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The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. V. Kinetic Studies of Enzyme-Catalyzed Hydrations of Aliphatic Aldehydes*

Y. Pocker† and D. G. Dickerson

ABSTRACT: The present investigation demonstrates that bovine carbonic anhydrase is a powerful catalyst for the reversible hydrations of propionaldehyde and isobutyraldehyde. The enzyme does not catalyze the hydration of pivalaldehyde, and, furthermore, it does not even appear to bind detectable amounts of this aldehyde. Hydrase activity rises with pH between 4.3 and 8; although the pH profiles for both propionaldehyde and isobutyraldehyde are sigmoidal, the former is characterized by an inflection around pH 6.6 whereas the inflection obtained with isobutyraldehyde is around 5.6. The profile for propionaldehyde reflects the variation in the turnover number with pH. Acetazolamide functions as a noncompetitive inhibitor of enzyme activity with propionaldehyde as substrate but serves as a competitive inhibitor of markedly diminished inhibitory capacity in the enzymic hydration of isobutyraldehyde. Thiocyanate and azide anions, while acting as noncompetitive inhibitors with propionaldehyde, are without detectable effect on the enzymic hydration of isobutyraldehyde.

Various aliphatic alcohols inhibit both reactions in a competitive fashion. Several similarities were noted between the binding of aldehydes and alcohols, most significant perhaps was the observation that *t*-butyl alcohol, in analogy with pivalaldehyde, does not appear to bind to bovine carbonic anhydrase. Observations relevant to the binding step are interpreted as being indicative of the importance of hydrophobic interactions in substrate binding. The data are analyzed in terms of a hydrating site of variable conformation depending on the nature of the substrate and containing an imidazole residue, the bound substrate, and protein-bound zinc–aquo complex.

It has been demonstrated in these laboratories that erythrocyte carbonic anhydrase (carbonate hydrolyase EC 4.2.1.1) (CA)[1] is not, as has been previously thought, an absolutely specific catalyst for the reversible hydration of carbon dioxide, a catalysis which does indeed embody its physiological function, but it also powerfully catalyzes the reversible hydrations of acetaldehyde and various pyridine aldehydes (Pocker and Meany, 1965a,b, 1967a; Pocker *et al.*, 1965). In addition, it has been demonstrated that carbonic anhydrase is a very potent esterase with respect to a variety of esters of *o*- and *p*-nitrophenol (Pocker and Stone, 1965, 1967; Pocker and Storm, 1968; *cf.* also Tashian *et al.*, 1964;

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase.

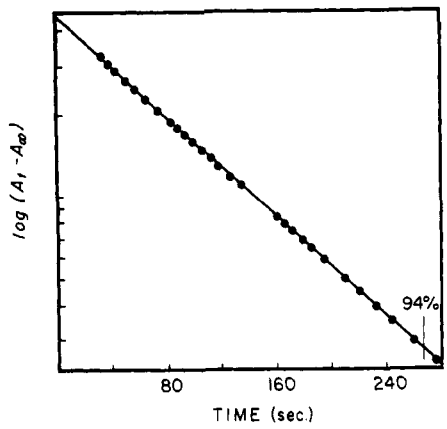


FIGURE 1: First-order rate plot for the hydration of 0.0833 M propionaldehyde at 0.0°: 15.4×10^{-6} M BCA_{II} in 0.0100 M diethylmalonate with 0.20 M *n*-butyl alcohol inhibitor (pH 7.25) followed at 302 mμ. Plot furnishes $k_{obsd} = 10.4 \times 10^{-3}$ sec^{-1}, $k_{enzyme} = 558$ M^{-1} sec^{-1}, $K_{eq} = 2.04$.

Malmström *et al.*, 1964; Armstrong *et al.*, 1966). The present study was undertaken in an effort to define the limit of this catalytic versatility with respect to one class of compounds, namely, aliphatic aldehydes.

There are many distinct advantages in studying this hydrase activity. Unlike carbon dioxide, the aldehydes are all liquids at room temperature, and as a consequence of their ultraviolet spectra, the concentrations of their respective unhydrated forms are readily ascertained throughout the course of the reaction. Because of the relatively simple kinetic technique utilized, very accurate kinetic data are furnished by the present systems. In addition, the general acid–general base catalyzed hydrations of these aldehydes are thoroughly understood (Pocker, 1960; Pocker and Meany, 1965a, 1967b; Y. Pocker and D. G. Dickerson, submitted for publication; *cf.* also Bell, 1966). Most significant of all, their hydrations involve no changes in pH, a complicating factor which is unavoidable in the hydration of CO_2 and the dehydration of HCO_3^-. This investigation considers the catalytic activity of BCA with respect to the hydrations of propionaldehyde, isobutyraldehyde, and pivalaldehyde. In the present study, propionaldehyde is shown to behave in a manner very similar to acetaldehyde with respect to the shape of the pH profile, the fact that this shape is dictated solely by the variation with pH of the turnover number, and the fact that acetazolamide functions as a potent noncompetitive inhibitor in both systems. The hydration of pivalaldehyde, at the other extreme, is shown not to be catalyzed by BCA, inasmuch as this aldehyde does not even appear to bind to the enzyme. Isobutyraldehyde appears to be intermediate in behavior: the pH profile, while similar to that of propionaldehyde, is displaced *ca.* 1.0 pH unit; acetazolamide inhibits in a competitive fashion; and noncompetitive anionic inhibitors of enzymic activity with respect to propionaldehyde (such as N_3^- and SCN^-) fail to detectably inhibit the enzymic hydration of isobutyraldehyde. An additional scrutiny of the steric requirements of the binding site is provided by an investigation of the influences of various alcohols which function as competitive inhibitors of enzyme activity with both substrates.

