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The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. I. Kinetic Studies of the Enzyme-Catalyzed Hydration of Acetaldehyde*

Y. Pocker† and J. E. Meany‡

ABSTRACT: The present investigation establishes that the catalytic activity of erythrocyte carbonic anhydrase is not limited to the reversible hydration of CO_2 but that the enzyme very powerfully and reversibly catalyzes the hydration of acetaldehyde. pH– and pD–rate profiles were obtained at 0.0° which show points of inflection at pH 7.0 and pD 7.5 for bovine carbonic anhydrase and pH 7.4 for human carbonic anhydrase-C. K_m and V_m values are deduced in both H_2O and D_2O; in the pH range studied, K_m remains essentially constant while V_m/[bovine carbonic anhydrase] dictates the over-all change in enzymatic activity.

Evidence is presented to show that acetazolamide is a powerful noncompetitive inhibitor for the hydration of acetaldehyde; the dissociation constant, K_i, = 6.1×10^{-7} mole $l.^{-1}$ at pH 7.22. It is suggested that the active site for CO_2 and for acetaldehyde hydration is the same.

The existence of a powerful catalyst in erythrocytes, promoting the hydration of CO_2 and the dehydration of HCO_3^-, was first clearly proven by the work of Meldrum and Roughton (1933). Unlike many enzymes, carbonic anhydrase (EC 4.2.1.1) (CA[1]) appears to reversibly promote a reaction which proceeds at an appreciable rate in the absence of any catalyst and is furthermore very susceptible to general base catalysis (Booth and Roughton, 1938; Kiese and Hastings, 1940). The physiological requirement for such a powerful catalyst becomes apparent if account is taken of the short time required by the blood to pass through the living capillaries (Henriques, 1928; H. Hartridge and F. J. W. Roughton, 1925, quoted by Henderson, 1925). CA is widely distributed in the animal kingdom, but it is most readily isolated from the red blood cells where it is present in about 0.1% concentration. Recently important advances have been made in the purification and characterization of bovine carbonic anhydrase (BCA) (Lindskog and Malmstrom, 1962; Lindskog, 1963) and human carbonic anhydrase (HCA) (Nyman, 1961; Rickli and Edsall, 1962; Laurent *et al.*, 1962; Rickli *et al.*, 1964). In purified form, it is a soluble, stable, colorless protein of molecular weight of about 30,000 and contains one atom of rather firmly bound zinc per molecule of enzyme. The zinc is indispensable for enzyme activity (Keilin and Mann, 1940; Lindskog and Malmstrom, 1962). It has often been claimed (Davis, 1961; White *et al.*, 1964) that the most remarkable property of CA is its *absolute* specificity, *i.e.*, a specificity with respect to a single substrate, CO_2. In

*From the Department of Chemistry, University of Washington, Seattle, Washington. *Recieved July 26, 1965*. Support of this work by the National Institutes of Health of the Public Health Service is gratefully acknowledged. Some of the results reported herein have been previously communicated (Y. Pocker and J. E. Meany, 1964, 1965).

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[1] Abbreviations used in this work: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; HCA, human carbonic anhydrase.

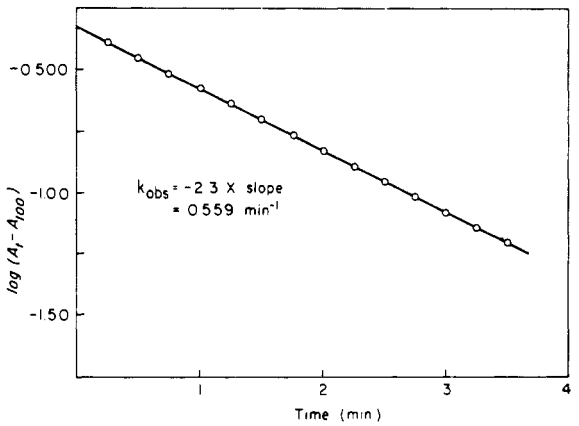

FIGURE 1: A typical run with 8.61×10^{-6} M BCA_I in 0.0020 M phosphate buffer (pH 6.76) at 0.0°.

contrast to this claim we have recently found in our laboratories (Pocker and Meany, 1964, 1965) that erythrocyte BCA and HCA promote not only the reversible hydration of CO_2 but in addition very effectively catalyze the reversible hydration of aldehydes and related carbonyl compounds and also the hydrolysis of certain esters (*e.g.*, *p*-nitrophenyl acetate). These findings have made possible a more comprehensive study of this enzyme which would allow a fuller appraisal of its mode of action. The present paper describes the enzymatically catalyzed hydration of acetaldehyde by both BCA and HCA.

