Brewers' Yeast Pyruvate Decarboxylase Produces Acetoin from Acetaldehyde: A Novel Tool To Study the Mechanism of Steps Subsequent to Carbon Dioxide Loss†

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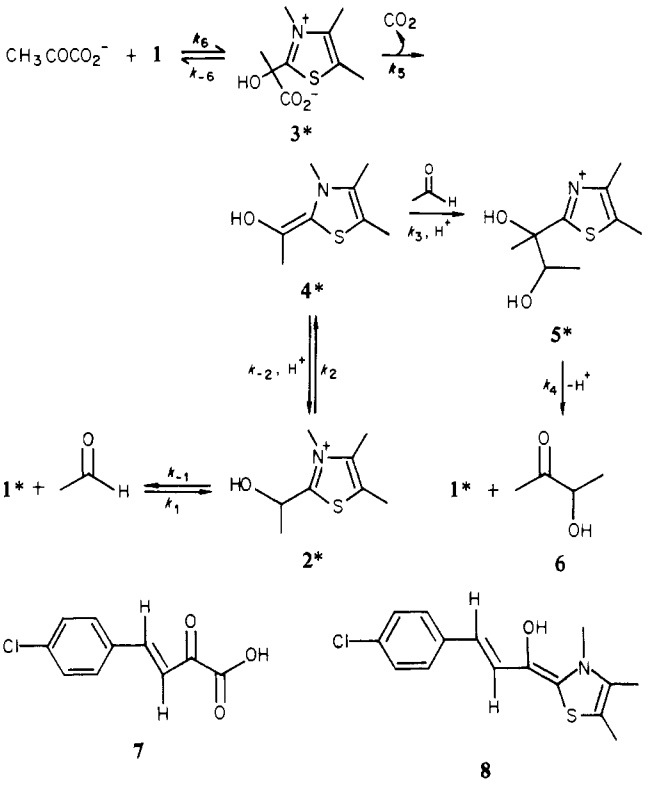
ABSTRACT: A gas–liquid chromatographic technique was developed for the determination of both acetaldehyde and the 3–4% acetoin side product that results from the brewers' yeast pyruvate decarboxylase (EC 4.1.1.1) catalyzed reaction of pyruvic acid. Employing this method enabled the demonstration of the catalysis of acetaldehyde condensation to acetoin by the enzyme. It was found that the acetoin produced enzymatically from pyruvic acid or from acetaldehyde was optically active, thus providing stereochemical information about the reaction. Deuterium kinetic isotope effects (employing CH_3CHO and CH_3CDO) were determined on the steady-state kinetic parameters to be 4.5 (V_{max}) and 3.2 (V_{max}/K_m^{app}), respectively. This enabled, for the first time, the estimation of relative kinetic barriers for steps past decarboxylation. It could be concluded that (a) C–H bond scission was part of rate limitation in the enzyme-catalyzed condensation of acetaldehyde to acetoin and that (b) among the steps leading to the release of acetaldehyde, protonation of the key enamine intermediate was part of rate limitation. This latter finding is also directly applicable to the mechanism of pyruvate decarboxylation.

Yeast pyruvate decarboxylase (PDC, EC 4.1.1.1)[1] catalyzes the irreversible decarboxylation of pyruvic acid to acetaldehyde and CO_2 (Lohmann & Schuster, 1937) employing thiamin diphosphate (TDP, **1**) and Mg(II) as tightly bound cofactors (Schellenberger, 1967). With the delineation of the role of the C2 atom of thiazolium in model catalytic reactions (Breslow, 1957, 1958) and with the confirmation of this role due to the isolation of 2-(1-hydroxyethyl)thiamin diphosphate (HETDP, **2**) from enzymatic reaction mixtures (Carlson & Brown, 1960; Holzer & Beaucamp, 1961; Krampitz et al., 1961), Scheme I has been accepted for the nonoxidative decarboxylation reaction (Krampitz, 1970; Kluger, 1982); **1*** represents enzyme-bound cofactor; other asterisked species are also enzyme bound. While three groups have confirmed that, of the steps culminating in CO_2 loss, the loss of pyruvate from **3*** (k_{-6}) is slower than the decarboxylation step (k_5) (O'Leary, 1976; DeNiro & Epstein, 1977; Jordan et al., 1978a,b); no detailed information is yet available concerning the relative rates of steps past decarboxylation. That such steps may be rate limiting overall could be concluded from the fact that **2** could be isolated from enzymatic reaction mixtures (Carlson & Brown, 1960; Holzer & Beaucamp, 1961; Krampitz et al., 1961), whereas **3** could not. Recently, in this laboratory, a PDC-bound species derived from a suicide substrate, (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid (**7**) (Kuo & Jordan, 1983a), was observed by UV–vis spectroscopy (Kuo & Jordan, 1983b). The spectral properties of the PDC-bound intermediate were consistent with those expected for **8**, an analogue of **4**, raising the possibility that the protonation of the enamine may participate in rate limitation. An entirely different approach to gain additional information about the forward pyruvate decarboxylation pathway is to study the reverse reaction, namely, acetaldehyde → **4** → **6**.

Scheme I



The steps indicated in eq 1 are reversible and common to both reactions, and the release of acetoin via this back-reaction

$$CH_3CHO + TDP^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathbf{2}^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathbf{4}^* + H^+ \qquad (1)$$

is irreversible (in our hands, at least). Knowledge of the relative size of the rate constants for this process will also provide insight to those steps that are common to pyruvate

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[1] Abbreviations: PDC, pyruvate decarboxylase; TDP, thiamin diphosphate; GLC, gas–liquid chromatography; SDS, sodium dodecyl sulfate.