Experimental Section

Materials. All aldehydes were purchased from J. T. Baker and fractionally distilled, under nitrogen, through a 20-cm column of Heli-Pak. Acid content was titrimetrically determined to be less than 0.05 mole % following distillation and remained below 0.20 mole % for several weeks when stored in the dark at −5° in nitrogen-flushed containers. Sodium salts of diethylmalonic acid, utilized as a buffering medium, were prepared from the diethyl ester by a method previously described (Pocker and Meany, 1965b, 1967a). Acetate buffers were prepared from the reagent grade materials of Baker and Adamson. Acetazolamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide) was obtained both in protonated form and as the disodium salt from the Lederle Laboratories Division of the American Cyanamide Co.

Bovine carbonic anhydrase (BCA) was a product of Mann Research Laboratories prepared and purified from bovine erythrocytes by the method of Keilin and Mann (1940). Standardization of BCA solutions was accomplished by zinc analysis employing both atomic absorption spectrophotometry and the dithizone technique of Lindskog and Malmström (1960). Concentrations based on zinc content, assuming a molecular weight of 30,000, were in accord with a molar extinction coefficient at 280 mμ of ε 54,000. The BCA was determined to contain a mixture of A and B isomers in a ratio of *ca.* 2:3. This was ascertained by examination of the electrophoretic pattern obtained in 7% polyacrylamide gels and by chromatography on DEAE-cellulose. Further confirmation of the standardization results was achieved by examining the effect of the specific inhibitor, acetazolamide, on the apparent enzymic activity of BCA with respect to the hydration of propionaldehyde. Indeed, a plot of relative activity as a function of the ratio of inhibitor concentration to enzyme concentration was shown to extrapolate to a concentration ratio of unity at zero activity. The absence of nonproteinic catalysts was demonstrated by the observation that the supernatant obtained by centrifugation of thermally denatured enzyme solutions hydrated both propionaldehyde and isobutyraldehyde at the same rate, within ±1%, as the buffer solution employed in this control experiment. Denaturation was affected in diethylmalonate buffers by heating the enzyme containing solutions to 60–70° for 30 min. The supernatant obtained by centrifugation had a pH of 6.99 and its catalytic efficiency was compared with that of diethylmalonate buffer of the same pH.

Apparatus. A Beckman Model DU ultraviolet spectrophotometer was used to monitor the rate of change in absorbance. The silica cells containing the reactants were placed in a specially constructed cell compartment containing a mixture of methanol and water which was thermostatted at 0.0° by circulation of a coolant through a coil located in the bottom of the compartment. The temperature of the water–ethylene glycol coolant was

TABLE I: Equilibrium Constants of Aldehyde Hydrations at 0.0°.

Aldehyde	λ_{max} (mμ)	ϵ_0[a]	K_{eq}	χ
CH_3CH_2CHO	277.5	18.5	1.98	0.665
$(CH_3)_2CHCHO$	284	22.5	1.58	0.613
$(CH_3)_3CCHO$	285.5	18.5	0.47	0.32

[a] Extinction coefficients (M^{-1} cm^{-1}) of unhydrated aldehydes in aqueous solution, as determined at λ_{max}.

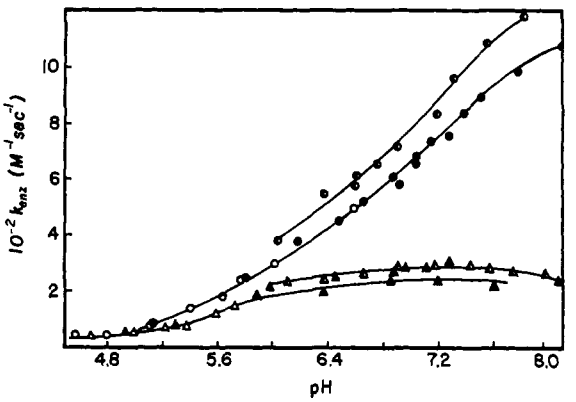


FIGURE 2: The BCA-catalyzed hydrations of propionaldehyde and isobutyraldehyde at 0.0° as a function of pH. (○) 0.0463 M propionaldehyde, 0.0100 M acetate buffer, and BCA_{II}; (●) 0.0463 M propionaldehyde, 0.0100 M diethylmalonate buffer, and BCA_{II}; (◐) 0.0463 M propionaldehyde, 0.0100 M diethylmalonate buffer, and BCA_I; (△) 0.0367 M isobutyraldehyde, 0.0100 M acetate buffer, and BCA_{II}; (▲) 0.0367 M isobutyraldehyde, 0.0100 M diethylmalonate buffer, and BCA_{II}; (◭) 0.0367 M isobutyraldehyde, 0.0100 M diethylmalonate buffer, and BCA_I.

maintained using a Forma-Temp Jr. refrigerating device Model No. 2095-1 which contained, in addition to the thermostatting mechanism, a pump to circulate the coolant. The phototube housing in the Beckman DU spectrophotometer required frequent change of desiccant in view of the condensation problem resulting from the low temperature utilized in this work. The pH of the reaction solutions were determined at 22° without dilution by using a Beckman Model H2 glass electrode pH meter. Suitable corrections were applied for each buffer system to deduce the pH corresponding to 0.0°.

Method. The technique used to measure reaction rates was essentially the same as that described by Pocker and Meany (1965a,b) for acetaldehyde. The aldehydes were injected into buffered aqueous solutions of BCA from calibrated Hamilton microliter syringes. The rate of approach to equilibrium was monitored by observing the rate of diminution of absorbance at the appropriate wavelength (see Results) and the pseudo-first-order rate constants, k_{obsd}, were evaluated by dividing the slope of the kinetic plots of log $(A_t - A_\infty)$ *vs.* time by -2.3 (Figure 1). The ratio of the extrapolated intercept $A_0 - A_\infty$ of such a kinetic plot to the equilibrium absorbance, A_∞, furnished a value of the equilibrium constant, $K_{eq} = (A_0 - A_\infty)/A_\infty$, which defines the ratio of equilibrium hydrate concentration to equilibrium aldehyde concentration. Because the rate of approach to equilibrium was determined, the values of k_{obsd} were equal to the sum of rate coefficients for the forward reaction, k_t, and for the reverse reaction, k_r: $k_{obsd} = k_t + k_r$. To obtain k_t, it is necessary to multiply k_{obsd} by the fraction of hydration, $\chi = K_{eq}/(K_{eq} + 1)$.