The reaction $CH_3CHO + H_2O \rightleftarrows CH_3CH(OH)_2$ is general-acid, general-base catalyzed (Bell and Darwent, 1950; Bell and Clunie, 1952; Pocker, 1960), and the experimental techniques necessary to obtain accurate rate data are far simpler than those involved in the similar studies pertaining to the reversible hydration of carbon dioxide. Furthermore, the solubility properties coupled with the molar absorption coefficient of acetaldehyde are such that conveniently measured amounts of substrate can be added to buffer solutions to obtain accurate spectrophotometric measurements. Acetaldehyde is a liquid at the temperatures studied, is easily handled and purified, hydrates more slowly than CO_2, and, in contrast to the latter, *involves essentially no pH change* during the course of the reaction.

Experimental Section

The hydration was followed on a Gilford high speed recording spectrophotometer, Model 2000. The cell compartment was thermostated to 0.0 ± 0.1° by means of a Sargent Model SV (S-82060) thermonitor. Acetaldehyde absorbs at a maximum at 278 mμ (ε 16.2), and the pseudo-first-order coefficients, k_{obsd}, were obtained by determining the rate of diminution of this peak. A plot of log $(A_t - A_\infty)$ *vs.* time gives a straight line with $k_{obsd} = -2.3 \times$ slope. Since the reaction is reversible, k_{obsd} is actually a sum of first-order rate coefficients for the forward, k_f, and for the reverse, k_r, processes: $k_{obsd} = k_f + k_r$. The reaction was generally followed for at least two half-lives, and the specific rates obtained were reproducible to about 1%.

Since this reaction is general acid–general base catalyzed, it is necessary to separate the enzymatically catalyzed component of the process from that accelerated by the various acidic and basic species present in the reaction solution. This was accomplished by determining k_{obsd} for a series of runs at constant pH and buffer concentration while varying only the amount of enzyme from about 10^{-6} to 10^{-5} M. The slope obtained from the linear plot of k_{obsd} *vs.* the enzyme concentration we define as k_{enz}. When the maximum concentrations of enzyme were used, the nonenzymic component of catalysis accounted for less than 15% of the total rate.

The acetaldehyde used in these experiments was fractionated immediately before use under nitrogen through a short Heli-Pak column. The aldehyde was introduced into the spectrophotometer cells containing 3 ml of the reaction solution by means of a calibrated Hamilton microliter syringe. The buffer solutions employed in these experiments were usually prepared from the commercially available compounds, analytical or reagent grade, without further purification. However, diethylmalonic acid ($K_2 = 5.1 \times 10^{-8}$) was synthesized by hydrolyzing the ethyl ester in strong alkali. It was purified by repeated recrystallization from ligroin (bp 60–70°).

The pH and pD values (pD = $pH_{reading}$ + 0.41; Glasoe and Long, 1960) of all buffer solutions were determined by means of a Beckman research pH meter, the *relative* accuracy of which is ±0.001 pH units.

Bovine carbonic anhydrase was obtained as a product of Mann Research Laboratories prepared and purified from bovine erythrocytes by the method of Keilin and Mann (1940). This enzyme was stored dry at −20° and its activity was periodically determined by observing its catalytic effect on acetaldehyde hydration in 0.002 M phosphate buffer at pH 7.12. During the course of the experiments described in this paper, no change in activity occurred. Samples of human carbonic anhydrases (HCA) "B" and "C" were generously donated by Drs. J. T. Edsall and J. Armstrong from the Biological Laboratories of Harvard University. The Wilbur–Anderson technique (1948) was used to determine the specific activities of the enzyme preparations employed. The assays are recorded as enzyme (specific activity): BCA_I (1600); BCA_{II} (1700); HCA-B (475); HCA-C (1160).

Although two different samples of BCA were used, BCA_I and BCA_{II}, the experimental results obtained from each, although essentially very similar, were nevertheless kept separate so that each "family" of experiments involved the use of only one of these enzyme samples.