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decarboxylation and acetoin formation (k_1, k_{-1}, k_2, k_{-2}). While acetoin formation from acetaldehyde had been reported as catalyzed by thiamin at higher values of pH (Krampitz, 1970), and by PDC from wheat germ (Singer & Pensky, 1952a) and some bacteria, an earlier paper claimed that PDC from brewers' yeast failed to convert acetaldehyde to acetoin (Juni, 1955, 1961). We now demonstrate [by employing a gas–liquid chromatographic (GLC) technique to quantify the amount of acetoin produced] that PDC isolated from brewers' yeast indeed synthesizes acetoin from acetaldehyde. A number of kinetic studies were performed to compare the rates of acetoin formation from pyruvic acid and from acetaldehyde. Also, primary deuterium kinetic isotope effects (KIE) were determined by comparing the rates of acetoin formation from CH_3CHO and CH_3CDO. The magnitude of the KIE's on the steady-state kinetic parameters allowed us to conclude that the protonation–deprotonation step (the step most sensitive to isotopic substitution) is at least partially rate limiting in acetoin formation. Finally, the acetoin produced from acetaldehyde or pyruvic acid was found to be optically active, thus confirming that the enamine intermediate **4** must be located in a chiral environment.

Experimental Procedures

Materials. Acetoin [*dl*-3-hydroxy-2-butanone, 85% (v/v) aqueous, lot TD-6614] was from Aldrich, acetaldehyde (99%, lot KJAT 2401) was from Mallinckrodt, acetaldehyde-d_1 (98 atom % D, lot 1905, F) was from Merck Isotopes, and thiamin diphosphate monochloride and pyruvic acid sodium salt were from Sigma. All buffers (acetate at pH 5, 0.1 M; citrate at pH 5.8–6.2, 0.1 M; phosphate at pH 6.7–7.0, 0.1 M) were prepared in doubly distilled, deionized water.

Purification of Brewers' Yeast Pyruvate Decarboxylase. The initial steps followed the procedure of Ullrich (1970). The activity was determined by pH-stat titration (Schellenberger et al., 1968) and is reported in units per milliliter of solution at 25 °C, where 1 unit represents the conversion of 1 μmol of pyruvate to acetaldehyde per min at pH 6.0, 25 °C. Further purification (Gounaris et al., 1971) of PDC was accomplished by chromatography through a DE-23 (Whatman) cellulose ion-exchange column (45 × 3 cm, packed to 30-cm height) that was equilibrated first with 0.01 M phosphate, pH 6.3, and that also contained 1 mM $MgCl_2$, 10 μM TDP, and 5 mM L-cysteine hydrochloride. The protein was eluted with a linear gradient of phosphate increasing in concentration from 10 to 100 mM at a flow rate of 20 mL/h. A total of 100 tubes with 5 mL/tube was collected. Fractions from tubes 55–65 were pooled and concentrated through ultrafiltration (PM-10). According to SDS–polyacrylamide slab gel electrophoresis, there was only one band present (at least 80%) whose molecular weight was 58 000–60 000. The purified enzyme had a specific activity, typically, of 40–50 units/mg of protein at pH 6.0, 25 °C.

Determination of Acetaldehyde and Acetoin by GLC. A method developed for the determination of acetoin content in wines was adopted (Shinohara et al., 1979). A Hewlett-Packard 5830 A gas chromatograph equipped with a multifunction digital processor and flame-ionization detector was used to collect all GC information. A column was made by packing 15% Carbowax 1000 on Chromosorb W Hp 60/80 (Supelco Co., University Park, PA) into a silanized 6 ft × 0.25 in. o.d. (2-mm i.d.) glass tubing. Prior to use, the packed column was conditioned overnight with approximately 10 mL/min N_2 gas flow at an oven temperature of 145 °C. The sample was introduced through the injection port with a 5- or 10-μL Hamilton syringe. Standard GLC conditions were as follows: programmed column temperatures of 40 °C initial and 90 °C final at a rate of 5 °C/min; isothermal conditions of 40 °C for acetaldehyde and 90 °C for acetoin; injection temperature of 170 °C; flame ionization detector temperature of 300 °C; N_2 carrier gas at a flow rate of 30 mL/min. The absolute retention times were 1 min for acetaldehyde and for acetoin 15.4 min with the temperature program and 9.3 min under isothermal conditions. Under the conditions described, the sensitivities were 0.02 mM for acetoin (SD 1.8%) and 0.16 mM for acetaldehyde (SD 2.0%). The standard curves were linear (0.04–0.3 mM for acetoin, $R = 1.000$; 0.6–5 mM for acetaldehyde, $R = 0.996$). Samples for GLC analysis were prepared by placing 2.1 mL of a buffer containing acetaldehyde or pyruvic acid sodium salt, thiamin diphosphate, and the enzyme solution into a screw-cap tube. The tube was incubated at 40 °C for 120 min. Periodically, 50-μL aliquots were removed and quenched with 200 μL of 0.01 N HCl. A 5- or 10-μL aliquot of this quenched solution was injected onto the GLC.

