α-Keto Esters as Substrates of Erythrocyte Carbonic Anhydrase. Kinetic Studies of Enzyme-Catalyzed Hydration of Methyl and Ethyl Pyruvate†

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ABSTRACT: The present work shows that bovine carbonic anhydrase powerfully and reversibly catalyzes the hydration of alkyl pyruvates to their corresponding 2,2-dihydroxy derivatives. These investigations were carried out in 2,2-diethylmalonate buffers at 5.0° using a spectrophotometric method. The sigmoidal pH–rate profiles associated with the enzymatic catalysis indicate increasing catalytic efficiency as a function of pH and exhibit inflection points around neutrality (methyl pyruvate, pH 7.0; ethyl pyruvate, pH 6.8). In the pH range studied, it appears that the profiles are predominantly dictated by variations in the respective turnover numbers rather than in K_m. While the turnover numbers favor methyl pyruvate (pH 7.85, k_2 = 53,000 min^{-1}) over ethyl pyruvate (pH 7.85, k_2 = 34,000 min^{-1}), the Michaelis constants are larger for the former substrate (methyl pyruvate, K_m = 0.39 M; ethyl pyruvate, K_m = 0.19 M). The enzymatically catalyzed hydrations are strongly inhibited by acetazolamide. For methyl pyruvate, it is demonstrated that this inhibitor acts in a noncompetitive fashion ($K_i = 2 \times 10^{-5}$ M). The reversible hydrations of the pyruvate esters are also catalyzed by divalent copper ions in acetate buffers (methyl pyruvate, $k_{Cu^{2+}}$ = 38 M^{-1} min^{-1}; ethyl pyruvate, $k_{Cu^{2+}}$ = 31 M^{-1} min^{-1}). The kinetic behavior of carbonic anhydrase in its catalysis of the hydration of the alkyl pyruvate esters is compared with that associated with its hydrase activity with aliphatic and heterocyclic aldehydes and its esterase activity as it pertains to various nitrophenyl esters.

The catalytic versatility of bovine carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) with respect to hydrase and esterase activity has often been demonstrated over the past decade from investigations carried out in these (Pocker and Meany, 1964, 1965a,b, 1967a, 1970; Pocker *et al.*, 1965; Pocker and Stone, 1965, 1967; Pocker and Storm, 1968; Pocker and Dickerson, 1968; Pocker and Watamori, 1971, 1973; Pocker and Beug, 1972; Pocker and Guilbert, 1972, 1974) as well as in other laboratories (Tashian *et al.*, 1964; Malmstrom *et al.*, 1964; Armstrong *et al.*, 1966; Duff and Coleman, 1966; Thorslund and Lindskog, 1967; Verpoorte *et al.*, 1967; Edsall, 1968; Kaiser and Lo, 1969; Lindskog *et al.*, 1971). The present work establishes that this enzyme strongly catalyzes the hydrations of methyl pyruvate ($CH_3COCO_2CH_3$) and ethyl pyruvate ($CH_3COCO_2C_2H_5$) to their corresponding 2,2-dihydroxypropionate esters ($CH_3C(OH)_2CO_2R$). The thermodynamics and the acid–base-catalyzed nature of these reversible hydrations have been studied in detail (Pocker *et al.*, 1971). However, the susceptibility of these processes to any form of enzymatic catalysis has not previously been considered and these substrates are the first of their type which show sensitivity toward the *hydrase* activity of erythrocyte carbonic anhydrase.

In an earlier publication, we reported that the dehydration of 2,2-dihydroxypropionate to pyruvate is catalyzed by carbonic anhydrase (Pocker and Meany, 1970). Unfortunately, accurate determinations of the rate of *hydration* of the pyruvate anion are precluded due to thermodynamic considerations (Pocker and Meany, 1970). On the other hand, the use of the pyruvate esters as substrates for carbonic anhydrase is advantageous not only because their fractions of hydration are favorable (Pocker *et al.*, 1971), but also because the introduction of the pyruvate esters into buffer solutions results in no change in pH.

