Acetoacetate Decarboxylase. Catalysis of Hydrogen–Deuterium Exchange in Acetone*

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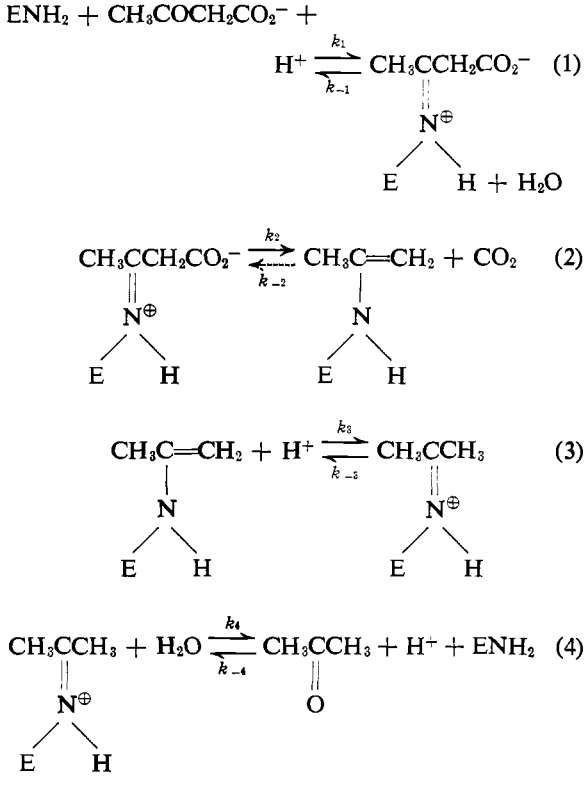
ABSTRACT: The mechanism for the decarboxylation of acetoacetic acid by acetoacetate decarboxylase proceeds by way of the enamine of acetone. The reverse reaction should then lead from acetone to this enamine, and therefore the mechanism requires that the enzyme catalyze the exchange of hydrogen atoms between acetone and solvent. This prediction has been realized; the rate of the reaction has been measured by nuclear magnetic resonance techniques. The interconversion of enamine and Schiff base salt is so rapid, compared to that for chemical analogs, that this step in the mechanism must be enzymic.

Acetoacetate decarboxylase from *Clostridium acetobutylicum* has been shown to catalyze the decarboxylation of acetoacetic acid by the following mechanism (Hamilton and Westheimer, 1959; Westheimer, 1963).



The reactions shown in eq 1 and 4 have previously been confirmed by demonstrating that the enzyme catalyzes the exchange of ^{18}O from both acetoacetate and from acetone with solvent (Hamilton and Westheimer, 1959). The Schiff base salt, produced as the product in eq 3, has been trapped by borohydride reduction (Fridovich and Westheimer, 1962; Warren *et al.*, 1966). Evidence that suggests the formation of enamines (such as that shown in eq 2) has been provided in an accompanying paper (Tagaki *et al.*, 1968).

The present paper is concerned with the protonation of the enamine to Schiff base salt, and the reverse reaction, as shown in eq 3. We have demonstrated this step and estimated a lower limit for its rate. The rate constant is sufficiently large to suggest that the protonation and deprotonation are enzymic, *i.e.*, the reaction is much more rapid than would have been anticipated for the corresponding thermal process. In order to observe the reactions shown in eq 3, we have allowed acetone-d_6 to react with water in buffered solution in the presence of enzyme, and have followed the loss of deuterium from the deuterioacetone by the increase in the appropriate nuclear magnetic resonance signal as hydrogen atoms were incorporated into the methyl groups of acetone. Conversely, we have measured the rate (by the same technique) at which hydrogen is replaced by deuterium when acetone reacts, in the presence of the enzyme, with D_2O.

Experimental Section

Materials. Acetoacetate decarboxylase was prepared by a modification of the method of Zerner *et al.* (1966) (*cf.* Westheimer, 1968), and was stored in 0.05 M potassium phosphate buffer (pH 5.98). Throughout this paper, the concentration of enzyme is given in terms of subunits (molecular weight 29,000) rather than in terms of the oligomer. The weight of enzyme in solution was calculated from the optical density, assuming that an optical density of 1.00 corresponds to 0.95 mg/ml (see Tagaki and Westheimer, 1968). Acetone (Fisher Scientific Co.) was distilled through a vacuum-jacketed 15-in. column packed with glass helices. 2-Picoline (Matheson Coleman and Bell) was distilled over potas-