The enzyme solutions were prepared directly before use. From 1 to 8 mg of the enzyme was weighed on a Cahn Model M-10 electrobalance and diluted with 25 ml of the appropriate buffer. The specific rate constants derived from the enzymatically catalyzed portions of acetaldehyde hydration are based on the concentrations

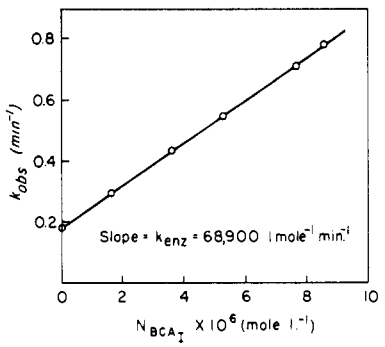

FIGURE 2: Determination of k_{enz} in 0.0020 M phosphate buffer at 0.0°.

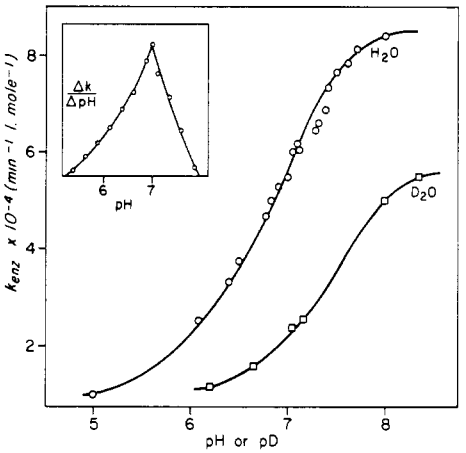

FIGURE 3: The BCA_I-catalyzed hydration of acetaldehyde as a function of pH and pD in 0.0020 M phosphate buffer at 0.0°.

of enzyme calculated from the *weight* of enzyme used. A molecular weight of 30,000 was used in calculating [E] for both BCA and HCA.

Results

The measurement of the rate of acetaldehyde hydration by the spectrophotometric method affords results which are reproducible to within ±1%. A first-order plot derived from spectrophotometric readings observed in a typical run is exemplified in Figure 1. The enzyme concentrations employed were regulated such that the first-order rate constants obtained usually ranged from about 0.1 to 1.0 min^{-1}. It is possible to obtain reproducible data on much faster runs using the equipment described in the Experimental Section.

The Activity of BCA as a Function of pH. One of our first considerations in studying both bovine carbonic anhydrase (BCA) and human carbonic anhydrase (HCA) was to determine their activities with respect to acetaldehyde hydration as a function of pH. For this reason, we evaluated the relative effectiveness of BCA at numerous pH values ranging from pH 5 to 8 in phosphate buffers at 0.0° using 0.0356 M acetaldehyde.

Since each acidic and basic species present in the reaction solution catalyzes the hydration process independently, the enzymatically catalyzed portion must be isolated from the contributions of other catalytically reactive molecules. The total catalysis may be represented by eq 1.

$$k_{obsd} = \Sigma k_i(HA)_i + \Sigma k_j(B_j) + k_{enz}(E) \quad (1)$$

In phosphate buffers, the observed first-order rate constant is a sum of terms as shown in eq 2.

$$k_{obsd} = k_0 + k_{H_3O^+}(H_3O^+) + k_{OH^-}(OH^-) + k_{H_2PO_4^-}(H_2PO_4^-) + k_{HPO_4^{2-}}(HPO_4^{2-}) + k_{enz}(E) \quad (2)$$

Several runs were made at each pH while varying only the concentration of enzyme so that k_{obsd} was determined as a function of enzyme concentration alone. It was observed that the enzymatic catalysis of acetaldehyde hydration was a linear function of BCA concentration from 1 × 10^{-6} M to 1 × 10^{-5} M in BCA. A typical plot of k_{obsd} *vs.* enzyme concentration is shown in Figure 2.

TABLE I: Hydration of Acetaldehyde Catalyzed by BCA_I.[a]

pH	k_{enz}	pH	k_{enz}
5.10	9,800	7.07	61,300
5.37	10,100	7.08	62,100
6.08	25,800	7.27	65,200
6.39	33,200	7.32	65,800
6.49	38,200	7.38	68,900
6.76	46,900	7.41	73,400
6.82	49,900	7.48	76,600
6.90	52,200	7.61	78,200
6.99	54,500	7.70	81,400
7.06	60,600	8.01	83,800

[a] Second-order catalytic constant, k_{enz} (l. mole^{-1} min^{-1}), at various pH values in 0.002 M phosphate buffer at 0.0°.