In the present study, the kinetic characteristics of the novel bovine carbonic anhydrase activity with respect to the hydration of methyl and ethyl pyruvate is compared to its catalytic behavior in the hydrations of various aliphatic aldehydes and the hydrolyses of nitrophenyl esters. Further, this novel hydrase activity toward the ketonic group of an α-keto ester is especially interesting in light of the fact that carbonic anhydrase shows little or no hydrase activity toward certain other substituted ketones, *e.g.*, $ClCH_2COCH_3$ and related compounds (Y. Pocker and J. E. Meany, unpublished observations). A thorough knowledge of the kinetic behavior of pyruvate systems is made even more important by the use of some of these compounds as enzyme modification agents, *e.g.*, bromopyruvate (Göthe and Nyman, 1972; Pocker and Watamori, 1973).

Experimental Section

Bovine carbonic anhydrase was obtained as a highly active lyophilized powder from Mann Research Laboratories. For kinetic purposes, further purification was effected by column chromatography on a DEAE-cellulose column (Pocker and Watamori, 1973). The method of standardization of enzyme solutions employed in the present work was described in a previous publication (Pocker and Meany, 1967a). The substrates, methyl and ethyl pyruvate, were purchased from Aldrich Chemical Co., and were three-times distilled directly

† From the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received September 28, 1973.* Support of this work by grants from the National Institute of Arthritis and Metabolic Diseases (AM 09221) and the Washington State Heart Association is gratefully acknowledged. This paper is part IV of a continuing thermodynamic and kinetic study of the reversible hydration of biologically important α-keto acids and their derivatives. Earlier papers in this study are part I (Pocker *et al.*, 1969), part II (Pocker and Meany, 1970), and part III (Pocker *et al.*, 1971).

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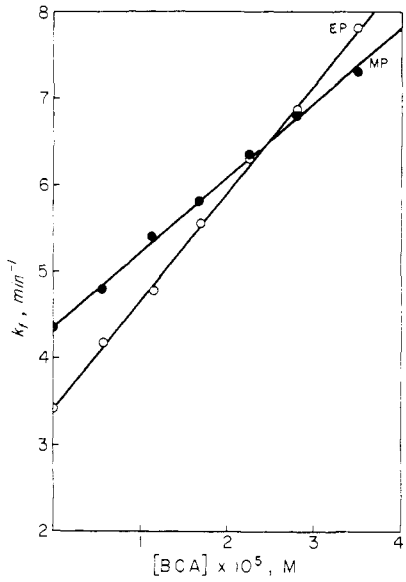

FIGURE 1: Determination of k_{enz} in 0.05 M 2,2-diethylmalonate buffer at pH 6.96 at 5.0°: closed circles (methyl pyruvate, 0.0376 M), slope = k_{enz} = 87,000 M^{-1} min^{-1}; open circles (ethyl pyruvate, 0.0304 M), slope = k_{enz} = 125,000 M^{-1} min^{-1}.

prior to use through a Vigreux column under an atmosphere of nitrogen: bp (methyl pyruvate) 43° (19 Torr) and bp (ethyl pyruvate) 55° (19 Torr). Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, obtained from American Cyanamid (Lederle Laboratory Division), was used without further purification. All buffer components, with the exception of diethylmalonic acid, were commercially available and obtained in reagent grade. The preparation and purification of diethylmalonic acid, as well as the advantages in using the diethylmalonate buffer system for carbonic anhydrase studies, was discussed in our earlier work (Pocker and Meany, 1967b). The ionic strength for all kinetic runs was adjusted to 0.2 M by the addition of the appropriate quantities of sodium chloride or sodium sulfate. Measurements of pH were carried out on a Beckman 101900 research pH meter, or an Orion Model 801 digital pH meter. The reaction kinetics were monitored either by means of a Beckman Kintrac recording spectrophotometer or a Gilford Model 2000 in conjunction with a Beckman monochromator. Results obtained using the two instruments were always in strict accord. The cell compartments of the instruments were maintained at constant temperature by means of a Forma-Temp Jr. refrigerating device, Model No. 2095-1.