Because the relationship between k_{obsd} and enzyme concentration is linear, values of a catalytic coefficient for the enzyme, k_{enzyme}, were obtained by subtracting from k_{obsd} the rate constant characteristic of the buffer system employed, k_{buffer}, and dividing the difference so obtained by enzyme concentration, $k_{enzyme} = (k_{obsd} - k_{buffer})/[E]_0$. Very careful scrutiny of the results obtained from about 200 runs performed in the absence of enzyme furnished very accurate values for k_{buffer}. These buffer rates typically accounted for 10–25% of the overall rate. Since nonenzymic hydrations of these aldehydes were found to be subject to both general acid and general base catalysis, most of the data reported in the present paper was obtained in diethylmalonate buffers ($pK_{a2} = 7.12$). This choice stems from our earlier observations that the rates of hydration of aldehydes in diethylmalonate buffers, in the absence of enzyme, are considerably lower than in any other buffers known to us to operate around physiological pH.[2] Two samples of BCA, designated BCA_I and BCA_{II}, were utilized during the course of this investigation. Because the activities of the two samples differed somewhat, specification of the sample used is included in the explanatory material accompanying figures and tables given in the Results section. Both BCA_I and BCA_{II} consisted of a mixture of isozymes A and B in the same ratio [BCA-A]:[BCA-B] $\simeq$ 2:3. Samples of the bovine isozymes A and B isolated from the same batch of BCA have been shown to have essentially the same activity, *i.e.*, $k_{BCA\text{-}A}/k_{BCA\text{-}B} \simeq 1.0$, with respect to propionaldehyde hydration. These observations accord with those made earlier with respect to CO_2 hydration (Lindskog and Malmström, 1960) and *p*-nitrophenyl acetate hydrolysis (Pocker and Stone, 1967). It is of interest to note here that the purified individual bovine isozymes A and B do not reveal any of the striking differences found to exist between the human isozymes B and C (Verpoorte *et al.*, 1967).

Results

Values of λ_{max}, ϵ, K_{eq}, and the fraction of hydration at equilibrium, χ, as determined at 0.0° for propionaldehyde, isobutyraldehyde, and pivalaldehyde, are sum-

[2] It was noted in these laboratories that the catalytic coefficient of diethylmalonic acid dianion is 0.8–1.4 log units below a line representing the Brønsted relationship as defined by catalytic coefficients for H_2O, $CH_3CO_2^-$, HPO_4^{2-}, and OH^- (Pocker and Meany, 1967b; Y. Pocker and D. G. Dickerson, submitted for publication). The monoanion of diethylmalonic acid furnished no detectable catalysis under the concentration conditions (0.0100 M) required to maintain constant pH.

TABLE II: Enzymic Activity as a Function of pH at 0.0°.

pH	k_{enzyme}[b]	pH	k_{enzyme}[b]	pH	k_{enzyme}[b]
	i. Propionaldehyde,[a] 0.0100 M Acetate Buffer, BCA_{II}				
4.30	0	5.11	71	5.78	242
4.60	42	5.41	140	6.03	300
4.79	46	5.56	186	6.58	503
	ii. Propionaldehyde,[a] 0.0100 M Diethylmalonate Buffer, BCA_{II}				
5.13	80	6.65	521	7.14	737
5.81	244	6.86	610	7.28	755
6.08	377	6.91	582	7.39	838
6.19	381	7.04	660	7.51	892
6.48	452	7.06	685	7.78	988
				8.11	1074
	iii. Propionaldehyde,[a] 0.0100 M Diethylmalonate Buffer, BCA_{I}				
6.05	378	6.62	617	7.20	832
6.37	547	6.78	648	7.32	961
6.61	573	6.92	716	7.57	1083
				7.84	1183
	iv. Isobutyraldehyde,[c] 0.0100 M Acetate Buffer, BCA_{II}				
4.30	0	5.01	58	5.40	82
4.71	44	5.25	75	5.61	125
				5.75	149
	v. Isobutyraldehyde,[c] 0.0100 M Diethylmalonate Buffer, BCA_{II}				
4.96	69	5.91	180	6.88	231
5.30	89	6.38	185	7.22	235
				7.62	210
	vi. Isobutyraldehyde,[c] 0.0100 M Diethylmalonate Buffer, BCA_{I}				
6.01	209	6.90	259	7.30	295
6.13	227	6.93	275	7.46	286
6.39	235	6.98	275	7.60	273
6.47	245	7.04	280	7.78	265
6.67	255	7.19	285	7.99	255
				8.10	235

[a] 0.0463 M. [b] M^{-1} sec^{-1}. [c] 0.0367 M.

marized in Table I. Enzymic activity was studied as a function of pH with both substrates (Table II) and the two pH–rate profiles are presented in Figure 2. The highest attainable concentrations of BCA did not result in detectable catalysis of pivalaldehyde hydration in the pH range 5–8, and it is concluded that k_{enzyme} for this aldehyde is less than 2 M^{-1} sec^{-1}.

The relationship between initial forward velocities and initial substrate concentrations is in accord with the Michaelis–Menten scheme (Figure 3) (eq 1).