Determination of Optical Activity of the Acetoin Produced. A Perkin-Elmer Model 241 polarimeter was employed. One experiment employed a mixture of 2.5 M acetaldehyde, 4.3 mM TDP, and 30 units of enzyme at pH 6.0. The mixture was incubated overnight at 40 °C. The acetoin concentration was checked by GLC employing authentic acetoin as the reference compound. The protein was next precipitated with $Zn(OH)_2$ at 0 °C, 0.5 mL of 0.5 M KH_2PO_4 was added, and the mixture was centrifuged. Vacuum transfer was next employed to remove the aqueous acetoin. The fraction of pure acetoin obtained upon vacuum transfer had a concentration of 7.44 mM by GLC and $[\alpha]^{27}_D = -23°$ (±1.5°). The second experiment started with a mixture of 0.135 M sodium pyruvate, 8.1 mM TDP, and 24 units of PDC at pH 6.0. After incubation of the mixture for 1 h at 40 °C, the acetoin was purified as in the first experiment. Upon vacuum transfer, the concentration of acetoin was 21.1 mM with a specific rotation $[\alpha]^{27}_D = -45°$ (±1°).

Results and Discussion

A Simultaneous GLC Determination of the Concentration of Acetaldehyde and Acetoin. A GLC method was developed for the simultaneous determination of both acetaldehyde and acetoin employing either a temperature-programmed technique (40 °C for acetaldehyde and 90 °C for acetoin) or separate isothermal injections (40 and 90 °C). Acetaldehyde could be assayed with routine detectability of 0.6 mM and a minimum sensitivity of 0.16 mM. Most significantly, however, acetoin could be reproducibly and routinely determined at concentrations as low as 37 μM, and a sensitivity of 20 μM could be obtained. Figure 1 represents a typical chromatogram obtained in the temperature-program mode for a mixture of authentic acetaldehyde (1.2 mM) and acetoin (0.07 mM). In control experiments, it was demonstrated that none of the components in the reaction mixtures (i.e., buffers, proteins, cofactors) affected the accuracy and reproducibility of the standard curves.

Demonstration of the Synthesis of Acetoin from Acetaldehyde by Brewers' Yeast Pyruvate Decarboxylase. First, is was necessary to demonstrate that no acetoin is formed from pyruvate or acetaldehyde in the absence of PDC under the conditions employed (pH, temperature, concentrations of substrates). Figure 2 demonstrates that acetaldehyde is produced from pyruvate only in the presence of PDC under the conditions employed. Figures 3 and 4 demonstrate formation of acetoin from pyruvic acid and from acetaldehyde, respectively, only in the presence but not in the absence of the en-

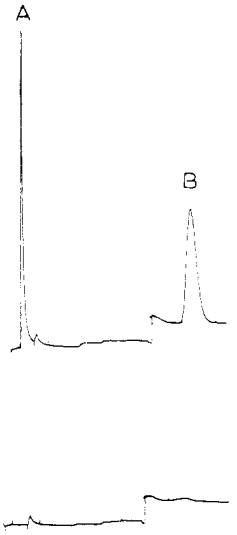


FIGURE 1: A typical chromatogram (GLC, the upper curve) of a mixture of acetaldehyde [(A) 1.2 mM)] and acetoin [(B) 0.07 mM] dissolved in 0.1 M citrate buffer (pH 6.0) obtained by a temperature-programmed method (from 40 to 90 °C). The absolute retention times under these conditions were 1 min for acetaldehyde and 15.4 min for acetoin. The acetoin was recorded at 8 times higher amplification. The lower curve is that of buffer alone.

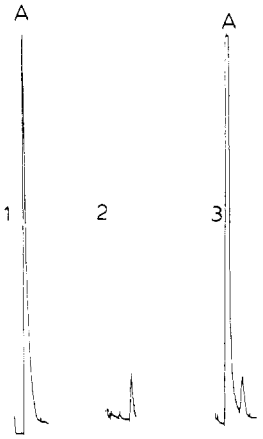


FIGURE 2: GLC traces demonstrating the formation of acetaldehyde (A) from pyruvate in the presence but not in the absence of PDC: (trace 1) 0.625 mM acetaldehyde; (trace 2) acetaldehyde is not formed from 0.01 M pyruvate at 40 °C and pH 6.0 in the presence of all components except PDC; (trace 3) acetaldehyde is formed from 0.01 M pyruvate at 40 °C in 30 min by 2 units of PDC. The GLC traces were collected under isothermal (T_c = 40 °C) conditions and an N_2 flow rate of 30 mL/min.

zyme. In order to demonstrate that the same protein catalyzed the formation of acetoin from acetaldehyde and from pyruvate, the initial rates of acetoin formation by relatively crude [$(NH_4)_2SO_4$ paste] and essentially homogeneous enzymes were compared. The crude and purified enzyme exhibited the same relative activities for conversion of pyruvic acid to acetaldehyde compared to condensation of acetaldehyde to acetoin. At 40 °C and pH 6.0, 1 unit of PDC produced 1.15 ± 0.1 μM acetoin per min for 1 M initial concentration of acetaldehyde.

The purified protein had a specific activity some 4 times greater than the cruder one, and the former appeared essentially homogeneous according to polyacrylamide–SDS slab gel electrophoresis. The molar ratio of acetaldehyde to acetoin produced from pyruvate by the crude and the purified enzyme was, within experimental error, the same. We therefore conclude that a single protein was responsible for the observed catalysis in the synthesis of acetoin from acetaldehyde, namely, PDC.