The hydrations were initiated by injecting appropriate volumes of the pyruvate esters by means of a calibrated Hamilton syringe, into 3 ml of the reaction solutions. Complete mixing was effected by rapid stirring at the time of injection. The hydration of the carbonyl group was monitored by the diminution of absorbancy either at 270 or 330 nm. Calculated rate constants were independent of the wavelength at which the reactions were followed. The hydrations of methyl and ethyl pyruvate obeyed excellent first-order kinetics for up to four half-lives. The observed rate constants were obtained from the slopes of linear plots of log ($A_t - A_\infty$) against time (where A_t and A_∞ denote the absorbancies of the respective alkyl pyruvates at time t and at equilibrium). The values thus obtained, k_{obsd} = −2.3 × slope, were reproducible to within about 2%. Duplicate runs were generally carried out under each given set of experimental conditions. Owing to the reversibility of the hydration reactions, the observed rate constants actually represent the sum of the forward, k_f and the reverse, k_r, rate constants, $k_{obsd} = k_f + k_r$. All of the rate data presented in the present paper are given in terms of *forward* rate constants, obtained by multiplying k_{obsd} by the fraction of hydration, χ, corresponding to the temperature at which the reactions were carried out: methyl pyruvate, χ = 0.867 (0.0°), 0.850 (5.0°); ethyl pyruvate, χ = 0.842 (0.0°), 0.825 (5.0°) (Pocker *et al.*, 1971).

It has previously been shown (Pocker and Meany, 1970; Kirrmann, 1934) that under the experimental conditions employed in the present work, the hydrolysis of the pyruvate esters is much slower than the hydration to the 2,2-dihydroxypyruvate esters, so much so that the hydration equilibration is essentially complete before any significant hydrolysis has taken place. It is interesting to note that the slower hydrolysis reaction which takes place subsequent to the hydration also appears to be catalyzed by carbonic anhydrase (Y. Pocker and J. E. Meany, unpublished results). This will be the subject of a future communication.

Results and Discussion

In the present work it was noted in all kinetic runs, including those in which substrate concentrations exceed K_m, that the first-order rate plots associated with the enzymatic hydrations of methyl and ethyl pyruvate were linear for up to four half-lives. This observation shows that as the hydrate concentration increases at the expense of that of the ketone, the level of the enzymatic catalysis remains the same. The same finding was reported earlier relative to investigations of the carbonic anhydrase catalyzed hydration of acetaldehyde (Pocker and Meany, 1964, 1965a,b) other aliphatic aldehydes (Pocker and Dickerson, 1968) as well as the pyridinecarboxaldehydes (Pocker and Meany, 1967a,b), and implies that for these systems the affinity of the hydrated and unhydrated species to carbonic anhydrase is similar. This being the case, it may be suggested that the unreactive hydrocarbon portions of the substrates under consideration are strongly implicated in enzyme to substrate binding and hence that hydrophobic interactions are important in the formation of the enzyme–substrate complexes.

The forward rate constants obtained as described in the Experimental Section contain, in addition to the enzymatic component, contributions from water (the "spontaneous" rate constant, k_0) as well as from the basic species present in the reaction solutions (Pocker and Meany, 1970). Thus in 2,2-diethylmalonate buffers, the total catalysis may be represented by eq 1.

$$\chi k_{obsd} = k_f = k_0 + k_{DEM^{2-}}[DEM^{2-}] + k_{OH^-}[OH^-] + k_{enz}[enz] = k_{buffer} + k_{enz}[enz] \quad (1)$$

where DEM^{2-} refers to the 2,2-diethylmalonate dianion, $(C_2H_5)_2C(CO_2^-)_2$. For a series of runs at constant buffer concentration and pH in which the concentration of carbonic anhydrase is the only variable, it may be shown that a plot of k_f against the enzyme concentration results in a straight line, the slope of which is defined as k_{enz}. The intercept values reflect the remaining catalytic components associated with water and the basic buffer components (Figure 1). With the knowledge that indeed k_f is a linear function of enzyme concentration, values of the catalytic coefficient for the enzyme, k_{enz}, may be obtained by subtracting from k_f, the other catalytic components associated with the aqueous buffer employed,