The slope of the resulting straight line is defined as k_{enz}. Numerically, k_{enz} is very nearly[2] k_2/K_m since the substrate concentration used in all runs for these particular determinations was only about 1/20 as large as K_m, the Michaelis constant for the enzymatically catalyzed process. The catalytic coefficient k_{enz} was evaluated at numerous pH values (Table I) so that a rather de-

[2] 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 may be considerably more complex than indicated by the three-step formulation.

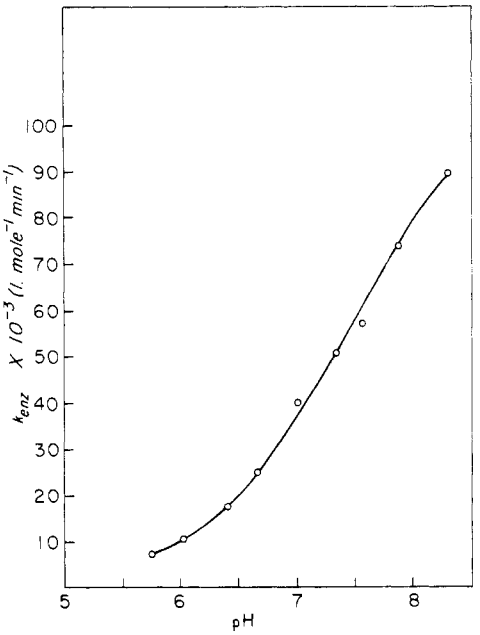

FIGURE 4: The HCA_c-catalyzed hydration of acetaldehyde as a function of pH in 0.01 M diethylmalonate buffer at 0.0°.

tailed pH–rate profile evolved. The resulting sigmoid curve, when k_{enz} is plotted *vs.* pH, is shown in Figure 3. Also shown in this figure is a plot of $\Delta k_{enz}/\Delta pH$ *vs.* pH in order to more accurately locate the pH at which the point of inflection occurs (pH 7.0). Since the point of inflection of a sigmoid curve resulting from such data indicates the vicinity of the pK of an ionizable group which is at least in part responsible for the catalytic activity, the possible importance of histidine residues or a water ligand of protein bound zinc was suggested.

A slight perturbation is apparent around pH 7.4. We believe this variation, although slight, to be real rather than an artifact since approximately 50 runs were made to determine the exact curvature in this region.

The Activity of BCA as a Function of pD. KINETIC ISOTOPE EFFECTS. A procedure similar to that described above was followed using D_2O as the solvent (Table II). The enzymatic activity as a function of pD again is represented by a sigmoid curve. The point of inflection now appears at approximately pD 7.5. This result is to be expected[3] since the pK of an ionizable group of about pK = 7 (such as imidazole) changes by 0.55 pH unit as one substitutes D_2O as solvent for H_2O (Li *et al.*, 1961). Hence, the entire curve in D_2O is displaced toward higher pD by about 0.55 pD unit. Interesting results were obtained when enzymic activity in H_2O was compared to that in D_2O at pH values corresponding to the proper pD values (Table II). In all these comparisons, in the pH range from 5 to 8, the kinetic isotope effect, $k_{enz(H_2O)}/k_{enz(D_2O)}$, is about 1.5.

The Activity of HCA as a Function of pH. The enzymatic activity of HCA "C" was also determined as a function of pH. Table III summarizes the various values of k_{enz} obtained at the pH values studied. A plot of k_{enz} *vs.* pH gives a sigmoid curve with a point of inflection at about pH 7.4, as may be observed from Figure 4. These runs were made in 0.01 M diethylmalonate buffer. This buffer was chosen since its activity as a catalytic acid–base pair is much smaller than that for $H_2PO_4^-$ and HPO_4^{2-}. A similar series of runs was made utilizing phosphate buffers rather than diethylmalonate buffers, and a similar pH–rate profile with a point of inflection at neutral pH emerged.