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

Initial forward rate coefficients, k_f, were calculated from the forward component of k_{enzyme}; $k_f = k_{enzyme}\,\chi$. As discussed by Pocker and Meany (1967a), a more plausible scheme for these reversible hydrations is given in eq 2.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (2)$$

However, under the condition [P] = 0, eq 1 and 2 predict the same kinetic behavior. The chief difference between eq 1 and 2 is that the turnover number and Michaelis constant deduced from a Lineweaver–Burk plot are of a more complex character when interpreted in terms of eq 2 (Pocker and Meany, 1967a). Values of the turnover number, designated simply as k_2, and the Michaelis constant, K_M, were obtained at several pH values for both aldehydes and are summarized in Table III. The constancy in K_M with changing k_2 (Table III) indicates k_2 (and k_{-2}) to be significantly smaller than the

TABLE III: pH Dependence of Michaelis Constants and Turnover Numbers for Enzymic Hydrations of Propionaldehyde and Isobutyraldehyde at 0.0°.[a]

Enzyme[b]	pH	$10^{-1}\ k_2$[c]	K_M[d]
	i. Propionaldehyde		
BCA_{II}	6.35	6	0.20
BCA_{II}	6.40	6	0.19
BCA_{II}	6.61	8	0.19
BCA_{II}	7.04	11	0.21
BCA_I	6.62	10	0.20
BCA_I	7.45	17	0.20
BCA_I	7.81	21	0.19
BCA_I	8.15	22	0.22
			0.20 ± 0.01
	ii. Isobutyraldehyde		
Enzyme	pH	k_2[c]	K_M[d]
BCA_I	6.47	25	0.17
BCA_I	6.60	30	0.16
BCA_I	7.20	30	0.15
BCA_I	7.22	29	0.14
BCA_I	7.26	25	0.15
BCA_I	7.46	30	0.14
BCA_I	8.15	26	0.14
BCA_{II}	7.30	27	0.15
		28 ± 2	0.15 ± 0.01

[a] Determined in 0.0100 M diethylmalonic acid. [b] BCA_I and BCA_{II} are two different batches of enzyme used during the course of this work. [c] Derived from forward initial velocities (sec^{-1}). [d] Derived from forward initial velocities (M).

other coefficients in eq 1 and 2, *i.e.*, $K_M \sim k_{-1}/k_1 = K_S$ in eq 3. Figure 4 depicts Lineweaver–Burk plots obtained with propionaldehyde as substrate and with various inhibitors present: acetazolamide, azide, thiocyanate, *n*-propyl alcohol, and *n*-butyl alcohol. Figure 5 displays, in terms of the same kind of plot, the influence of acetazolamide and *n*-propyl alcohol on the enzymic hydration of isobutyraldehyde. Acetazolamide was found to

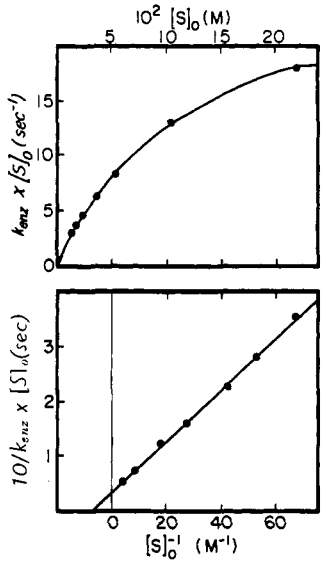


FIGURE 3: Relation between initial forward velocity at 0.0° and initial substrate concentration: S is isobutyraldehyde, $[BCA_I] = 10.6 \times 10^{-6}$ M and 0.0100 M diethylmalonate at pH 7.46. Upper graph is velocity *vs.* [S], lower graph is velocity^{-1} *vs.* $[S]^{-1}$ and furnishes $k_2 = 30\ sec^{-1}$ and $K_m = 0.14$ M.

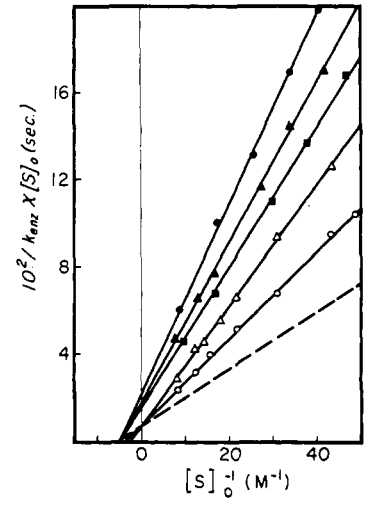


FIGURE 4: Lineweaver–Burk plot for BCA-catalyzed hydration of propionaldehyde in 0.0100 M diethylmalonate buffer at 0.0°: (----) Uninhibited hydration plot calculated using k_{enzyme} for BCA_I at pH 7.35 (Figure 3) and the appropriate K_m (Table III); (○) 0.84 M *n*-propyl alcohol, BCA_I at pH 7.3; (△) 0.35 M *n*-butyl alcohol, BCA_I at pH 7.4; (■) 2.28×10^{-3} M KSCN, BCA_{II} at pH 7.2; (▲) 9.92×10^{-4} M NaN_3, BCA_{II} at pH 7.2; (●) 1.5×10^{-5} M acetazolamide, BCA_I at pH 7.3.

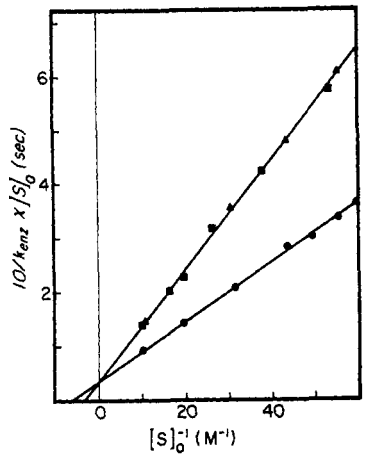
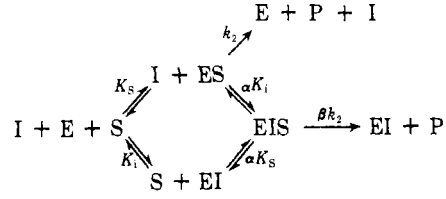