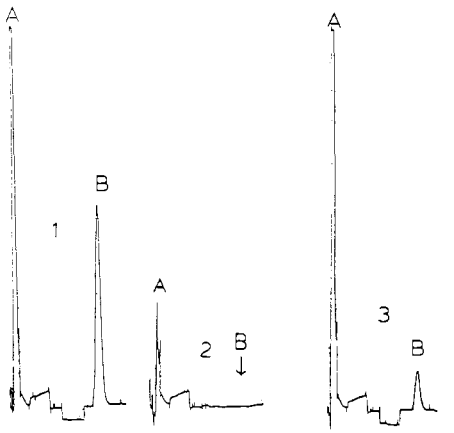


FIGURE 3: GLC traces demonstrating the formation of acetoin (B) from pyruvate in the presence but not in the absence of PDC: (trace 1) 37 μM authentic acetoin; (trace 2) 60-min incubation at 40 °C of 0.01 M pyruvate in 0.1 M citrate, pH 6.0, in the presence of all components but PDC; (trace 3) 30-min incubation at 40 °C of 0.01 M pyruvate in 0.1 M citrate, pH 6.0, in the presence of 2 units of PDC. The GLC traces were collected under isothermal (T_c = 90 °C) conditions at an N_2 flow rate of 30 mL/min. The acetoin was recorded at 8 times higher amplification.

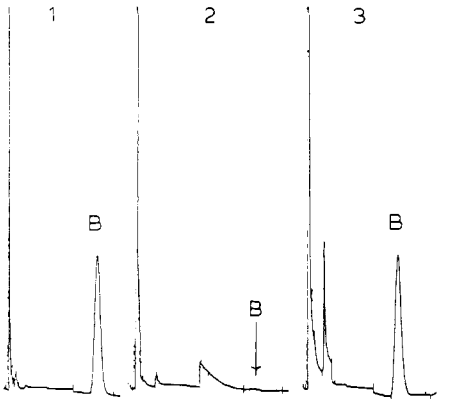


FIGURE 4: GLC traces demonstrating that PDC isolated from brewers' yeast forms acetoin (B) from acetaldehyde: (trace 1) authentic acetoin (19 μM); (trace 2) 2-h incubation of 0.2 M acetaldehyde at 40 °C in 0.1 M citrate, pH 6.0, in the presence of all components but PDC; (trace 3) 1-h incubation of 0.2 M acetaldehyde at 40 °C in 0.1 M citrate, pH 6.0, in the presence of 10 units of PDC. The GLC traces were collected under isothermal conditions (T_c = 90 °C) at a gas flow rate of 30 mL/min.

In addition to the enhanced sensitivity, the important advantage of the GLC method compared to the colorimetric one employed in previous studies (Westerfield, 1945) for determination of acetoin content is that acetaldehyde even at 1 M concentrations does not interfere with the GLC method. Apparently, PDC from all sources so far examined is capable of producing acetoin from acetaldehyde to some limited extent.

Kinetics of Acetoin Formation by Different Pathways. It was demonstrated earlier by several groups that acetoin is produced by PDC's from various sources as a side product (ca. 3%) of pyruvate decarboxylation (Singer & Pensky, 1952a; Juni, 1955, 1961). Those results were confirmed in this laboratory and will not be presented in detail. Acetoin can be produced by two pathways employing brewers' yeast PDC as catalyst: (a) starting with pyruvate (k_6, k_5, k_3[CH_3CHO], k_4 pathway), a reaction that is known to be greatly accelerated by added acetaldehyde [Singer & Pensky (1952a), Juni (1955, 1961), and confirmed in this laboratory], and (b) starting with acetaldehyde (k_1, k_2, k_3[CH_3CHO], k_4 pathway). Table I lists the amounts of acetoin formed from acetaldehyde and the percent conversion to this product at pH 5–7 by 10 units of

Table I: PDC-Catalyzed Synthesis of Acetoin from Acetaldehyde[a] at pH 5–7

	acetoin formed and % conversion					
	pH 5.0		pH 6.0		pH 7.0	
incubation time (min)	concn (mM)	%	concn (mM)	%	concn (mM)	%
0.5	*b*	*b*	0.0225	0.02	*b*	*b*
15	0.100	0.11	0.389	0.41	*b*	*b*
30	0.198	0.21	0.716	0.75	0.060	0.06
60	0.293	0.31	1.28	1.35	0.140	0.15
120	0.316	0.33	1.54	1.61	0.158	0.17

[a] The incubation mixture contained 190 mM acetaldehyde and 5 mM TDP in 0.1 M buffer (acetate for pH 5.0, citrate for pH 6.0, and phosphate for pH 7.0) along with 10 units of PDC at 40 °C. At the indicated times aliquots were removed, quenched, and analyzed according to the protocol under Experimental Procedures. [b] Trace amounts.