TABLE II: Relative Effectiveness of BCA_I in D_2O as Compared to H_2O.[a]

pD	k_{enz} (D_2O)	pH	k_{enz} (H_2O)	k_H/k_D
6.19	11,600	5.64	16,000	1.4
6.64	15,800	6.09	25,000	1.6
7.05	23,700	6.50	38,000	1.6
7.14	23,400	6.59	41,000	1.6
7.97	50,500	7.42	72,500	1.4
8.46	55,000	7.91	84,000	1.5

[a] Second-order catalytic constant k_{enz} (l. mole^{-1} min), at various pH values in 0.002 M phosphate buffer at 0.0°

TABLE III: Hydration of Acetaldehyde Catalyzed by HCA-C.[a]

pH	k_{enz}
5.75	7,400
6.02	10,700
6.40	17,300
6.66	24,900
7.01	40,300
7.34	50,600
7.57	57,100
7.88	73,900
8.33	88,400

[a] Second-order catalytic constant, k_{enz} (l. mole^{-1} min^{-1}), at various pH values for HCA-C in 0.01 M diethylmalonate buffer at 0.0°.

[3] When one compares the catalytic activity of an enzyme at a given pH to that at the corresponding pD, it is necessary to choose a pH and pD such that at the values selected the enzyme molecule exists in exactly the same amounts of its ionized forms. That is, if a basic group is catalytically important, the ratio of this group as the free base to the conjugate acid must be the same in D_2O as it is in H_2O when one compares enzymic activity in these two solvents. Since there is a shift in the pK of the catalytically reactive site in BCA of 0.55 unit when one alters the solvent from H_2O to D_2O, this occurs when pD = pH + 0.55.

TABLE IV: Results from Lineweaver–Burk Plots[a] for BCA_{II} in 0.01 M Diethylmalonic Buffers at 0.0°.

pH	7.08	7.22	7.64	7.22[b]	pD = 7.85
K_m(mole $l.^{-1}$)	0.65	0.65	0.61	0.48	2.0
V_m/(BCA) (min^{-1})[c]	42,000	48,000	53,000	4,700	87,000

[a] In general, six different concentrations were used to determine the reciprocal plots using a concentration range $[CH_3CHO]$ = 0.036–0.36 M in H_2O. Fourteen different concentrations with $[CH_3CHO]$ = 0.07–0.55 M determine the reciprocal plot in D_2O. Duplicate runs were often made at each concentration. [b] With 4.11×10^{-6} M acetazolamide. [c] As defined earlier all values of k_{obsd} (and k_{enz}) refer to the sum of the forward and reverse rate constants ($k_f + k_r$). Strictly speaking, V_m obtained from these values are experimental constants which may be corrected to give values corresponding to the forward process by multiplying each by the fraction of hydration (χ_{H_2O} = 0.70 in H_2O; χ_{D_2O} = 0.73 in D_2O).

Michaelis–Menten Kinetics. The enzymically catalyzed hydration of acetaldehyde was also studied as a function of substrate concentration in order to determine whether or not it followed Michaelis–Menten kinetics. The application of this type of data to a Lineweaver–Burk plot is exemplified in Figure 5, and Table IV summarizes the results obtained. These experiments were designed to show (a) the variance of K_m and V_m with pH; (b) the possible inhibitory effect of a sulfonamide on the enzymatically catalyzed hydration; and (c) the relative magnitude of K_m and V_m in H_2O as opposed to the corresponding values in D_2O.

(a) Although studied over a narrow pH range, K_m appears to remain constant and V_m/(BCA_{II}) reflects the expected increase in k_{enz} with increasing pH. From this observation, it would appear that the ionizable group responsible for the point of inflection in the pH–rate profile (Figure 3) is not the binding site of the substrate in the active enzyme–substrate complex.

(b) Acetazolamide appears to act basically as a noncompetitive inhibitor as witnessed by the small variation in K_m but rather large change in V_m/(BCA_{II}). This could reflect the lack of binding between zinc and substrate in the active enzyme–substrate complex. The dissociation constant K_i at pH 7.22 was found to be 6.1×10^{-7} mole $l.^{-1}$.

(c) The Michaelis constant, K_m, is greater in D_2O than in H_2O, indicating greater enzyme to substrate binding in H_2O than in D_2O. However, V_m/(BCA_{II}) is favored in D_2O (Table IV). From the arguments presented earlier, enzyme activity at pD 7.85 should be strictly compared with that at pH 7.30. The value of V_m/(BCA_{II}) at pH 7.30 can be interpolated from the corresponding values at pH 7.22 and 7.64 to be roughly 4.9×10^4 min^{-1}. However, K_m remains essentially constant in this pH range.