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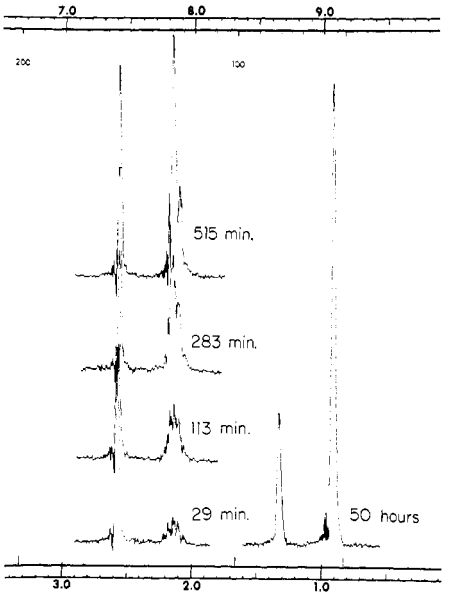

FIGURE 1: The nuclear magnetic resonance spectra, at various times, for 0.5 M deuterioacetone in 0.15 M 2-picoline–0.15 M 2-picolinium sulfate buffer at pH 6.04, in the presence of 7×10^{-8} M (subunits) acetoacetate decarboxylase. The spectrum at 50 hr has been displaced about 2.6 ppm upfield for clarity of presentation.

TABLE I: Kinetics of D–H Exchange Reaction of Acetone-d_6 Catalyzed by Acetoacetate Decarboxylase.[a]

Reaction Time (min)	r^b	ln ($r_\infty - r$)
11	0.15	1.0854
29	0.30	1.0333
64	0.44	0.9822
106	0.61	0.9163
113	0.67	0.8921
209	1.11	0.6932
283	1.30	0.5934
335	1.47	0.4946
410	1.70	0.3436
515	2 00	0.1043
612	2.18	−0 0725
698	2.41	
875	2.66	
1302	2.95	
2340	3.11	
3000	3.11	

[a] Acetone-d_6, 0.5 M; enzyme, 7.01×10^{-6} M in subunits; enzyme concentration based on the molecular weight of 29,000 for the subunits; see Experimental Section; 2-picoline, 0.15 M; and 2-picolinium sulfate, 0.15 M. [b] r = (peak area for acetone-h)/(peak area for picoline-methyl-h).

sium hydroxide pellets through this same column. It boiled at 128° and showed essentially no impurities in vapor phase chromatography analysis with a 5 ft × 0.25 in. column packed with Carbowax 20M on 60–80 Chromosorb W treated with dichlorodimethylsilane.

Acetone-d_6 was prepared by heating 14.5 g of acetone with 50 g of 99.8% deuterium oxide and 500 mg of anhydrous sodium carbonate in a sealed tube for 24 hr at 60–65°. The reaction mixture was fractionated, and the partially deuterated acetone dried with calcium chloride. The exchange reaction was repeated. Almost 12 g of deuterated acetone (bp 56°) was obtained. The nuclear magnetic resonance spectrum of this material in D_2O showed only a small quintet centered at δ 1.91, with J = 2 cycles/sec (*cf.* Bernstein and Sheppard, 1962); comparison of this peak (which of course arises from a trace of residual hydrogen) with that of the methyl group of 2-picoline (see Methods below) indicated that the exchange with deuterium was at least 95% complete.

Deuterated sulfuric acid was prepared by mixing 5 g of reagent grade sulfuric acid with 11 g of D_2O, and removing water (about 8 g) on a vacuum line at 10^{-4}-mm pressure. The procedure was repeated twice more, and the resulting acid standardized by titration.

Methods. The isotopic composition of solutions of acetone or acetone-d_6 was determined in picoline–picolinium ion buffers by nuclear magnetic resonance spectroscopy with a Varian A-60 spectrometer. The signal from the methyl group of acetone appears in D_2O at δ 2.19, and that of the methyl group of 2-picoline at δ 2.64. (The latter applies to solutions at pH 5.90; the position of the peak is pH sensitive, *i.e.*, it depends on the ratio of picoline to picolinium ion.) Control experiments showed that, in the buffer solutions here employed, the hydrogen atoms of the methyl group of 2-picoline exchange with solvent only slowly at 100° (*cf.* Zatsepina *et al.*, 1963); at room temperature, exchange is negligible.