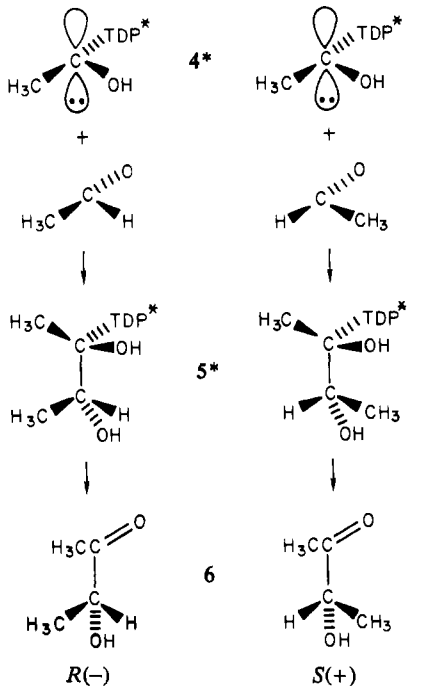
PDC at 40 °C. At pH 6 and 40 °C, in two separate sets of experiments that employed 7 and 14 units of PDC, respectively, the rates of acetoin formed from pyruvate or acetaldehyde were compared. Extrapolated to zero time, acetoin was formed some 80 ± 20 times faster from pyruvate than from acetaldehyde. Given that steps k_3 and k_4 are common to both reactions, this number suggests that the enamine is produced at least 80 ± 20 times faster from pyruvate than from acetaldehyde. The K_m and V_{max} for acetoin from acetaldehyde are 1 M and 1.4×10^{-4} M/min, respectively, at 40 °C and pH 6.0 compared to the K_m of 2.0 mM for pyruvate decarboxylation (Hübner et al., 1978). In addition, at pH 6.0 and 40 °C, the initial rates of acetoin formation starting with 10 mM pyruvic acid in the presence of 5, 10, or 15 mM acetaldehyde were determined. This allowed a calculation (double-reciprocal plot of $1/v_0$ vs. $1/[\text{acetaldehyde}]$) of apparent steady-state kinetic constants for the trapping of the enamine by acetaldehyde: $K_m^{app} = 10.4$ mM and $V_{max} = 0.9 \times 10^{-4}$ M/min for 10 mM initial concentration of pyruvic acid.

The initial rate of acetoin formation from pyruvate compared to that from acetaldehyde (80 ± 20) is expressed in the K_m's, that is, the K_m for trapping of the enzyme-bound enamine by acetaldehyde, compared to the K_m for binding acetaldehyde to enzyme-bound TDP. The pH dependence of the rate of acetoin formation from acetaldehyde (Table I) or from pyruvate, as well as the rate of acetaldehyde formation from pyruvate, is very similar. While the relative rate constants differ, the general shape and optimum pHs of 6–6.2 (Green et al., 1941; Singer & Pensky, 1952b; Schellenberger et al., 1968; Jordan et al., 1978c) are the same for all three processes. Very likely, the same groups on the enzyme are responsible for all pathways, and all processes may follow the same rate-limiting step(s), k_1 (k_{-1}) and k_2 (k_{-2}) in Scheme I.

Optical Activity of the Acetoin Produced by PDC: Stereochemical Implication. The acetoin produced by PDC from acetaldehyde had $[\alpha]^{27}_D = -23°$ (±1.5°), i.e., consisted of 64 (−) and 36% (+) enantiomer. The acetoin produced by PDC from pyruvate had $[\alpha]^{27}_D = -45°$ (±1°), hence consisted of 77 (−) and 23% (+) enantiomer. These numbers are based on the $[\alpha]^{27}_D = -84°$ (±2°) obtained for (−)-acetoin isolated from a number of bacteria and tissues (Tanko et al., 1940; Berl & Bueding, 1951; Juni, 1951b) and confirmed by Singer & Pensky (1952a). No evidence for racemization was obtained under the reaction and purification conditions.

The facts that (a) the acetoin produced by PDC was optically active and (b) as much as 18% acetoin could be produced from pyruvate by simply adding acetaldehyde (extrapolating to 1 M acetaldehyde concentration) to the enzymatic reaction mixture should make this a useful procedure

Scheme II



for the enantioselective synthesis of α-ketols with a substantial enantiomeric excess. The findings that the acetoin produced from pyruvate or from acetaldehyde is optically active demonstrate several points. First of all, it clearly demonstrates that the observations refer, at least mostly, to enzyme-catalyzed processes. In the reaction catalyzed by TDP, measurable amounts of acetoin were only produced above pH 8.0 (Krampitz, 1970). Starting with pyruvate, there could be two pathways for acetoin synthesis: (i) addition of the acealdehyde released to the enamine or (ii) addition of a second pyruvic acid molecule to the enamine to produce acetolactate [$CH_3COC(CH_3)(OH)CO_2H$], a β-keto acid that would thermally decarboxylate to acetoin. The second of these pathways is likely to be important in the nonenzymatic TDP-catalyzed reaction. The fact that the acetoin produced enzymatically is optically active is only consistent with the first pathway being more significant for the PDC-catalyzed condensation when starting with pyruvate. The thermal decarboxylation of acetolactate should yield racemic acetoin. Scheme II accounts for all the observed stereochemistry. The active aldehyde (enamine) is protected on one side, as is also evident from the fact that **2** isolated from an enzymatic reaction mixture was optically active [see Holzer (1961)]. Its nucleophilic carbon can react with acetaldehyde on both its *re* and *si* faces to form two diastereomeric **5***'s (one erythro and one threo) with the simultaneous creation of two chiral centers. Upon release of these diastereomers, the chirality at one of the carbons is abolished, and a pair of enantiomeric **6**'s results. On the basis of the recent report of the synthesis of the pure enantiomers of acetoin (Crout & Morrey, 1983), the absolute stereochemistry of the condensation reaction can be assigned as depicted in Scheme II. It is premature to speculate on the origins of the preference for enamine addition to the *si* face of acetaldehyde.