Using a different sample of the enzyme (BCA_I), runs were made in order to establish if the reaction obeys Michaelis–Menten kinetics in other buffers. Michaelis–Menten kinetics are obeyed in phosphate buffers, and Table V reveals some exemplifying data, which incidentally also show that K_m does not appreciably change with increasing total buffer concentration from 0.002 to 0.01 M in the pH range in which these data were obtained.

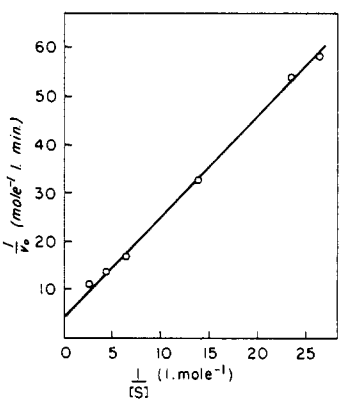

FIGURE 5: A typical Lineweaver–Burk plot for a series of hydration runs in 0.01 M phosphate buffer, pH 7.09, 0.0°.

Discussion

Perhaps the most significant observation which evolved from our research in these laboratories is the fact that both bovine and human carbonic anhydrase are excellent catalysts for the reversible hydration of carbonyl systems other than CO_2. The enzymatically catalyzed hydration of acetaldehyde shows many similarities to the enzymatic hydration of CO_2.

Our quantitative pH profile determinations (Figures 3 and 4) indicate that an ionizable group having pK = 7.0 (BCA) and pK = 7.4 (HCA) is important in the enzymatically catalyzed hydration of CH_3CHO, and the constancy in K_m as a function of pH suggests that the group under consideration is not the binding site. These observations are in accord with those made in the enzymatic hydration of CO_2 (Kiese, 1941; Roughton and Booth, 1946; De Voe and Kistiakowsky, 1961; Kernohan, 1964). Since zinc is essential for catalytic activity (Keilin and Mann, 1940; Lindskog and Malmström, 1962), it has been suggested that either metal-

TABLE V: Results from Lineweaver–Burk Plots[a] for BCA_I in Phosphate Buffers at 0.0°.

pH	7.09	7.68
Buffer	Phosphate	Phosphate
Concentration	0.01	0.002
K_m (mole $l.^{-1}$)[b]	0.49	0.55
$V_m/(BCA_I)$ (min^{-1})[b]	32,000	50,000

[a] Six different concentrations were used to determine the reciprocal plots, using a concentration range $[CH_3CHO] = 0.036–0.36$ M in H_2O. [b] These are experimental constants and in a reversible reaction should be treated as indicated in footnote *c* in Table IV.

bound water or hydroxide is directly transferred to the substrate. It has further been suggested that the pK_a of this zinc aquo complex might occur at neutral pH (Davis, 1958, 1961). The pH–rate profile is also consistent with the participation of an unprotonated imidazole group in the active site. We envisage the imidazole as a promotor (possibly as a general base) of the direct transfer of water (formally OH^-) from the zinc to the substrate. Our suggestion accords with the observation that the binding in the active enzyme–substrate complex is apparently unaffected by pH in the region under consideration. Additionally, it has been shown that the catalytic effectiveness of zinc ions to promote the hydration of acetaldehyde is strongly enhanced in imidazole buffers (Pocker and Meany, 1964, 1965).

It has been observed at pH 7.22 (Table IV) that the addition of 4.11×10^{-6} M acetazolamide reduces $V_m/(BCA_{II})$ by a factor of 10 while causing only a slight reduction in K_m (from 0.65 to 0.48). Since $K_m = (k_{-1} + k_2)/k_1$,[2] the relatively small change in its value with added inhibitor is consistent with the observed decrease in k_2. Acetazolamide is found to be a noncompetitive inhibitor in the hydration of CO_2 (Forrest, 1953; Keller *et al.*, 1959), and our work shows that it behaves as a noncompetitive inhibitor in the hydration of acetaldehyde. The above inhibitory effects of acetazolamide suggest that the active site of CA in the hydration of acetaldehyde is essentially the same as in the corresponding CO_2 reaction.