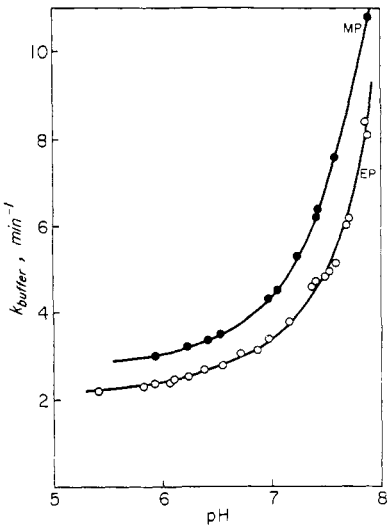

FIGURE 2: Catalysis of the ethyl pyruvate, 0.0304 M (open circles) and methyl pyruvate, 0.0376 M (closed circles) hydration by 0.05 M 2,2-diethylmalonate buffer as a function of pH 5.0°, μ = 0.2 M.

and dividing the difference so obtained by the enzyme concentration.

$$k_{enz} = (k_f - k_{buffer})/[E_0] \quad (2)$$

The pH–rate profiles for the nonenzymatic hydrations of methyl and ethyl pyruvate are shown in Figure 2. When the total amount of diethylmalonate buffer was varied, but the ratio of buffer components was held constant, only negligible differences in k_{buffer} were observed. Thus, the threshold of catalysis associated with the term $k_{DEM^{2-}}[DEM^{2-}]$ can be disregarded in the study of pyruvate ester hydration.

The catalytic rate constant for carbonic anhydrase was determined as a function of pH (Figure 3). The sigmoidal pH–rate profiles show points of inflection at pH 7.0 and 6.8 for methyl pyruvate and ethyl pyruvate, respectively, suggesting that for these substrates, as with several others studied in this laboratory, an ionizable group in the enzyme, having a pK_a around neutrality is involved in catalysis. In contrast to other substrates studied, it should be noted that on the acid side of the sigmoidal pH–rate profile, k_{enz} does not approach zero around pH 6, as has been observed with other hydrase (Pocker and Meany, 1965a,b; Pocker and Dickerson, 1968) and esterase activities (Pocker and Stone, 1965, 1967; Pocker and Watamori, 1971). The "residual" activity decreases further at lower values of pH (k_{enz} = 4.0 M^{-1} min^{-1} at pH 5.81, but k_{enz} = 2.6 M^{-1} min^{-1} at pH 5.40). As one might have predicted, inhibition of this "residual" activity by acetazolamide is not as effective as at neutral pH. With two and one-half times as much inhibitor as enzyme only 56% reduction in activity occurs at pH 5.83, while less than 3% activity remains at pH 7.1. Neither the poorer binding of acetazolamide at low pH (Lindskog, 1969) nor the general protein catalysis can account for *all* of the remaining activity at pH values below 6.0. Control experiments with apoenzyme and bovine serum albumin show that equivalent weights of these proteins produce little or no catalysis.

The enzymatically catalyzed hydration of the pyruvate esters was also studied as a function of substrate concentration. It was observed that in the case of both substrates, Michaelis–Menten kinetics were obeyed. The application of the data to Lineweaver–Burk plots is presented in Figure 4 and the Michaelis constants and turnover numbers deduced from these plots are summarized in Table I. It will be noted

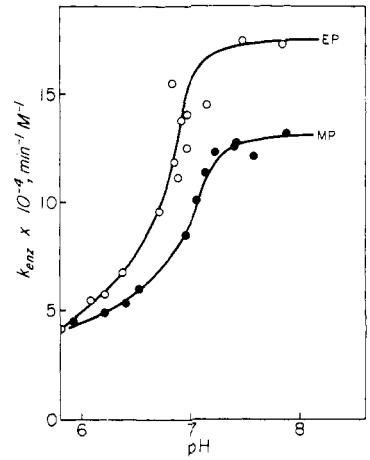

FIGURE 3: The bovine carbonic anhydrase catalyzed hydration of ethyl pyruvate, 0.0304 M (open circles) and methyl pyruvate, 0.0376 M (closed circles) as a function of pH in 0.05 M 2,2-diethylmalonate buffer at 5.0°, μ = 0.2 M.

for methyl pyruvate that the value of K_m in the pH range studied appears to be invariant and consequently the variation in enzymatic activity as a function of pH as illustrated in the pH–rate profile (Figure 3) can be presumed to be due to the actual hydration step rather than the binding step. This result parallels those obtained in the cases of the aldehydic substrates previously studied in this laboratory (Pocker and Meany, 1965a, 1967a; Pocker and Dickerson, 1968).