Deuterium Kinetic Isotope Effects. The concentration and purity of acetaldehyde-d_1 were determined by GLC employing the protiated substrate as the reference compound. In separate experiments, 10 or 14 units of PDC was employed at 40 °C, and aliquots were removed after 15 and 25 min. The initial rate of acetoin formation is defined as the average rate of

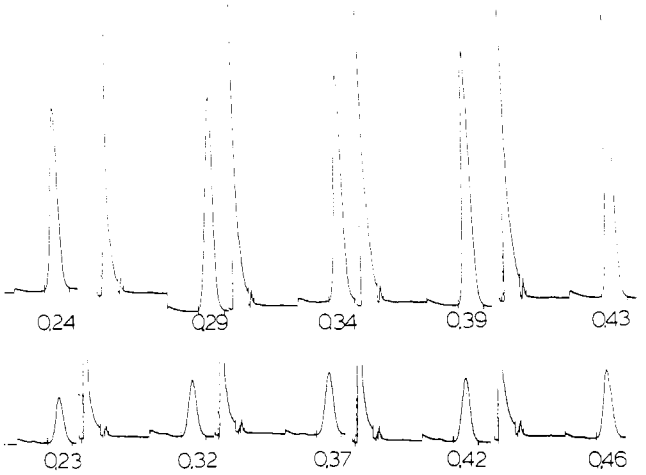


FIGURE 5: GLC traces of acetoin synthesized from CH_3CHO (upper traces) and CH_3CDO (lower traces) at increasing initial molar concentrations of substrate indicated under each acetoin peak. The traces were obtained from 25-min incubations at pH 6.0 (0.1 M citrate) and 40 °C in the presence of 10 units of PDC.

acetoin production in the quoted time interval. Figure 5 demonstrates the amounts of acetoin formed from increasing initial concentrations of CH_3CHO and CH_3CDO. Even at the pH optimum of 6–6.2, only very small quantities of acetoin were produced from CH_3CDO. The concentration dependence of v_0 for CH_3CHO and CH_3CDO could only be determined at pH 6 and 6.2. On either side of the optimum pH, only v_0 ratios could be determined for equal concentrations of protiated and deuterated substrates and at equal concentrations of PDC. Because of the complexity of the kinetic expression (due to the second-order dependence of v_0 on substrate concentrations), the isotope effects will be discussed in terms of ${}^{D}V$ and ${}^{D}V/A$ or ${}^{D}V/B^2$ (where A and B are a collection of kinetic constants) rather than ${}^{D}V/K_m$, the quantity that is presented ordinarily.

The kinetic scheme adopted is

$$\text{TDP}^* + \text{S} \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} \underset{1}{\text{TDP}^*\cdot\text{S}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{2^*}{\text{HETDP}^*} \underset{k_{-2},\,\text{H}^+}{\overset{k_2}{\rightleftharpoons}} \underset{4^*}{\text{En}^*} \underset{k_{-3}}{\overset{k_3,\,\text{S}}{\rightleftharpoons}} \underset{5^*}{\text{P}\cdot\text{TDP}^*} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \underset{6}{\text{P}} + \text{TDP}^* \quad (2)$$

where TDP* is tightly enzyme-bound TDP[3] that remains on the enzyme throughout the catalytic turnover, S is acetaldehyde, P is acetoin, En* is the active aldehyde (α-carbanion, enamine), and P·TDP* is 2-(2,3-dihydroxybutyl)-TDP*—the bound form of acetoin. Although reversibility of steps k_0 (k_{-0}), k_1 (k_{-1}), and k_2 (k_{-2}) is obvious (since the rate constants for the reverse reactions are those for pyruvate decarboxylation), no evidence is available for the reversibility of k_4; therefore, k_{-4} will be assumed to equal 0. If one writes an equilibrium constant K_s (=[TDP*][S]/[TDP*·S]) for the dissociation of the noncovalent TDP*·S complex and steady states on HETDP*, enamine*, and P·TDP*, the following format results for v_0:

$$v_0 = \frac{V_{max}}{A/[S]^2 + B/[S] + 1.0} \quad (3)$$

where

$$A = \frac{k_{-1}k_{-2}'(k_{-3} + k_4)}{k_3(k_{-1}k_4 + k_2k_4 + k_1k_2 + k_1k_4)} \quad (4)$$

$$B = [(k_1k_2 + k_{-1}k_{-2}' + k_1k_{-2}')(k_{-3} + k_4) + Kk_3k_4(k_{-1} + k_2)]/[k_3(k_{-1}k_4 + k_2k_4 + k_1k_2 + k_1k_4)] \quad (5)$$

where

$$k_{-2}' = k_{-2}[\text{H}^+]$$

and V_{max} (obtained from v_0 by allowing [S] $\rightarrow \infty$) is

$$V_{max} = \frac{k_1k_2k_4E_t}{k_{-1}k_4 + k_2k_4 + k_1k_2 + k_1k_4} \quad (6)$$

In double-reciprocal form, eq 3 is converted to eq 7. Barring

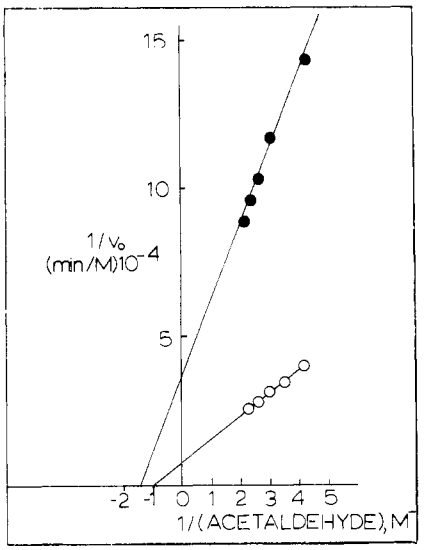


FIGURE 6: Lineweaver-Burk double-reciprocal plots of the initial rates (v_0) of acetoin formation from CH_3CHO (○) and CH_3CDO (●) a 40 °C and pH 6.0 (0.1 M citrate) with 10 units of PDC. The v_0 values were obtained as the average amount of acetoin formed per minute over a 15-min incubation period.