FIGURE 5: Lineweaver–Burk plots for BCA-catalyzed hydration of isobutyraldehyde in 0.0100 M diethylmalonate buffer (pH 7.3) at 0.0°: (●) uninhibited, BCA_I; (■) 0.43 M *n*-propyl alcohol, BCA_I; (▲) 1.00×10^{-4} M acetazolamide, BCA_I.

be a noncompetitive inhibitor of enzyme activity with propionaldehyde as substrate and a competitive inhibitor with isobutyraldehyde as substrate. Furthermore, both inhibitions were found to be partial rather than complete (Figure 6). The description of an inhibition mechanism, in terms of its being partial or complete, is most readily understood in terms of eq 3 (Webb, 1963). Having established the basic mechanism



(3)

of inhibition as competitive or noncompetitive by Lineweaver–Burk or similar plots, the values α or β may be obtained from the intercept of a plot of $1/i$ *vs.* 1/[I], where i stands for (1-fractional activity) and [I] stands for total inhibitor concentration. Partially noncompetitive inhibitors are characterized by $0 < \beta < 1$ and partially competitive inhibitors by $\infty < \alpha < 1$.

Both azide and thiocyanate anions were also found to be partially noncompetitive inhibitors of the enzymic hydration of propionaldehyde. Concentrations of these anions 1000 times greater than those required to cause 20% inhibition with propionaldehyde failed to result in detectable inhibition of enzymic activity with respect to isobutyraldehyde. In this case both Lineweaver–Burk plots (Figure 4) and plots of $1/i$ *vs.* 1/[anion] (Figure 7) are presented. Figure 4 furnishes a value of K_i only if β is known (Figure 7), and Figure 7 furnishes values of both β and K_i only if the basic mechanism of inhibition is known (noncompetitive, from Figure 4). Thus it is apparent why both of these plots are essential unless it is demonstrated that a large concentration of inhibitor causes 100% inhibition (thereby unambiguously proving the inhibition to be complete rather than partial).

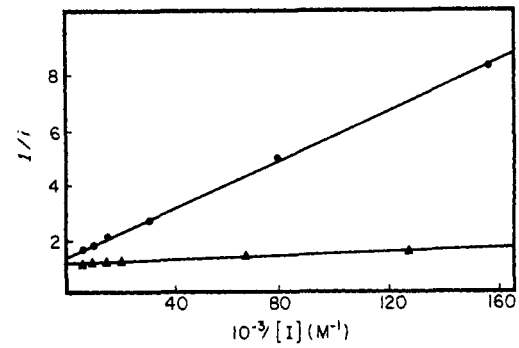


FIGURE 6: Acetazolamide inhibition of BCA-catalyzed hydrations of aldehydes in 0.0100 M diethylmalonate buffer (pH 7.2) at 0.0°. Reciprocal fractional inhibition as a function of reciprocal inhibitor concentration: (▲) propionaldehyde; (●) isobutyraldehyde.

By preparing $1/i$ *vs.* 1/[I] plots (Figure 8) it was shown that *n*-propyl alcohol and *n*-butyl alcohol are complete inhibitors of enzymic activity with both substrates. Lineweaver–Burk plots indicate both alcohols to be competitive inhibitors with propionaldehyde, and *n*-propyl alcohol was similarly shown to competitive with isobutyraldehyde. Also, isopropyl alcohol and isobutyl alcohol were shown to be complete inhibitors with isobutyraldehyde but only partial inhibitors with propionaldehyde. Quantitative characterization of the influence of these two alcohols with respect to propionaldehyde could not be achieved because concentrations as high as 1 M failed to cause more than 20% inhibition. Methyl alcohol and ethyl alcohol were not investigated because the exceptionally low equilibrium absorbancies obtained in the presence of these materials and the associated nonlinear kinetics were indicative of a reaction between these alcohols and the aldehydes. Concentrations of *t*-butyl alcohol as high as 1 M resulted in less than 2% inhibition with both substrates. Values of K_i, α, β, and the mechanism of inhibition for all inhibitors studied are summarized in Table IV.[3]

Neither pivalaldehyde nor equilibrated solutions of pivalaldehyde and its hydrate were capable of inhibiting the enzymic hydration of either propionaldehyde or isobutyraldehyde. The concentrations of pivalaldehyde and its hydrate employed in these attempted inhibitions were large enough so that if the respective binding constants were as small as 0.2 and 0.3 M, at least 10% inhibition would have resulted. Different results might be

[3] Verpoorte *et al.* (1967) have recently reported alcohol inhibition of the esterase activity of HCA-B and HCA-C. They conclude that the mechanism of inhibition with respect to esterase activity seems to be noncompetitive. The inhibitory effects of various agents containing hydroxyl groups, notably alcohols, on the esterase activity of BCA have been analyzed in some detail in our laboratory and were also found to be noncompetitive (Y. Pocker, J. T. Stone, and M. Beug, unpublished results). In contrast to anion inhibition which decreases at high pH values, the alcohol inhibition does not seem to be influenced by pH. Also, while anionic inhibition is noncompetitive with respect to both propionaldehyde hydrase activity and *p*-nitrophenyl acetate esterase activity, alcohol inhibition seems to be competitive with respect to aldehyde hydration but noncompetitive with respect to ester hydrolysis.

TABLE IV: Inhibition of Enzymic Aldehyde Hydration at 0.0°.

