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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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* From the James Bryant Conant Laboratory of the Chemistry Department of Harvard University, Cambridge, Massachusetts. *Received August 28, 1967.* This work was supported by GM 04712 from the Institute of General Medical Sciences of the National Institutes of Health.

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‡ J. P. G. gratefully acknowledges a National Research Council (Canada) Special Scholarship.

acetopyruvate reacts at the same lysine residue (Laursen and Westheimer, 1966) as does the substrate. The enzyme is an oligomer (dodecamer; see Tagaki and Westheimer, 1968) but, as shown in this paper, only one lysine residue in each two subunits participates in formation of Schiff base or enamine.

The chemistry shown in eq 1 is here supported by two general lines of evidence: (a) the isolation and determination of the properties of the enzyme–substrate compound, and (b) preparation of enamines that can serve as models for the enzymic one. For (a), radioactive acetopyruvate was prepared, and the compound it forms with the enzyme was isolated by precipitation and analyzed radiochemically. The compound shows an intense absorption band at 325 mμ that also allows its quantitative estimation and permits a determination of the rate at which the enzyme–inhibitor compound is formed. For (b), the reactions of acetopyruvate and pivaloylpyruvate with aminoacetonitrile ($H_2NCH_2C{\equiv}N$) were investigated; the resulting enamines were identified by their analyses and nuclear magnetic resonance and ultraviolet spectra. In particular, the absorption band near 325 mμ helps establish the relationship between the model compounds and the enzymic one.

Experimental Section

Materials. The crystalline decarboxylase was prepared according to Zerner *et al.* (1966; *cf.* Westheimer, 1968). Throughout this paper, the concentration of enzyme is calculated on the basis of a molecular weight of 29,000, *i.e.*, the concentrations are given in terms of the ultimate subunit of acetoacetate decarboxylase (Tagaki and Westheimer, 1968) rather than in terms of the oligomer.

Labeled acetopyruvate (APY)[1] was prepared starting from 0.1 mCi of oxalic acid-^{14}C (New England Nuclear Corp.). The oxalic acid (6.5 mg, 1.35 mCi/mmoles) was diluted with about 400 mg of unlabeled oxalic acid dihydrate (Merck) and converted to diethyl oxalate (yield, 300 mg) according to the method of Marvel and Dreger (1932). The ester was further diluted with 100 mg of unlabeled diethyl oxalate and condensed with acetone according to Lehninger and Witzemann (1942). The hydrolysis of the ester was inefficient: after sublimation and recrystallization of the acid (from carbon tetrachloride), only 24 mg of acid, mp 99° (lit. mp 98°), was obtained. Further recrystallization and sublimation did not change the melting point. The specific activity of the acid was 3.85×10^5 dpm/mg (0.02 mCi/mmole). Ethyl acetopyruvate (unlabeled) was prepared on a large scale by the method given above and hydrolyzed by the method of Meister and Greenstein (1948). Methyl pivaloylpyruvate was prepared according to Royals (1945). The ester was hydrolyzed by the same method as that used for the ester of acetopyruvate; the acid crystallizes as the monohydrate (Mumm and Hornhardt, 1937), or from chloroform–petroleum ether (bp 39–52°) to give material melting at 57–58.5°. Aminoacetonitrile was obtained by neutralizing Aldrich aminoacetonitrile bisulfate (Cook *et al.*, 1948).

TABLE I: Formation of Enamine at 30°.

Initial Concn (M)[a]			
Amino-acetonitrile	Aceto-pyruvate ($\times 10^5$)	Equilibrium OD_{310}	$\frac{OD_{310} \times 10^5}{[\text{Acetopyruvate}]_{initial}}$
0.0445	13.9	1.788	0.1286
0.0249	13.9	1.682	0.1210
0.0178	13.9	1.580	0.1137
0.0142	13.9	1.520	0.1094
0.0107	13.9	1.433	0.1031

[a] In 0.0815 M phosphate buffer, pH 5.80, μ 0.3 M.