TABLE I: Michaelis Parameters for the Carbonic Anhydrase Catalyzed Hydration of Pyruvate Esters at 5.0°.[a]

Substrate	MP	MP	MP	MP[b]	EP
pH	6.52	6.96	7.40	6.97	6.96
K_m (M)	0.39	0.39	0.39	0.39	0.19
k_2 (min^{-1})	24,000	34,000	50,000	17,300	25,400

[a] Hydrations monitored in 0.05 M 2,2-diethylmalonate buffers, an ionic strength, μ, of 0.2 M using 5.25 × 10^{-5} M bovine carbonic anhydrase. [b] In the presence of 4.2 × 10^{-5} M acetazolamide.

The substrate concentrations of methyl pyruvate (0.0376 M) and ethyl pyruvate (0.0304 M) used in the determination of the pH–rate profiles illustrated in Figure 3 are much lower than their respective values of K_m. Accordingly the values of k_{enz} in the figure are very nearly equal[1] to k_2/K_m.

The final observation regarding the pH–rate profiles which is of interest is that for the substrate containing the larger hydrocarbon fragment, *viz.*, ethyl pyruvate, the inflection point (pH 6.8) is slightly *lower* than that associated with methyl pyruvate (pH 7.0). This is consistent with the trend noted in the enzymatic hydrations of acetaldehyde, propionaldehyde, and isobutyraldehyde where the pH values at inflection decrease as the sizes of the aldehydes increase.

[1] The constants k_1, k_{-1}, and k_2 are the specific rate constants in the simplified mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

It is realized that the kinetic parameters determined experimentally are considerably more complex than indicated by the three-step formulation (Pocker and Meany, 1967a).

TABLE II: Comparison of Binding Constants or Reciprocal Michaelis Constants of Bovine Carbonic Anhydrase with *p*-Nitrophenyl Esters, Aliphatic Aldehydes, and Pyruvate Esters.

p-Nitrophenyl Esters	k_1/k_{-1} (M^{-1})	Aliphatic Aldehydes	K_m^{-1} (M^{-1})	Pyruvate Esters	K_m^{-1} (M^{-1})
Acetate[a]	500	Acetaldehyde[c]	1.5	Methyl	2.6
Propionate[b]	1,850	Propionaldehyde[d]	5.0	Ethyl	5.2
Isobutyrate[b]	5,630	Isobutyraldehyde[d]	6.7		
n-Caproate[b]	50,000				

[a] In 10% (v/v) acetonitrile at 25.0° (Pocker and Stone, 1967). [b] In 1% (v/v) acetonitrile at 25.0° (Pocker and Storm, 1968). [c] At 0.0° (Pocker and Meany, 1965a). [d] At 0.0° (Pocker and Dickerson, 1968).

The turnover number (Table I) for methyl pyruvate is larger than that for ethyl pyruvate throughout the pH range studied, while the K_m value for ethyl pyruvate is smaller. For carbonic anhydrase, the turnover numbers for both substrates are very high; that for methyl pyruvate at pH 7.40, k_2 = 50,000 min^{-1}, second *only* to that associated with the reversible hydration of CO_2 catalyzed by this enzyme.[2]

Since K_m values appear to be insensitive to changes in k_2 (Table I), the reciprocals of the Michaelis constants should at least approximate the binding constants between the respective substrates and carbonic anhydrase. Thus, it would appear that ethyl pyruvate (K_m = 0.19 M) the substrate having the larger hydrocarbon fragment binds more strongly to the enzyme than does methyl pyruvate (K_m = 0.39 M). Parallel observations have been made regarding the enzymatic hydrolyses of *p*-nitrophenyl esters (Pocker and Stone, 1967; Pocker and Storm, 1968) and aliphatic aldehydes (Pocker and Meany, 1964, 1965a,b; Pocker and Dickerson, 1968) and are summarized in Table II.