Inhibitor (aldehyde)[a]	Mechanism[b]	α or β	K_i	pH
Acetazolamide (P)	PNC	$\beta = 0.145$	$2.7 \pm 0.8 \times 10^{-6}$[c]	7.2
Acetazolamide (I)	PC	$\alpha = 5.3$	$3.5 \pm 0.5 \times 10^{-5}$[d]	7.2
NaN_3 (P)	PNC	$\infty = 0.34$	$2.05 \pm 0.15 \times 10^{-4}$[d]	7.2
KSCN (P)	PNC	$\beta = 0.37$	$6.4 \pm 1.2 \times 10^{-4}$[d]	7.2
n-Propyl alcohol (P)	CC	$\alpha \sim \infty$	1.2 ± 0.2[d]	7.3
n-Propyl alcohol (I)	CC	$\alpha \sim \infty$	0.59 ± 0.2[d]	7.2
Isopropyl alcohol	CC	$\alpha \sim \infty$	0.57[e]	7.2
n-Butyl alcohol (P)	CC	$\alpha \sim \infty$	0.25 ± 0.02[d]	7.3
n-Butyl alcohol (I)	CC	$\alpha \sim \infty$	0.08[e]	7.2
Isobutyl alcohol (I)	CC	$\alpha \sim \infty$	0.80[e]	7.2

[a] P is propionaldehyde, I is isobutyraldehyde. [b] PC is partially competitive, PNC is partially noncompetitive, CC is completely competitive. [c] Average of three determinations. [d] Average of two determinations. [e] Mechanism assigned by analogy with other alcohols. Other inhibitors of enzymic propionaldehyde hydration were isopropyl alcohol, isobutyl alcohol, and an equilibrated solution of isobutyraldehyde–isobutyraldehyde hydrate. The highest attainable concentrations of these species produced less than 20% inhibition, and could therefore not be quantitatively evaluated. Similar remarks pertain to the inhibition of enzymic isobutyraldehyde hydration by propionaldehyde–propionaldehyde hydrate. *t*-Butyl alcohol at concentrations of 1.0 M did not inhibit enzymic hydration of either aldehyde.

anticipated if propionaldehyde were utilized as an inhibitor of enzymic isobutyraldehyde hydration or *vice versa*. If these two aldehydes were to bind to totally independent sites on the enzyme, it would be reasonable to assume that no inhibition would result. However, if these two aldehydes used precisely the same binding sites, competitive inhibition would be expected, and K_i values calculated from such experiments should be identical with the independently obtained Michaelis constants. A third possibility arises if the two aldehydes share some but not all points of attachment to the protein matrix. In this case, simultaneous coordination of the two substrates, S_1 and S_2, would not be precluded, but the affinity of ES_1 for S_2 might well be detectably less than the affinity of E for S_2. In view of the following results, this third possibility would appear to be the most reasonable characterization of the mode of binding of propionaldehyde and isobutyraldehyde. It was observed that concentrations of an equilibrated mixture of propionaldehyde and its hydrate as low as 0.04 M resulted in *ca.* 15% inhibition of the enzymic hydra-

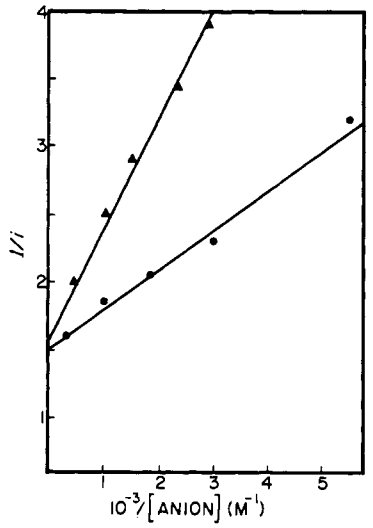


FIGURE 7: Anionic inhibition of the BCA-catalyzed hydration of propionaldehyde in 0.0100 M diethylmalonate buffer, (pH 7.2) at 0.0°. Reciprocal fractional inhibition as function of reciprocal anion concentration: (●) NaN_3; (▲) KSCN.

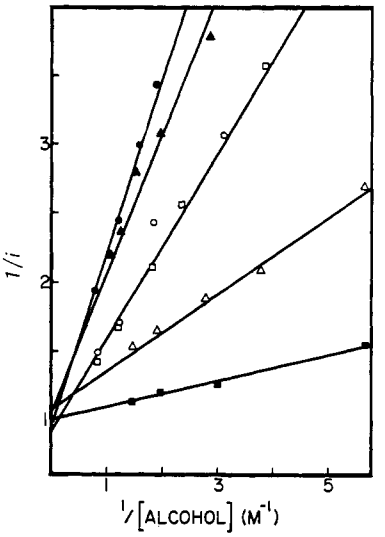


FIGURE 8: Alcohol inhibition of BCA-catalyzed hydrations of aldehydes in 0.0100 diethylmalonate buffer (pH 7.2) at 0.0°. Reciprocal fractional inhibition, i^{-1}, as a function of reciprocal inhibitor concentration, $[I]^{-1}$: (■) *n*-butyl alcohol isobutyraldehyde hydration; (△) *n*-butyl alcohol, propionaldehyde hydration; (□) *n*-propyl alcohol, isobutyraldehyde hydration; (○) isopropyl alcohol, isobutyraldehyde hydration; (▲) isobutyl alcohol, isobutyraldehyde hydration; (●) *n*-propyl alcohol, propionaldehyde hydration.

tion of isobutyraldehyde. Similarly, a concentration of 0.04 M of isobutyraldehyde–isobutyraldehyde hydrate caused *ca.* 10% inhibition of the enzymic hydration of propionaldehyde. However, increasing these concentrations to values as high as 0.20 M did not result in any greater degree of inhibition. Thus, the two binding sites, although not identical, would appear to overlap to some extent. This suggestion is confirmed by the observed difference in K_i which is obtained when the inhibitory capacity of a given alcohol is tested with respect to the enzymic hydration of the two different substrates (Table IV).