Table II: Primary Deuterium Kinetic Isotope Effects on v_0, V, and V/B in the PDC-Catalyzed Conversion of CH_3CHO and CH_3CDO to Acetoin at 40 °C

pH	${}^{D}v_0$[a]	${}^{D}V$[d]	${}^{D}V/B$[d]
5.8	4.81[b]		
	4.54[c]		
6.0		4.53[b]	3.37[b]
		4.70[c]	3.13[c]
6.0	4.82[b]		
	4.84[c]		
6.2		4.10[c]	3.09[c]
6.2	4.50[b]		
	4.05[c]		
6.7	4.85[b]		
av	4.63 ± 0.34	4.44	3.2

[a] Determined at single identical concentrations of substrates and PDC. Substrate concentrations were 2–3-fold lower than K_m. [b] The rate ratios represent average rates over 15-min incubations. [c] The rate ratios represent average rates over 25-min incubations. [d] Obtained from [S]/v_0 vs. [S] or $1/v_0$ vs. $1/[S]$ plots; B is from the limiting slope according to eq 7 and represents the collection of rate constants described in eq 5.

[2] Throughout the following discussion, the abbreviations recommended by Northrop (1975) will be employed, i.e.

$${}^{D}v_0 = \frac{v_0(\text{protonated substrate})}{v_0(\text{deuterated substrate})}$$

at the same concentrations of enzyme and substrates. ${}^{D}k_2 = {}^{H}k_2/{}^{D}k_2$; ${}^{D}V = V_{max}(\text{protonated substrate})/V_{max}(\text{deuterated substrate})$; ${}^{D}V/A = (V_H/A_H)/(V_D/A_D)$; ${}^{D}V/B = (V_H/B_H)/(V_D/B_D)$.

[3] Employing the ${}^{D}V$ and ${}^{D}V/B$ values, one can estimate (Northrop, 1975) K_s (=k_{-0}/k_0, the dissociation constant of the PDC–acetaldehyde noncovalent complex) at 40 °C and pH 6.0 to equal 0.88 M, in reasonable agreement with the K_m^{app} reported above.

$$1/v_0 = (1/V_{max})(A/[S]^2 + B/[S] + 1.0) \quad (7)$$

experimental limitations, three parameters, A, B, and V_{max}, should be accessible for the protiated and deuterated substrates. With our current technique, v_0 could be determined only in a limited and rather high (0.2–0.5 M) substrate concentration range. A plot of $1/v_0$ vs. $1/[S]$ was linear (see, for example, Figure 6), allowing the determination of V_{max} and B but not of A. Table II summarizes ^DV, $^Dv_0$, and $^DV/B$ values. Within experimental uncertainty, the $^Dv_0$ ratios and ^DV ratios are essentially the same (~4.50) and pH independent in the region 5.8–6.7, at and on either side of the pH optimum for both the pyruvate decarboxylation and acetoin formation.

The overall equilibrium isotope effect, $^DK_{eq}$ (eq 8), for eq 2 can be estimated from isotope fractionation factors (Cleland,

$$^DK_{eq} = [CH_3CHO]^2/[\text{acetoin}] \quad (8)$$

1980) to be between 0.90 and 0.94 on the basis of 40% unhydrated and 60% hydrated acetaldehyde ($0.4 \times 0.83 + 0.6 \times 1.14 = 1.016$) and a fractionation factor of 1.10 or 1.16 for acetoin (the hydration of which is not very extensive). This relatively small magnitude of $^DK_{eq}$ and the following factors allow us to place qualitative limits on some key rate constant ratios in the mechanism. (a) The rate constant k_2 is assumed to be the only one that is sensitive to isotopic substitution. (b) $k_{-2}' \ll k_3K$. This involves no assumption; rather, it reflects the fact that under conditions comparable to those employed in this study, **2***, when added to a large amount of apo-PDC for prolonged periods of time, failed to lose its tritium label from the 2α-carbon (Ullrich & Mannschreck, 1967). This comparison demonstrates that the step k_{-2}' is slower than the subsequent condensation here studied. (c) The rate constants k_{-3} and k_{-4} are 0 since no back reaction is observable under the reaction conditions. (d) $k_3K \ll k_4$. The rate of acetoin formation from pyruvate was found to increase linearly with increasing concentrations of added acetaldehyde (Singer & Pensky, 1952a; Juni, 1955, 1961; this study).

The expressions for the isotope effects can now be recast in their usual format as follows:

$$^DV = \frac{^Dk_2 + C_1}{1 + C_1} \quad (9)$$

where

$$C_1 = k_2\frac{1/k_4 + 1/k_1}{1 + k_{-1}/k_1} \quad (10)$$

and

$$^DV/B = \frac{^Dk_2 + C_2}{1 + C_2} \quad (11)$$

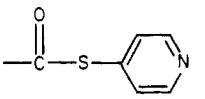
where

$$C_2 = k_2\frac{1/(k_3K) + 1/k_1}{k_{-2}'/(k_3K) + k_{-1}/k_1} \quad (12)$$

It is important to point out that, for the mechanistic scheme depicted in eq 2, $^DV/A$ (that we have no access to currently) would provide $^Dk_2$ directly! Lacking $^Dk_2$, we can bracket it between 4.5 ($^DV_{max}$ in this study) and 10 [based on a number of examples: Jencks (1969) and Klinman (1978)]. On the basis of the C_1 and C_2 values so calculated, one can next search for acceptable values that simultaneously satisfy the experimental data, yielding $k_{-2}' < k_3K$ (reaffirming point b above), $0.1 < k_{-1}/k_2 < 10$, and $0.1 < k_{-1}/k_{-2}' < 10$.