The methyl group of 2-picoline serves as an internal standard for the nuclear magnetic resonance measurements. As the reaction with acetone in D_2O proceeds, and hydrogen atoms are replaced by deuterium atoms, the area of the peak from the methyl groups of acetone diminishes, relative to that of picoline. Conversely, in the measurements made with acetone-d_6 in H_2O, the area of the peak associated with the methyl groups of acetone increases with time as the deuterium atoms are replaced by hydrogen atoms. The absolute areas of the peaks vary with changes in the sensitivity and performance of the nuclear magnetic resonance spectrometer, but the relative areas can be determined accurately. The measurements in H_2O can be made despite the enormous peak from the solvent itself, which totally obscures the spectrum in the region around δ 5 and produces massive spinning side bands that may come as low as δ 3. Fortunately, the measurements here made were concerned only with the region between δ 1.8 and 2.8. The areas of the methyl peaks from 2-picoline and acetone were obtained by integration,

TABLE II: Summary of Hydrogen-Exchange Kinetics of Acetone at 25°.

Acetone (0.5 M)	Buffer	(Enzyme), $\times 10^6$ M[a]	Aminoaceto-nitrile (M)	k_{obsd} $\times 10^5$ sec^{-1}	k_{obsd}/(Enzyme), M^{-1} sec^{-1}
d_6		3.51		1.54	4.39
d_6	*b*	7.01		3.15	4.49[d]
d_6		14.0		6.38	4.56
d_6	*b*		0.3	1.58	(5.3×10^{-5})[e]
h_6	*c*	7.01		4.22	6.02
h_6	*b*	7.40		5.41	7.31

[a] Based on the molecular weight of 29,000 for the subunits and the weight factor; see Experimental Section. [b] 2-Picoline (0.15 M)–2-picolinium sulfate (0.15 M) in H_2O. [c] 2-Picoline (0.1 M)–2-picolinium sulfate (0.2 M) in D_2O. [d] Average 4.48. [e] Rate constant for amine catalysis.

and good first-order rate constants were calculated from the ratio of these areas. Solutions for rate measurements were thermostated at 25°; the nuclear magnetic resonance measurements were performed at room temperature, and the brief minor variations in temperature during the measurements did not affect the rates.

Results

The enzyme strongly catalyzes the exchange of hydrogen atoms between acetone and the solvent. A series of nuclear magnetic resonance spectra obtained at various times for deuterioacetone in a picoline–picolinium sulfate buffer is shown in Figure 1. The area of the peak for the methyl groups of acetone increases relative to that for the methyl group of picoline from a small multiplet centered on δ 2.15 at 29 min, through peaks of increasing size at intermediate time values, to the final singlet at 50 hr that completely dominates the methyl peak from picoline. (The splitting in the peaks obtained at intermediate times arises from spin–spin coupling between hydrogen and deuterium atoms.)

Data for an individual experiment for the exchange of protons into acetone-d_6 are shown in Table I and in Figure 2. A summary of the data for forward and for reverse reactions is given in Table II. In the absence of enzyme, the reaction rate in picoline–picolinium sulfate buffers at pH 5.90 is too slow to measure. In one experiment, aminoacetonitrile was added at 0.3 M. This compound was chosen because it is the best model for the enzyme that we have found so far (Tagaki *et al.*, 1968), that is, the compound is the most effective nonenzymic catalyst for the decarboxylation. The rate of reaction with this catalyst is low; it requires 50,000 times as much aminoacetonitrile as enzyme to achieve a comparable rate (see Figure 2).

Discussion

The catalysis by acetoacetate decarboxylase of the exchange of hydrogen atoms into acetone is obvious from the nuclear magnetic resonance spectra presented in Figure 1. The reaction rate is large; the long times for reaction result from turning over 0.5 M acetone with about 5×10^{-6} M enzyme. The second-order rate constant observed is about 4.5 M^{-1} sec^{-1} for the dedeuteration of acetone-d_6 in H_2O, calculated from a concentration of enzyme, based on 29,000 as the molecular weight of the subunit. However, accompanying papers (Tagaki *et al.*, 1968; O'Leary and Westheimer, 1968) suggest that two subunits of the enzyme may be required for the formation of an active site, so that a rate constant of 9 M^{-1} sec^{-1} at 25° is probably preferable.