Enamine from Ethyl Acetopyruvate and Aminoacetonitrile. A solution of 3.39 g of aminoacetonitrile in 30 ml of methylene chloride was added to 9.28 g of ethyl acetopyruvate (EAPY) in 10 ml of methylene chloride and 40 ml of petroleum ether (bp 39–52°). The reaction mixture was cooled in ice, and the abundant white precipitate was removed by filtration and allowed to stand overnight. The resulting brown and sticky mass was dissolved in chloroform and chromatographed on a 2 × 28 cm column of Florisil, using chloroform as eluent; the progress of the elution was monitored by thin-layer chromatography on Eastman chromatogram sheet, developed with chloroform–methanol (1:1) as solvent and using ultraviolet light to make the spots visible. The enamine appeared with the solvent front in about 350 ml of chloroform; after the solvent had been removed by evaporation, the product was crystallized from ethyl acetate and then further purified by decolorization with activated charcoal in methylene chloride. The product from this treatment, after recrystallization from chloroform–petroleum ether, melted at 134–136° (cor); yield of crude material, 20%; nmr spectrum in chloroform: triplet (3 protons) at δ 1.3, singlet (3 protons) at δ 2.2, quartet at δ 4.3 and doublet at δ 4.5 (4 protons), singlet (1 proton) at δ 6.0, and broad triplet (1 proton) at δ 11.4. This spectrum is accounted for adequately by either enamine, and in particular by $CH_3COCH{=}C(CO_2C_2H_5)NHCH_2CN$. The infrared spectrum in $CHCl_3$ showed 3.1, 3.31, 4.04, 4.43, 5.75, 6.2 (sh), 6.30, 7.34, 8.79, 9.05, 9.84, and 10.29 μ. The mass spectrum of the compound, determined with an Associated Electrical Industries, Ltd. MS-9 mass spectrometer, showed a molecular ion peak at 196 and base peak (molecular ion minus $CO_2C_2H_5$) at 123, with an accompanying metastable peak as expected at 77.2. The next strong peaks, at 96, 81, and 54, correspond to loss of HCN, ketene, and HCN plus ketene

[1] Abbreviations used: APY, acetopyruvate; EAPY, ethyl acetopyruvate.

TABLE II: Composition of Solution for Radiochemical Assay (4 hr at 1°).

Enzyme Soln (ml)[a]	APY Soln (ml)[b,c]	Buffer (ml)[d]	Enzyme Concn of Subunits × 10^5 (M)	APY Concn × 10^5 (M)	(APY)[e]/(Enzyme)
0.25	1.39	0.00	5.54	110	20
0.25	0.70	0.69	5.54	56	10
0.25	0.28	1.11	5.54	22	4.0
0.20	0.17	0.92	5.64	17	3.0

[a] 12 mg/ml, 3.64×10^{-4} M in subunits, specific activity 44. [b] APY, acetopyruvate. [c] 1.3078×10^{-3} M. [d] Potassium phosphate buffer, 0.05 M, pH 5.98. [e] Ratio of the concentration of APY to that of the enzyme subunits.

from the base peak. The ultraviolet absorption in ethanol has λ_{max} 335 mμ (ε 15,000).

Anal. Calcd for $C_9H_{12}N_2O_3$: C, 55.08; H, 6.17; N, 14.28. Found: C, 55.17; H, 6.20; N, 14.23.