Those results are very significant to our understanding of the relative barrier heights in steps past decarboxylation. They demonstrate, for example, that the barrier heights on the enzyme for several steps are very similar (k_{-1}, k_2, k_{-2}').

The following is a summary of our current status of knowledge of the relative energetics of the reaction steps depicted in Scheme I. (i) **4*** is produced 80 ± 20 times faster from pyruvate than from acetaldehyde (this study). (ii) $k_{-6} < k_5$ (O'Leary, 1976; DeNiro & Epstein, 1977; Jordan et al., 1978a,b). (iii) k_3K (or k_4) > k_{-2}' [this study and comparison with results of Ullrich & Mannschreck (1967)].[4] (iv) $k_2 \sim k_{-1}$ (within a factor of 10); hence, C–H bond scission is at least part of rate limitation in acetoin synthesis from acetaldehyde. (v) $k_{-2}' \sim k_{-1}$ (within a factor of 10); i.e., on the forward decarboxylation pathway of the steps beyond CO_2 loss, protonation of the enamine is at least partially rate limiting. These last two rate constant ratios also suggest that the enzyme has an important role in stabilizing the enamine such that **4*** becomes the central intermediate on the reaction pathway. The spectroscopic observation of a PDC-bound chromophore with λ_{max} and ϵ consistent with those expected for **8**, analogous to **4***, the product of decarboxylation (Kuo & Jordan, 1983b), and the ability of the oxidizing electrophile 4,4′-dithiodipyridine to trap **8** to produce the thiol ester (unpublished results)



also strongly support stabilization of such enamines on the enzyme. At least in the decarboxylation reaction catalyzed by brewers' yeast PDC, the release (upon protonation) of the enamine is slower overall than steps through decarboxylation.

It is tempting to speculate, finally, that the subsequent reaction of such enamines (protonation or oxidation) is part of rate limitation on pyruvate decarboxylation pathways requiring TDP, whereas the formation of the enamine is part of rate limitation in those reactions leading to condensations (such as transketolases and phosphoketolase).

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[4] The implications of $k_{-2}' < k_3K$ are that to avoid production of acetoin during fermentation, the subsequent enzyme (alcohol dehydrogenase) must perform its function very efficiently.

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Azasterol Inhibition of Δ^{24}-Sterol Methyltransferase in *Saccharomyces cerevisiae*†

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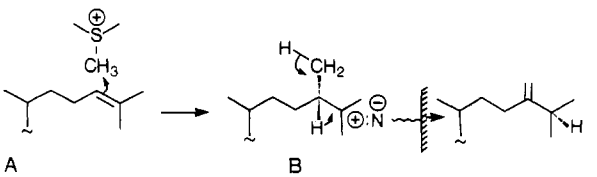
ABSTRACT: The inhibition of the Δ^{24}-sterol methyltransferase (24-SMT) of *Saccharomyces cerevisiae* by side-chain azasterols is related to their nuclear skeleton and side chain nitrogen position. Inhibitory power [I_{50} (μM)] was found to be in the order of 25-azacholesterol hydrochloride salt (0.05) > 25-aza-24,25-dihydrozymosterol (0.08) > 25-azacholesterol ≈ 25-azacholestanol (0.14) > (20*R*)- and (20*S*)-22,25-diazacholesterol (0.18) > 24-azacholesterol (0.22) > 25-aza-24,25-dihydrolanosterol (1.14) > 23-azacholesterol (4.8). In the presence of azasterols, *S. cerevisiae* produces increased amounts of zymosterol, decreased amounts of ergosterol and ergostatetraenol, and the new metabolites cholesta-7,24-dienol, cholesta-5,7,24-trienol, and cholesta-5,7,22,24-tetraenol. Kinetic inhibition studies with partially purified 24-SMT and several azasterols suggest the azasterols act uncompetitively with respect to zymosterol and are competitive inhibitors with respect to *S*-adenosyl-L-methionine (SAM). These results are consistent with at least two kinetic mechanisms. One excludes competition of azasterol and zymosterol for the same site, whereas a second could involve a ping-pong mechanism in which 24-SMT is methylated by SAM and the methylated enzyme reacts with sterol substrate.

The C-24 alkylation of Δ^{24}-sterols is viewed as a nucleophilic attack of the Δ^{24}-π electrons on the *S*-methyl group of SAM.[1] This generates an intermediate possessing a cationic site at C-25 in a C-24-methylated sterol. Migration of hydrogen from C-24 to C-25 and subsequent loss of a hydrogen from the C-24 methyl yields the 24-methylenesterol in yeast and higher plants (Arigoni, 1978):



The enzyme mediating this process might be inhibited by mimics of either the substrate A or the intermediate B. Recent work with the yeast *Saccharomyces cerevisiae* (Avruch et al., 1976) has shown that 25-aza-24,25-dihydrozymosterol (**1**) is

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[1] Abbreviations: 24-SMT, Δ^{24}-sterol methyltransferase; 24(28)-MSR, 24-methylenesterol $\Delta^{24(28)}$-reductase; SAM, *S*-adenosyl-L-methionine; MS, mass spectrum; TLC, thin-layer chromatography; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane; GLPC, gas–liquid partition chromatography.

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