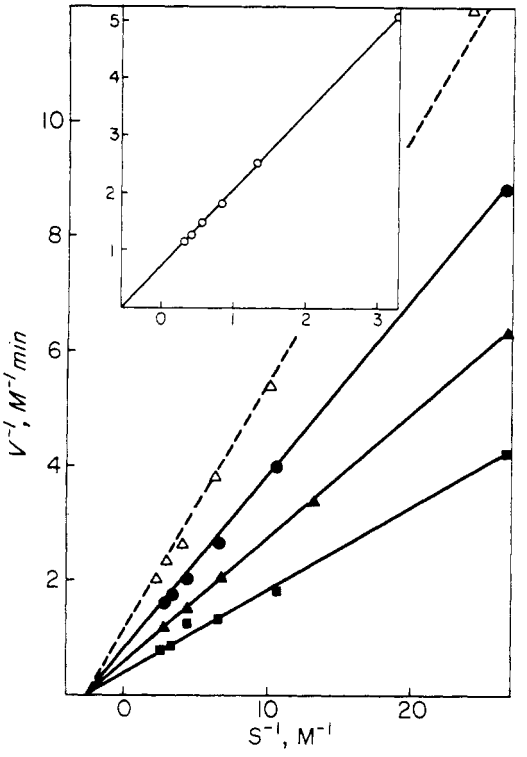

FIGURE 4: Lineweaver–Burk plots of the carbonic anhydrase catalyzed hydration of methyl pyruvate in 0.05 M 2,2-diethylmalonate buffer, at 5.0°, μ = 0.2 M: (●) pH 6.52, (▲) pH 6.96; (■) pH 7.40, (△) (dashed line) methyl pyruvate, pH 6.96 in the presence of 4.2×10^{-5} M acetazolamide, $K_i = 2 \times 10^{-5}$ M. Insert: Lineweaver–Burk plot of the carbonic anhydrase catalyzed hydration of ethyl pyruvate in 0.05 M 2,2-diethylmalonate buffer at 5.0°, μ = 0.2 M (○) ethyl pyruvate, pH 6.96.

The enzymatically catalyzed hydrations of ethyl and methyl pyruvates are strongly and completely inhibited by acetazolamide. For methyl pyruvate, the quantitative kinetics analysis is depicted by Figure 4 in which a comparison of the Lineweaver–Burk plots associated with the inhibited and uninhibited enzymatic processes indicate that acetazolamide behaves as a noncompetitive inhibitor. It is known that acetazolamide binds with carbonic anhydrase at or near the zinc atom (Tilander *et al.*, 1965; Fridborg *et al.*, 1967; Liljas *et al.*, 1969; Kannan *et al.*, 1971; Lindskog *et al.*, 1971). Thus, methyl pyruvate, like carbon dioxide (Forrest, 1953; Keller *et al.*, 1959; Liebman *et al.*, 1961), acetaldehyde (Pocker and Meany, 1965a), propionaldehyde (Pocker and Dickerson, 1968), *p*-nitrophenyl acetate (Pocker and Stone, 1967), and *p*-nitrophenyl propionate (Pocker and Storm, 1968), is not bound to the zinc atom in carbonic anhydrase.

It should be noted that acetazolamide does not inhibit pyruvate ester hydration ($K_i = 2 \times 10^{-6}$ M, pH 6.97, for methyl pyruvate) as potently as acetaldehyde hydration ($K_i = 6.1 \times 10^{-7}$ M, pH 7.2). Anomalous behavior has also been observed in the inhibition of the hydration of other bifunctional substrates, *e.g.*, pyridinecarboxaldehydes. The unusual behavior of pyruvate esters, both with respect to acetazolamide inhibition and relatively high residual activity below pH 6, may be instructive. One possible explanation is that we are dealing with a secondary catalytic site which has a weaker affinity for the sulfonamide inhibitor, and which also possesses residual activity at low values of pH. A second possible interpretation is that the first molecule of pyruvate ester binds nonproductively to the enzyme, giving rise to a modified enzyme system possessing the kinetic characteristics delineated above. The latter explanation has a parallel in the substrate-induced conformational change suggested by Koshland (1958, 1962).

