained in this study with L-chlorosuccinate and soluble enzyme are identical with those obtained by Dervartanian (1965) with succinate and soluble enzyme,[3] albeit the kinetic data for succinate was obtained in the presence of a competitive inhibitor, malonate, the presence of a competitive inhibitor being required to emphasize small differences in the slopes of the $1/v$ *vs.* 1/(S) plots. In the absence of the competitive inhibitor, the slopes of $1/v$ *vs.* 1/(S) plots appear parallel, as found by Massey *et al.* (1961), and lead to a different formulation for the reaction.[4] With regard to the interaction of succinate with the particulate preparation, kinetic relationships, similar to those of L-chlorosuccinate with particulate, have been found (unpublished work, this laboratory).

Of relevance to the present discussion are the observations of Giuditta and Singer (1959). These investigators noted with a Keilin–Hartree particulate, a linear $1/v$ *vs.* 1/(dye) plot, in the presence of 0.02 M succinate, with phenazine methosulfate as the electron-transfer agent to oxygen and a nonlinear $1/v$ *vs.* 1/(dye) relationship, similar to that observed in this study with particulate enzyme, with brilliant cresyl blue as the transfer agent. On the basis of the present concepts, the phenazine curve is considered similar to that for i_p *vs.* 1/(A) (Figure 8) while the brilliant cresyl blue curve corresponds to one of the $1/v$ *vs.* 1/(A) curves of Figure 4. It may also be noted that with a soluble succinic dehydrogenase (Neufeld *et al.*, 1954) Giuditta and Singer found a linear $1/v$ *vs.* 1/(A) relationship with dichlorophenolindophenol as the oxidant, suggesting that with this preparation and oxidant and, as for the other reported observations, over the concentration ranges studied, that this situation corresponds either to the initial or final linear segment of the $1/v$ *vs.* 1/(A) curves of Figure 2. The absence of data on the effect of electron-transfer agent or oxidant concentration on the slopes of $1/v$ *vs.* 1/(A) curves does not permit a definite interpretation of the above results.

The closely related flavoprotein, DPNH[5] dehydrogenase of the respiratory chain, is also of interest. Minkami *et al.* (1962) have noted that an ETP particle (beef heart) gives a $1/v$ *vs.* 1/(ferricyanide) plot, at 0.6 mM DPNH, that consists, as presented, of two linear segments with positive slopes. At 0.15 mM DPNH, however, the $1/v$ *vs.* 1/(ferricyanide) plot is linear. Further, high concentrations of DPNH at a fixed ferricyanide concentration are inhibitory. These observations suggest that substrate reactions such as reactions 7 and 10 and/or substrate inhibition must also be taken into account with this preparation. With soluble beef heart enzyme $1/v$ *vs.* 1/(ferricyanide) plots are linear at all DPNH concentrations, with DPNH being a competitive inhibitor of ferricyanide. Again this makes for a situation complicated by substrate effects. Similar effects have been observed by King and Howard (1962).

Acknowledgments

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[2] We are grateful to one of the referees, Dr. W. J. Ray, Jr., for suggesting this and for sending a detailed kinetic analysis based on these equations.

[3] Dervartanian's data, presented as $1/v$ *vs.* 1/(S) plots, give $1/v$ *vs.* 1/(A) plots that are similar to the corresponding plots (Figure 2) found for L-chlorosuccinate.

[4] The $1/v$ *vs.* 1/(S) data obtained by Massey *et al.* (1961) for the oxidation of D-alanine by oxygen, catalyzed by D-amino acid oxidase, give $1/v$ *vs.* $1/(O_2)$ plots which may be fitted by curves. The mechanism might then be formulated in other terms.

[5] Abbreviations used: DPNH, reduced diphosphopyridine nucleotide; ETP, electron transport particle.

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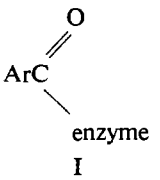
The Influence of pH on the Rate of Hydrolysis of Acylchymotrypsins*

S. A. Bernhard, E. Hershberger, and J. Keizer

ABSTRACT: The pH-dependent first-order rates of hydrolysis of a series of aroyl- and β-arylacryloylchymotrypsins have been examined. Over the range of acyl substituents examined, nonlinear structural correlations are observed in plots of log k_d *vs.* the pK_A of the corresponding carboxylic acid (where k_d is the maximal specific rate of hydrolysis at high pH). The specific rate at any pH, k_{obsd}, can be correlated (in every case examined) with k_d by the expression $k_{obsd} = k_dK_A/(K_A + [H^+])$. The magnitude of K_A, the apparent protonic dissociation constant of the acyl enzyme, is dependent upon the electronic structure of the particular acyl moiety. Plots of log K_A *vs.* the pK_A of the corresponding carboxylic acid exhibit linear structural correlations. Arguments in favor of a chemical interaction between the acyl group and an activity-linked proton dissociable residue of the enzyme are presented.

The influence of pH on the rate of hydrolysis of aroylchymotrypsins of the type I have been previously reported (Bender *et al.*, 1962; Caplow and Jencks, 1962; Bernhard *et al.*, 1965). These hydrolyses can be



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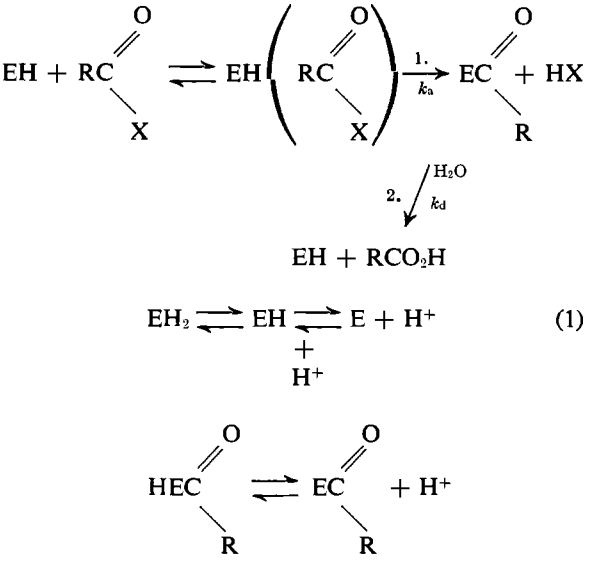
conveniently and directly followed spectrophotometrically at neutral pH, owing to the shifts in the ultraviolet spectra of the acylate anion products relative to the corresponding acyl enzymes. Previous reports concerning the influence of pH on the hydrolytic rate all agree that the hydrolytic rate–pH profile is of the pure sigmoid type illustrated in Figure 1. In an attempt to generalize the mechanistic pathway of chymotryptic hydrolysis of all substrates, the acyl enzyme model (eq 1) has been proposed (Wilson *et al.*, 1950; Hartley and Kilby, 1954; Bender, 1962), and pK_A values have been assigned to catalytic groups within the enzyme site (Bender *et al.*, 1962, 1964).



(1)

The initial *chemical* step in the catalytic sequence (step 1) has been assumed to involve two dissociating groups with pK_A values of approximately 6.7 and 8.7 (Bender *et al.*, 1962; Bender *et al.*, 1963, 1964; Bender and Kézdy, 1964). The monoprotonated species is assumed to be the catalytically active component, in correspondence with the observed "bell-shaped" pH–rate profiles in cases where there is suggestive evidence that this step is rate controlling (Bender *et al.*, 1963). This interpretation of "bell-shaped" curves as an indication of rate-controlling "acylation" has recently been

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