Enamine from Acetopyruvate Ion and Aminoacetonitrile. Solutions of aminoacetonitrile and acetopyruvate were equilibrated in buffered solutions at 30°, and their spectra were recorded with a Cary 15 spectrophotometer. From the data for 310 mμ (the wavelength of maximum absorption for the enamine) values of the equilibrium constant and the extinction coefficient for the enamine can be calculated. The data in Table I were fitted to eq 2 by a rigorous least-squares method (Wentworth, 1965), using a program for an IBM 1620 computer and adjusting two parameters, ($\epsilon_{enamine} - \epsilon_{acetopyruvate}$) and K, to give the best fit. In eq 2, K is the dissociation constant

$$\frac{OD_{310\,m\mu}}{(acetopyruvate)_{initial}} = \epsilon_{acetopyruvate} + \frac{\epsilon_{enamine} - \epsilon_{acetopyruvate}}{1 + \frac{K}{(amine)}} \quad (2)$$

of the enamine, ε's represent extinction coefficients, and the simple form of the equation can be derived to apply to those data where the concentration of amine greatly exceeds that of acetopyruvate and $\epsilon_{amine} = 0$. Using the data of Table I and 2120 ± 50 for the extinction coefficient for acetopyruvate at 310 mμ, the extinction coefficient of the enamine was calculated as 14,000 ± 300, with $K = 5.0 \pm 0.5 \times 10^{-3}$ M.

Enamine from Methyl Pivaloylpyruvate and Aminoacetonitrile. Acetic acid (4 drops) was added to a solution of 3.72 g of methyl pivaloylpyruvate and 1.14 g of aminoacetonitrile in 15 ml of methylene chloride. After 1.5 hr, evaporation of the solvent and seeding led to the formation of crystalline enamine. This material, however, was impure, and could be purified only with difficulty. When 0.64 g of the crude enamine was dissolved in benzene and eluted from a 2 × 10 cm column of Florisil with benzene, benzene–chloroform mixtures, and finally with chloroform, the various chloroform fractions could be pooled to yield 0.38 g of purified enamine. (The course of the chromatography could be followed by thin-layer chromatography on Eastman chromatogram sheet using 1:1 chloroform–methanol as solvent and iodine vapor to make the spots visible.) Four recrystallizations of the purified enamine from carbon tetrachloride–petroleum ether or cyclohexane–petroleum ether gave material of mp 87.5–89.5°; nmr in chloroform: singlet at δ 1.3 (9 protons), singlet at δ 4.1 (3 protons), doublet at δ 4.5 (2 protons), singlet at δ 6.26 (1 proton), and a broad, low peak at δ 10.2 (0.6 proton) (presumably the integration for the NH proton was inaccurate); infrared spectrum in chloroform: strong bands at 3.32, 5.74, 6.12, 6.29, 7.30, 7.71, 8.85, 9.18, 9.82, and 10.04 μ.

Anal. Calcd for $C_{11}H_{16}N_2O_3$: C, 58.91; H, 7.19; N, 12.49. Found: C, 58.70; H, 7.64; N, 12.52.

Preparation of the Enzyme-^{14}C-Acetopyruvate Compound. A reaction mixture (1.6 ml) was made up of enzyme (10^{-5} M in subunits), acetopyruvate (3×10^{-5} to 2×10^{-4} M): and buffer in a disposable plastic centrifuge tube. The mixture was allowed to stand for 4 hr in an ice bath, and the protein was precipitated by the addition of two volumes of 90% saturated ammonium sulfate solution in 0.05 M phosphate buffer (pH 5.98). After 30 min at 0°, the mixture was centrifuged, and the supernatant was decanted for measurement of radioactivity. The precipitate was then dissolved in

TABLE III: Radioactivity of Supernatant from Precipitations of Acetopyruvate–Enzyme Compounds.

(APY)/(Enzyme)[a]	Supernatant[b] 1st	2nd	3rd
20	1130	29	2
10	550	13	3
4.0	194	5	3
3.0	126	7	0

[a] Ratio of concentration of acetopyruvate to subunits of enzyme in solution from which compound was prepared; see Table II. [b] Counts above background; cpm/0.1-ml sample.

TABLE IV: Properties of the Acetopyruvate–Enzyme Compound.