Although a rate for the reaction has also been recorded for the exchange of hydrogen atoms from acetone in D_2O, the data so far obtained are not sufficient to establish the magnitude of the isotope effect. In

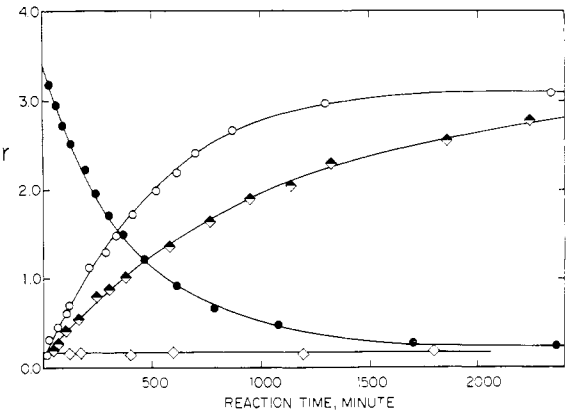

FIGURE 2: The change with time of the ratio (r), where r = peak area (nuclear magnetic resonance signal from the methyl groups of acetone)/peak area (nuclear magnetic signal from the methyl groups of 2-picoline). The conditions for the reaction in water, and for the control experiment, are the same as those given in the legend of Figure 1. The experiment in D_2O was conducted in a buffer containing 0.10 M 2-picoline and 0.20 M picolinium sulfate. (◇) Control, (●) acetone-h_6 and 7×10^{-6} M enzyme (subunits) in D_2O, (○) acetone-d_6 and 7×10^{-6} M enzyme (subunits) in H_2O, and (▲) acetone-d_6 and 0.3 M aminoacetonitrile in H_2O.

order to do so, it will be necessary to determine the entire pH– and pD–rate profiles for the reaction; otherwise the effect of the change in solvent on the enzyme may obscure the kinetic isotope effect.

The rate constant given above applies to the over-all reaction between acetone and enzyme, and is represented by eq 3 and 4. For purposes of comparison with the rate of nonenzymic processes, we wish to obtain k_{-3}, the rate constant for the removal of a proton from the Schiff base salt of acetone. In order to calculate this constant, both the value of the equilibrium constant between acetone and the enzyme, to form Schiff base and water, and the ionization constant for the Schiff base salt are required. Although these constants are not available, a lower limit can be established for k_{-3}. In particular, the gross equilibrium between enzyme (protonated plus unprotonated) and acetone to give Schiff base (protonated plus unprotonated) can be estimated from the inhibition constant for acetone.

Acetone is a poor inhibitor of the enzyme; for the exchange of deuterioacetone with water the Michaelis constant is about 2 M, and in unpublished research S. M. Coutts has found that K_I is about 0.3 M. (With enzyme from a different bacterial strain, Davies (1943) found that 1 M acetone depressed the rate only 10%.) A consideration of the data given above suggests that, in 0.5 M acetone solution, an appreciable fraction of the enzyme is still free, and therefore k_{cat} (in sec^{-1}) must exceed the numerical value of the second-order rate constant of 9 M^{-1} sec^{-1}. Furthermore, k_{-3} must exceed k_{cat}, perhaps by a sizable factor, since (a) the formation of enamine may not be the rate-limiting step in the enzymic process and (b) anyway, only a fraction of the bound enzyme will be present as Schiff base salt. We then take k_{cat} as a lower limit to k_{-3}, and evaluate a lower limit for k_{cat} as 9 sec^{-1} by making the (conservative) assumption that the enzyme is completely bound by acetone. The value of k_{-3} (or, more properly, the lower limit here established for k_{-3}) should now be compared with the rate of nonenzymic exchange of deuterium ions between Schiff bases and water.

The literature on the rates of nonenzymic formation of enamines from Schiff base salts is not clear. Hine and his coworkers (Hine *et al.*, 1966, 1967; Hine and Mulders, 1967) have studied the rate of production of the enamine formed by the loss of deuterium from the Schiff base salt, $CH_3N^+H{=}CHCD(CH_3)_2$. They have obtained an approximate value for a kinetic parameter that is related to the first-order rate constant (k_w) for catalysis of the removal of D^+ by water (as well as parameters related to the catalytic constants for hydroxide ion, methylamine, and other bases). Unfortunately, the evaluation of k_w from their kinetic parameter depends upon an estimate of the (unknown) ionization constant of the Schiff base salt shown above. However, assuming 10^{-7} M for this ionization constant, we calculate that k_w is less than 10^{-5} sec^{-1} at 35°. (Here $k_w = k_{w'}(H_2O)$, or $55.5k_{w'}$). Furthermore, Hine *et al.* (1965) have shown that isobutyraldehyde is more reactive in enolization than is acetone, so that quite possibly the first-order rate constant for the water-catalyzed loss of a deuteron from a Schiff base salt of deuterioacetone is small compared to 10^{-5} sec^{-1}.