The relative effectiveness of BCA in H_2O as compared to D_2O in the hydration of CH_3CHO is instructive. There is evidence suggesting the generality of a deuterium isotope effect of 2–3 when reaction mechanisms involve general acid–general base catalysis, while lower values seem to be observed when nucleophilic catalysis is operative (Bender *et al.*, 1962). For the catalysis of CH_3CHO hydration by BCA, we observed a value of $k_{enz(H_2O)}/k_{enz(D_2O)} = 1.5$ for the over-all catalysis, which was constant throughout the pH range studied. A more detailed analysis separates the components of k_{enz} into K_m and V_m. By studying the effect of substrate concentration on enzymatic catalysis at pH 7.30 *vs.* pD 7.85, we were able to show that formation of enzyme–substrate complex is favored in H_2O over D_2O ($K_{m(D_2O)}/K_{m(H_2O)} \sim 3$). Such an isotope effect could suggest the formation of the active enzyme–substrate complex through the participation of hydrogen bonding (possibly *via* tyrosine). It is known that the dissociation constants of phenols are about 3–4 times greater in H_2O than in D_2O (Martin and Butler, 1939). However, it is realized that dissociation constants are not *entirely* analogous to the ability of a compound to bond its hydrogen.

We also observed that the breakdown of the enzyme–substrate complex is slightly more facile in D_2O as opposed to H_2O ($V_{m(D_2O)}/V_{m(H_2O)} \sim 2$). In the present paper we have analyzed our H_2O *vs.* D_2O effects in terms of a simplified hydration mechanism involving a single intermediate. However, the detailed roles of the enzyme-bound Zn and of imidazole in the hydration process are far from being fully understood and leave open the possibility that a rational interpretation may necessitate the inclusion of more intermediates in the hydration mechanism. Kinetic isotope effects of deuterium oxide must still be interpreted with the reserve with which respect for the long arm of coincidence should always engender. They provide at present no compelling evidence for the mode of hydration although they could be interpreted to imply the transfer of (nucleophilic attack by) H_2O (or ^-OH) from protein-bound zinc to enzyme-bound acetaldehyde. In this respect it is interesting to note that ^-OD is a better base than ^-OH by a factor of about 1.5 (Pocker, 1959a,b). It is of course fully realized that as it exists in the enzyme an incipient ^-OH (or ^-OD) must be considered and that nucleophilicity is not always axiomatic with basicity. However, due to the structural similarities between OH^- and OD^- it seems reasonable to assume that OD^- may likewise be a slightly better nucleophile than OH^-.

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Trifluoroacetylated Cytochrome *c**

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ABSTRACT: The lysine residues of horse heart cytochrome *c* can be trifluoroacetylated by reaction with ethyl thioltrifluoroacetate and the blocking groups subsequently removed by mild alkaline hydrolysis. The regenerated material displays full electron-transfer activity in the succinate oxidase system and moves on Amberlite CG-50 in the same manner as fraction I of unmodified preparations.

Trifluoroacetylated cytochrome *c* containing no amino groups detectable by dinitrophenylation or treatment with nitrous acid is without activity in the succinate oxidase system, but displays, over the range of pH 6–10, Soret and visible spectra essentially unchanged from those of nontrifluoroacetylated samples. By tryptic hydrolysis of the trifluoroacetylated protein, a heme peptide of 38 amino acid residues, representing approximately one-third of the cytochrome molecule, has been prepared.

Treatment of horse heart cytochrome *c* with acetic anhydride, under conditions leading to acylation of about half the lysine residues, has been reported to result in complete inactivation of the molecule (Minakami *et al.*, 1958; Takemori *et al.*, 1962). It is not known whether all groups other than lysine remained unmodified in these experiments, but the inference generally drawn, and consistent with various other observations, is that the loss of appreciable numbers of positively charged groups leads to disruption of essential structural features of the protein and impairs its binding to other components of the electron-transfer chain (*e.g.*, Margoliash, 1961, 1962).

Reaction with ethyl thioltrifluoroacetate (Schallenberg and Calvin, 1955) would thus be anticipated also to bring about loss of activity. This reaction is, however, of special interest in that trifluoroacetylamide linkages can be cleaved hydrolytically under conditions where peptide bonds remain intact, a circumstance which makes possible the use of trifluoroacetyl (TFA)[1] groups

* From the Department of Biochemistry, Yale University, New Haven, Conn. *Received June 21, 1965.* This work was supported by grants from the National Institutes of Health, U. S. Public Health Service (GM-07317), and the National Science Foundation (GB-1556).

† Predoctoral fellow supported by a research training grant from the National Institutes of Health, U. S. Public Health Service (5T1-GM-53).

[1] Abbreviations used in this work: TFA, trifluoroacetyl; TFA-cytochrome *c*, trifluoroacetylated cytochrome *c* prepared as described.