(APY)/ (Enzyme)[a]	A_{320}/A_{280}[b]	% Inhibn	Complex (mmoles × 10^5)[c]	Cpm ^{14}C[d]	APY (mmoles × 10^5)[e]	(APY)/ (Complex)
20	0.41	94.9	1.66	290	0.82	0.49
10	0.41	92.5	1.64	319	0.91	0.56
4.0	0.41	94.9	1.64	303	0.86	0.53
3.0	0.40	93.7	1.36	267	0.75	0.55

[a] Ratio of concentration of acetopyruvate to subunits of enzyme in solutions from which compound was prepared; see Table II. [b] Ratio of optical absorption at 320 mμ to that at 280 mμ; see text. [c] Millimoles for a 0.1-ml sample of the final solution calculated from the optical density at 280 mμ, on the assumption that the enzyme and the complex (enamine) have the same absorption at this wavelength; see Figure 2. [d] Counts per minute including background (*ca.* 25) for a 0.1-ml sample. [e] Millimoles of APY calculated from the radioactivity with the aid of a calibration curve that specifically allows for 0.1 ml of aqueous solution in scintillation fluid; see Methods.

1 ml of 0.05 M potassium phosphate buffer (pH 5.98), and the precipitation procedure was repeated, this time with 2 ml of 90% ammonium sulfate. This cycle was again repeated. The final precipitate was dissolved in 0.4 ml of pH 5.98 buffer for assay and determination of radioactivity. The data are shown in Table II–IV.

The reaction of acetopyruvate with the enzyme at 0° leads to the production of a single product, but at 30° the stoichiometry is not well defined, and the incorporation of radioactivity continues as is shown in Figure 1. The reaction was therefore conducted at low temperatures.

Methods. Ultraviolet spectra were determined either with a Zeiss PMQ II spectrophotometer or with a Cary 15 recording spectrophotometer. When the latter was used for low-temperature experiments, thermostated cooling water was circulated through a special block that held the cells. Dry nitrogen was circulated through the cell compartment to prevent condensation of moisture on the cell faces.

Radiochemical counting was conducted with a Nuclear-Chicago Model 720 scintillation counter, with the scintillation solutions previously described (Warren *et al.*, 1966). The absolute values of radioactivity were determined by comparison with standard toluene, lot no. 61-41-6c from the New England Nuclear Corp. For determination of the ratios of acetopyruvate to enzyme, however, only relative values were needed; they were determined as follows. A weighed sample of acetopyruvic acid was serially diluted. Equivalent quantities of acetoacetate decarboxylase were added to each portion and 0.1-ml samples of each dilution were counted. The resulting calibration curve is strictly linear and takes full account of the slight quenching from the buffer and from the protein. When, therefore, a sample of the enamine from acetopyruvate and enzyme was counted in 0.1 ml of this same buffer solution, the correct acetopyruvate concentration could be read from the calibration curve.

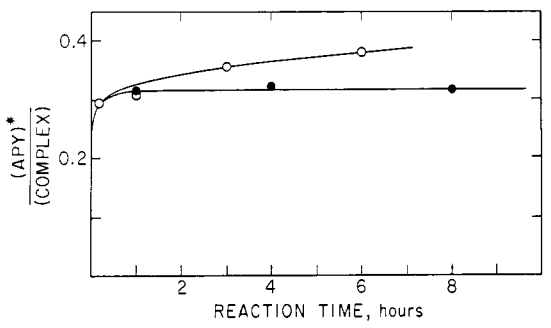

FIGURE 1: Effect of temperature on the reaction between acetoacetate decarboxylase and acetopyruvate, for enzyme of specific activity 20. (The low values of APY per mole of complex relate to the low specific activity of the enzyme.) (●) 0–1° and (○) 30°.

The concentration of the enamine from the reaction of enzyme and acetopyruvate was determined from the optical density at 280 mμ. As can be calculated from data such as those in Figure 2, the absorption at this wavelength is almost unaffected by the presence of acetopyruvate.