On the other hand, Bender and Williams (1966) report a rate constant (calculated for 55.5 M water) for the water-catalyzed enaminization of the Schiff base salt of acetone of about 1.4 sec^{-1} at 25°. If this rate constant is corrected for a deuterium isotope effect of 7 and for a statistical factor of 6, it is reduced to 0.03 sec^{-1}; this is the proper value to compare with the rate constants that we and Hine report.[1] Like the rate constant reported by Hine *et al.*, Bender and Williams' value depends upon an assumed (and unknown) ionization constant for the Schiff base salt. However, the differences and uncertainties in this ionization constant are small compared to the discrepancy in the projected rates for water-catalyzed hydrogen–deuterium exchange of the Schiff base salt of acetone derived from the results of the two laboratories. But if either value (or one between them) for the "spontaneous" (*i.e.*, water catalyzed) rate is approximately correct, then the exchange of hydrogen with deuterium catalyzed by acetoacetate decarboxylase is relatively so fast as to make it clear that the enzyme actively catalyzes the interconversion of enamine and Schiff base salt.

The enolization of dihydroxyacetone phosphate, catalyzed by muscle aldolase (Rose *et al.*, 1965; Rose and Reider, 1958), proceeds by way of a Schiff base salt with k_{cat} of about 70 sec^{-1}. This rate constant, however, may not represent that for the deprotonation process. The latter may be much greater (Rose *et al.*, 1965). Similarly, of course, the value of k_{cat} of 9 sec^{-1} presented here for the hydrogen–deuterium exchange in deuterioacetone, catalyzed by acetoacetate decarboxylase, is a lower limit for the actual chemical process. The rate constant for the deprotonation of pyruvate, catalyzed by 2-keto-3-deoxy-6-phosphogluconate aldolase, is about 400 sec^{-1} (I. A. Rose, private communication). All of these enzymic rate constants are large compared to the probable rates of their purely chemical analogs.

The present work should also be compared to the preliminary communication by Kobes and Dekker (1967). They show that 4-hydroxy-2-ketoglutarate aldolase catalyzes the decarboxylation of oxaloacetate. Enzymic aldol condensations (Grazi *et al.*, 1962; Kobes and Dekker, 1966) proceed by way of Schiff bases and enamines, as does the enzymic decarboxylation of acetoacetic acid. (For an example of nonenzymic aldol condensation *via* a Schiff base, see Westheimer, 1940.) For both ketoglutarate aldolase and acetoacetate decarboxylase the dual functions of the enzyme reinforces the generally accepted mechanistic framework for enolization processes.

[1] No statistical factor should be employed in comparing Hine's rate constant with ours, since our rate was determined for the conversion of acetone-d_6 to acetone-h_6. Our measurements refer to six successive enolizations, and although a statistical factor of six attaches to each step, the over-all rate constant we measure should be compared directly to that for the enolization of the single deuterium atom of deuterioisobutyraldehyde

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Acetoacetate Decarboxylase. Reaction with Acetopyruvate*

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ABSTRACT: Acetoacetate decarboxylase is inhibited by acetopyruvate with an inhibition constant (dissociation constant) of about 10^{-7} M. One molecule of inhibitor binds to about 60,000 mol wt units (possibly two subunits) of the enzyme, as shown by spectrophotometric titration, by the relationship between activity and inhibitor concentration, and by isolation and analysis of the compound formed between enzyme and inhibitor. The isolated material shows an intense ultraviolet absorption at 325 mμ. Comparison of its spectrum with that of model compounds, prepared from α,γ-diketo acids or esters and aminoacetonitrile, identifies the enzyme–inhibitor compound as an enamine, presumably obtained by the tautomerization of the Schiff base initially formed from inhibitor and enzyme. The rapid reaction between acetopyruvate and the decarboxylase is shown to be catalyzed by the enzyme.

Acetoacetate decarboxylase isolated from *Clostridium acetobutylicum* (Hamilton and Westheimer, 1959a; Zerner *et al.*, 1966) is strongly inhibited by salts of acetopyruvic acid (Davies, 1943; *cf.* Colman, 1962). This study of this inhibitor shows that it reacts reversibly with the enzyme to form an enamine (II), probably according to eq 1. (Some uncertainty exists as to which carbonyl group is involved in the formation of the Schiff base and enamine.) Acetoacetate reacts with the amino group of a specific lysine residue in the enzyme to form a Schiff base salt that serves as the essential intermediate in the decarboxylation (Hamilton and Westheimer, 1959b; Fridovich and Westheimer, 1962; Warren *et al.*, 1966). Presumably,

$$CH_3COCH_2COCO_2^- + ENH_2 \rightleftharpoons$$

$$CH_3CCH_2CCO_2^- \rightleftharpoons CH_3CCH{=}CCO_2^- \quad (1)$$

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