Discussion

An interesting feature of these systems is the fact that even at high aldehyde concentrations, [S] $\geqslant K_m$, the value of k_{enzyme} for any given run remained the same from the time the reaction was initiated until 90% of the equilibrium hydrate concentration had been formed. This indicates that the concentration of free enzyme is not influenced by the ratio of aldehyde to hydrate, *i.e.*, starting material and product have very similar affinities for the enzyme although the only structural feature common to both aldehyde and hydrate is the relatively unreactive hydrocarbon portion of the molecule. This constancy of k_{enzyme}, invariably observed in more than 1000 enzymic runs, could be interpreted to mean that hydrophobic interactions are very important in the binding of both aldehydes and aldehyde hydrates. Such a suggestion receives strong support from the observation that aliphatic alcohols are competitive inhibitors of enzyme activity with both propionaldehyde and isobutyraldehyde as substrates. Furthermore, the Michaelis constants for acetaldehyde (0.65 M), propionaldehyde (0.20 M), and isobutyraldehyde (0.15 M) are in accord with their relative hydrophobic character. In discussing the role of hydrophobic bonds in the maintenance of protein conformation, Tanford (1962) points out that the difference in hydrophobicity between two molecules can be computed in terms of $\Delta F°$ from a knowledge of how the two differ in the number of methyl, isopropyl, and methylene substituents. Tanford suggests that the $\Delta F°$ values associated with various structural moieties, which he records at 25°, likely contain only a minor enthalpy contribution so that they could be employed at 0° following multiplication by 273°:298°. These values would predict a difference between acetaldehyde and propionaldehyde of $\Delta F° = 670$ cal mole^{-1}; between acetaldehyde and isobutyraldehyde a difference of $\Delta F° = 880$ cal mole^{-1}. The corresponding differences obtained by evaluating $-RT \ln K_M = \Delta F°_{binding}$ are in close accord, 640 and 800 cal mole^{-1}, respectively. In view of the fact (Table III) that the value of the Michaelis constant ($K_M = (k_{-1} + k_2)/k_1$, in terms of eq 1) for propionaldehyde is unaffected by changes in k_2, it is concluded that these K_M values are reasonably accurate estimates of the ratio k_{-1}/k_1 defining [E][S]/[ES]. Two facts must be kept in mind before drawing further inferences from the alcohol inhibition data. (1) Such a hydrophobic cavity in the enzyme would presumably have a reasonably well-defined geometry as indicated by the observations that pivalaldehyde, pivalaldehyde hydrate, and *t*-butyl alcohol, all of which contain a hydrophobic residue of the type $(CH_3)_3C$, do not appear to bind to the enzyme. (2) The portions of such a cavity utilized for binding propionaldehyde and isobutyraldehyde are not identical, as neither aldehyde is capable of completely inhibiting enzymic activity with respect to the other. In view of these considerations, two apparent anomalies are resolved. (i) The relative inhibitory capacities of the alcohols, *n*-butyl > *n*-propyl > isobutyl ~ isopropyl with propionaldehyde; *n*-butyl > *n*-propyl ~ isopropyl > isobutyl with isobutyraldehyde, are not in exact agreement with the relative hydrophobic character of the alcohols, *n*-butyl > isobutyl > *n*-propyl > isopropyl. (ii) The K_i of a given alcohol is dependent on the aldehyde with respect to which inhibition is tested. Certain observations recently reported by other workers may also be pertinent to the above suggested importance of hydrophobic binding. X-Ray structure studies on the human enzyme, HCA-C, have revealed that sulfonamide inhibitors such as acetazolamide not only coordinate *via* the zinc atom, but the bulk of such inhibitors is positioned in a cavity near the zinc (Fridborg *et al.*, 1967). Hydrophobic interactions between this cavity and a molecule such as acetazolamide would not require the participation of zinc. Coleman (1967) has reported that acetazolamide does indeed bind to zinc-free HCA-B, and the dissociation constant of this complex is 10^{-4}–10^{-5} M. It is interesting to note that acetazolamide, which inhibits the enzymic hydration of isobutyraldehyde in a *competitive* fashion, is characterized by an inhibition constant of very similar magnitude $3.5 \pm 0.5 \times 10^{-5}$ M.

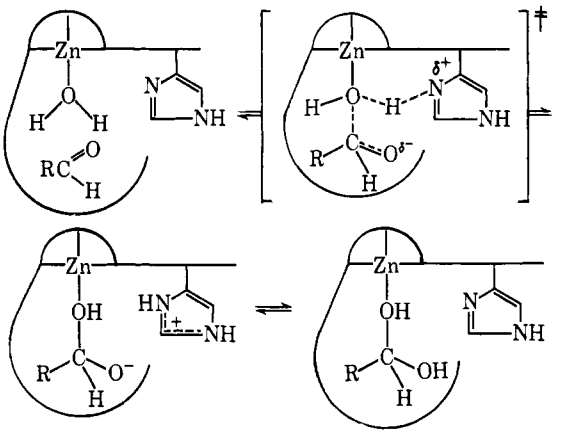
The mechanism whereby hydration of the complex between enzyme and either propionaldehyde or isobutyraldehyde is accomplished will depend on the conformation of the complex rather than on the conformation of free enzyme. There is evidence that the conformation of the propionaldehyde–enzyme complex is different from that of the isobutyraldehyde–enzyme complex. In the first place, it has already been pointed out that the binding sites utilized by the two aldehydes appear to be slightly different. Secondly, the point of inflection in the pH profiles for the two substrates differs by a pK unit, *i.e.*, it changes from an apparent pK_a of around 6.6 for propionaldehyde to an apparent pK_a of around 5.6 for isobutyraldehyde. These inflections are consistent with the titration of a group whose basic form is associated with enzymic activity, a feature reasonably attributed to imidazole. A ΔpK_a of *ca.* 1.0 might be the consequence of an altered conformation, and hence environment in the protein matrix, induced by substrates differing in spacial requirements. Because the elements essential to hydration may be situated differently in the propionaldehyde–enzyme complex, and in the isobutyraldehyde–enzyme complex, it is not imperative that the same mechanism of hydration be invoked for both complexes. Indeed, the fact that the hydration of the propionaldehyde–enzyme complex, but not the hydration of the isobutyraldehyde–enzyme complex, is inhibited by acetazolamide, azide, and thiocyanate would appear

to preclude rationalization in terms of the same mechanism.