The near proximity of the basic oxygens in the pyruvate esters would suggest the possibility that these compounds could serve as bidentate ligands. The hydration of 2-pyridinecarboxaldehyde, in which the ring nitrogen and the aldehydic oxygen can form a five-membered ring with various divalent metal ions, was shown to be very powerfully catalyzed by a series of divalent metal ions (Pocker and Meany, 1968). Similarly, the same metal ions strongly catalyzed the dehydration of 2,2-dihydroxypropionate (Pocker and Meany, 1970). It was noted in an earlier publication (Pocker and Meany, 1968) that the stability constants of metal complexes of

[2] As reported in an earlier publication, the turnover number for the carbonic anhydrase catalyzed hydration of acetaldehyde is 53,000 min^{-1} at pH 7.64. The reaction is also reversible and when corrected to correspond to the forward process k_2 becomes 37,000 min^{-1} for this aldehydic hydration.

TABLE III: Copper, Water, and Hydronium Ion Catalysis for the Hydrations of Alkyl Pyruvates, and Pyridinecarboxaldehydes[a] and the Dehydration of 2,2-Dihydroxypropionate[b] at 0.0°.

	Methyl Pyruvate	Ethyl Pyruvate	2-Pyridinecarboxaldehyde	2,2-Dihydroxypropionate
$k_{Cu^{2+}}$ (M^{-1} min^{-1})	38	13	1,100,000	10,300
k_{H_2O} (M^{-1} min^{-1})[c]	1.98/55.5	1.55/55.5	0.25/55.5	0.05/55.5
$k_{H_3O^+}$ (M^{-1} min^{-1})	42	41	1.1×10^4	4.2×10^4

[a] Pocker and Meany (1968). [b] Pocker *et al.* (1971). [c] $k_{H_2O} = k_0/55.5$, where k_0 is the spontaneous rate.

N,N'-di(2-hydroxybenzyl)ethylenediamine-*N,N*-diacetic acid (Eplattenier *et al.*, 1967) follow the order $Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Cd^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$, an order which is very similar to that followed by the ability of these same divalent ions to catalyze the hydration of 2-pyridinecarboxaldehyde, the dehydration of 2,2-dihydroxypropionate, the hydrolysis of amino acid esters (Kroll, 1952; Bender and Turnquest, 1957; Koltun *et al.*, 1960) and amides (Meriwether and Westheimer, 1963; Alexander and Busch, 1966), and the tautomerization of acetylacetone and enol (Meany, 1969). The hydrations of the alkyl pyruvates are also catalyzed by divalent metal ions. Illustrated in Figure 5 is the catalytic effect caused by copper ions, so chosen because of their relatively strong catalytic effectiveness for such reactions (Pocker and Meany, 1968, 1970). The figure represents a series of kinetic runs in which the concentration of copper acetate was varied in acetate buffers (pH 4.5) at an ionic strength μ = 0.1 M. The slope of the straight line obtained from a plot of k against $[Cu^{2+}]$ is defined as $k_{Cu^{2+}}$. In Table III is a comparison of the sensitivity of the hydration of 2-pyridinecarboxaldehyde, the dehydration of 2,2-dihydroxypropionate and the hydration of methyl and ethyl pyruvates toward catalysis by divalent copper. The respective spontaneous rates and other catalytic rate coefficients are also included in the table to serve as a basis for comparison. It will be noted that relative to the other substrates, the sensitivity of the alkyl pyruvates toward copper ions is actually quite small. Thus unlike 2-pyridinecarboxaldehyde, a compound in which its carbonic anhydrase catalyzed hydration might possibly involve some interaction with the zinc atom of the enzyme in the formation of the enzyme–substrate complex, one would anticipate that such interaction between the enzyme bound metal and the alkyl pyruvate esters would be relatively insignificant. Again the noncompetitive inhibition brought about by acetazolamide would seem to bear this out.