Before attempting to explain the differing mode of action of these inhibitors, consideration should be given to a plausible scheme for the uninhibited hydrations. The evidence that the pH profiles reflect titration of an imidazole residue has been reviewed in earlier papers of this series (Pocker and Meany, 1965a,b, 1967a,b; Pocker and Stone, 1965, 1967) and has recently received independent support (Whitney *et al.*, 1967; Edsall *et al.*, 1967; Nilsson and Lindskog, 1967; Y. Pocker, J. T. Stone, and M. Beug, unpublished observations). Zinc has been unambiguously demonstrated as an essential component of the hydration mechanism and the water and aldehyde are obviously necessary components.

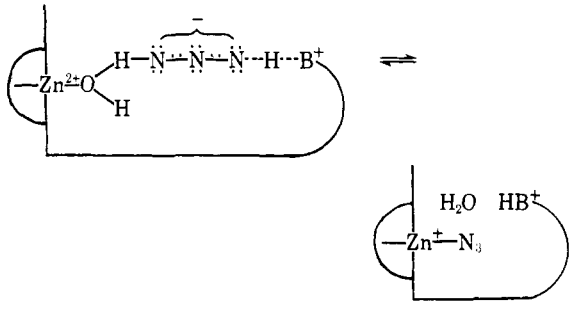
In a study of the acid–base-catalyzed hydration of acetaldehyde, Pocker and Meany (1967c) have postulated a mechanism involving a cyclic transition state in which protons are transferred to and from the catalyst at a site effectively removed from the substrate by several water molecules. It is difficult at present to assess the importance of such cyclic transition states in the enzymatic hydration of CO_2 and of aldehydes. The remarkable catalytic activity of carbonic anhydrase may be attributed in part to a zinc-coordinated water molecule and in part to a conveniently located imidazole. It is easy to envisage water molecules acting as bridges between the protein-bound aldehyde, zinc, and imidazole. Such water molecules may be hydrogen bonded into a structure which allows a rapid proton transfer by a synchronous making and breaking of rigidly held hydrogen bonds in the enzyme–substrate complex. For simplicity, we follow our earlier more condensed model and regard hydration in the pH region 5–8 as consisting essentially of an imidazole (general base) promoted attack by the zinc-coordinated H_2O (formally, zinc-coordinated ^-OH). This simplified model does not of course preclude either the presence of additional water molecules or the formation of a cyclic transition state. It has not escaped our attention that the association between a conveniently located Zn^{2+} and the carbonyl oxygen would facilitate a general base (*e.g.*, imidazole) assisted attack by H_2O on the carbonyl carbon thereby providing a very attractive mechanism for certain metal ion assisted hydrations (Pocker and Meany, 1967*a*–c, 1968). It should be noted, however, (a) that in the enzymatic hydrations of the "more normal" substrates (*e.g.*, CO_2, CH_3CHO, and *p*-nitrophenyl acetate) monovalent anions and sulfonamides act noncompetitively; (b) that the pH and spectral changes associated with the binding of anions to the Zn- and Co–enzymes fit with the assumption that a *coordinated* H_2O is being displaced near a very strong anion binding site of p*K*a = 7.5; and (c) that X-ray data indicate that sulfonamides bind at or near the zinc. These and related observations strongly suggest that the electrophilic capacity of Zn^{2+} is being employed by the enzyme in the "activation" of H_2O rather than in the "activation" of the carbonyl substrate.

The only difference between propionaldehyde and isobutyraldehyde, presumably, would be the fashion in which the various components of this scheme are arranged. The degree to which acetazolamide, azide, and



thiocyanate are capable of noncompetitively inhibiting the enzymic hydration of propionaldehyde depends both on the manner in which they become associated with the elements of this hydration scheme and on the relative positions of these components. In order to effect noncompetitive inhibition, it is not essential that one of the elements of this scheme be displaced, only that the cycle of events involved in hydration be broken at some stage. For example, it is not necessary that these inhibitors displace zinc-bound water entirely. It would be sufficient to have these inhibitors interact with the zinc through the water which would function as a bridge. If the formation of such a bridged complex or ion pair sterically interfered with effective participation of imidazole as a base, noncompetitive inhibition would be observed. Although such water-bridged ion pairs are commonly found to be in equilibrium with the covalent complex (Eigen and Wilkins, 1965) in which water is entirely displaced, the position of equilibrium will be a function of the relative stabilities of the ion pair and covalent complex. It is possible that within the protein matrix the ion pair would receive additional stabilization as a consequence of two-point attachment of the inhibitor. Thus, we visualize that $Zn^{2+}(OH_2)N_3^-$ and the $Zn^{2+}(OH_2)SCN^-$ ion pair complexes are stabilized by additional interaction with a neighboring BH^+ group in the enzyme. Indeed the inhibitory effect of anions decreases with increasing pH as if the cationic binding site lies close to the zinc and has an apparent pK_a value between 7 and 8. (Kernohan, 1965; Pocker and Stone, 1967). We have shown earlier that the Hofmeister lyotropic series is followed, *i.e.*, that there is no direct parallelism between the association constants of anions with zinc ions and their inhibition constants (Pocker and Stone, 1967). Apparently the cationic binding site lies close to zinc and the bound anion interferes with the water structure in the immediate vicinity of the metal.

The extent to which formation of such complexes would inhibit enzymic activity would depend on the extent to which the enzyme–inhibitor complex interfered with the normal hydration cycle. This in turn would depend on the relative position of the elements involved in hydration, a feature determined by the size and shape of the substrate bound to the enzyme. Thus it is apparent



that radical changes in the hydration mechanism are not necessarily required in order to explain the observed differences between the enzymic hydrations of isobutyraldehyde and propionaldehyde; rather these differences can be rationalized in terms of a flexible active site whereby conformational changes are induced by substrate (Koshland, 1963).

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