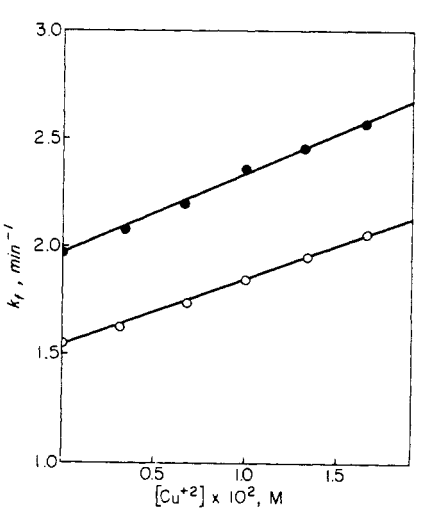

FIGURE 5: Copper ion catalysis of the hydration of ethyl pyruvate, 0.0304 M (open circles), slope = $k_{Cu^{2+}}$ = 38 M^{-1} min^{-1}) and methyl pyruvate, 0.0376 M (closed circles, slope = $k_{Cu^{2+}}$ = 31 M^{-1} min^{-1}) in acetate buffer, pH 4.5, 0.0°, μ = 0.1 M. Concentrations of divalent copper refer to the *total* concentrations of $Cu(OAc)_2$ *formally* present in each reaction mixture.

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pH Dependence of Chymotrypsin Catalysis†

Appendix: Substrate Binding to Dimeric α-Chymotrypsin Studied by X-Ray Diffraction and the Equilibrium Method

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ABSTRACT: The pK_a of the active site of α-chymotrypsin at 25° and ionic strength 0.1 was found to be 6.80 ± 0.04 from accurate determinations of the pH dependence of k_{cat}/K_M for several substrates in conjunction with the pH dependence of the conformational equilibria for the enzyme. Direct measurement of the proton release on binding certain hydrazide substrates reveals that the pK_a of the active site is definitely and significantly lowered in the enzyme–substrate complex. This is accounted for by extending a recently described model for peptide binding to include a hydrogen bond between the hydrazide leaving group and the oxygen of Ser-195. Rapid stopped-flow experiments show that if an intermediate accumulates on the reaction pathway it must do so with a rate constant of greater than 2000 sec^{-1}. The partitioning of acetylphenylalanine-δ-chymotrypsin between formylhydrazine and water at high ionic strength (to minimize primary salt effects due to varying surface charges) increases by about 30% below pH 6.8. This is consistent with an intermediate being on the reaction pathway. Log (k_{cat}/K_M) for the hydrolysis of AcPheONp by δ-chymotrypsin at high ionic strength decreases linearly with pH from pH 5 to pH 2 with unit slope when the conformational equilibria in the enzyme is taken into account. The first ionization of the charge-relay system must be below pH 2. An X-ray diffraction study of formyl-L-phenylalanine semicarbazide bound to dimeric α-chymotrypsin shows that the substrate is bound nonproductively.

The combination of crystallographic and chemical studies has led to a detailed model for the acylation of the serine proteases by specific polypeptide substrates. It is suggested that for subtilisin (Robertus *et al.*, 1972) and for chymotrypsin and trypsin (Fersht *et al.*, 1973) the substrate forms stronger noncovalent bonds with the enzyme when in the transition state rather than in the initial noncovalent complex. The transition state is thought to resemble the tetrahedral intermediate formed by the addition of the hydroxyl of Ser-195 to the substrate carboxyl. Rühlmann *et al.* (1973) have co-crystallized trypsin and the basic pancreatic trypsin inhibitor to give a complex which is thought to resemble an enzyme–substrate complex. They have interpreted their electron density map from X-ray diffraction studies as showing that the complex is the tetrahedral adduct of the enzyme and the reactive peptide bond. The otherwise unstable tetrahedral intermediate is suggested to be stabilized by the features proposed by Fersht *et al.* (1973) and by the relief of strain built into the native inhibitor. Similar results have also been obtained in this laboratory for the soybean inhibitor (Sweet *et al.*, 1974). The question is whether or not a tetrahedral intermediate occurs in the acylation of the enzyme by a normal substrate and whether or not the intermediate is thermodynamically

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