Assay. The assay procedure previously employed (Fridovich, 1963; Zerner *et al.*, 1966) was used throughout. Since the assay involves a dilution of the enzyme solution, usually by a factor of 30, dissociation of the enzyme–inhibitor compound accompanies the assay. This problem is treated in the discussion.

Results

Preparation of Enamine of Enzyme with Labeled Acetopyruvate. The results of experiments with radiochemically labeled acetopyruvate (Table III) show that three precipitations have removed all extraneous radioactive material. Regardless of the ratio of acetopyruvate to enzyme in the solutions used to prepare the compound, after three precipitations the radioactivity of the various supernatant solutions was equal, and only slightly above background.

On the assumption that the molecular weight of the

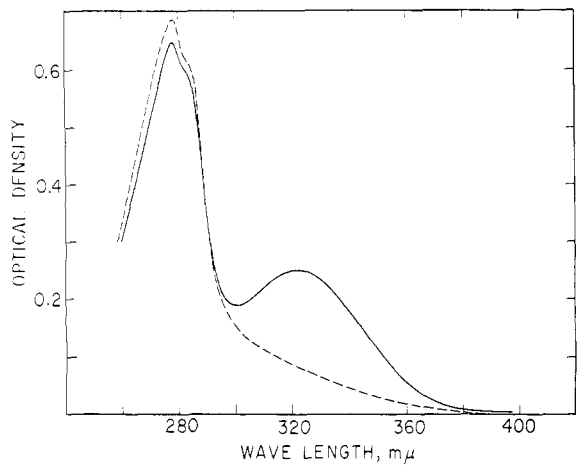

FIGURE 2: The absorption spectrum of the enamine. The upper curve (——) represents the spectrum of a mixture of 1.68×10^{-5} M enzyme (subunits) and 2.0×10^{-5} M acetopyruvate in 0.05 M phosphate buffer (pH 5.96); the lower curve (- - -) shows the sum of the curves for the components.

subunits is about 30,000, the ratio of moles of radioactive inhibitor to enzyme subunits approaches 1:2 (Table II). This ratio, however, was only obtained with enzyme of highest specific activity (45 or more arbitrary units/mg). With enzyme of lower specific activity (*e.g.*, 28 arbitrary units/mg) the ratio of acetopyruvate to subunits is somewhat less, and perhaps only 1:3. The less active enzyme then has inaccessible or inhibited sites, and perhaps even the best enzyme so far prepared has some inactive sites (see Discussion). When the proportion of radioactive inhibitor to enzyme subunits is less than 1:2, the incorporation of acetopyruvate is essentially quantitative, *i.e.*, the proportion of bound inhibitor to subunits is nearly the same as the proportion in which the inhibitor is added.

Spectrum of the Enzyme–Acetopyruvate Compound (Enamine). The ultraviolet absorption spectrum of the isolated enzyme–acetopyruvate compound is shown in Figure 2. The enzyme itself has some absorption at about 320 mμ. However, the enamine has a far higher absorption. Spectra very similar to the spectrum shown in Figure 2 are obtained from solutions of the purified radioactive enzyme inhibitor compounds prepared from the solutions listed in Table II. The extinction coefficient calculated on the basis of 1 mole of the chromophore for about 60,000 mol wt units is about 18,000–20,000; the exact value depends upon whether or not the absorption of the enzyme itself should be subtracted from that of the complex. This in turn depends upon whether the formation of the enamine destroys the absorption of the enzyme at 320 mμ; since the cause of this latter absorption is still unknown, the proper method of calculation cannot yet be specified. Furthermore, it is not certain that the reaction between acetopyruvate and 60,000 mol wt units of enzyme really signifies a combination of one molecule of inhibitor with two subunits (molecular weight about 29,000 each), and that the apparent stoichiometry is not an artifact.

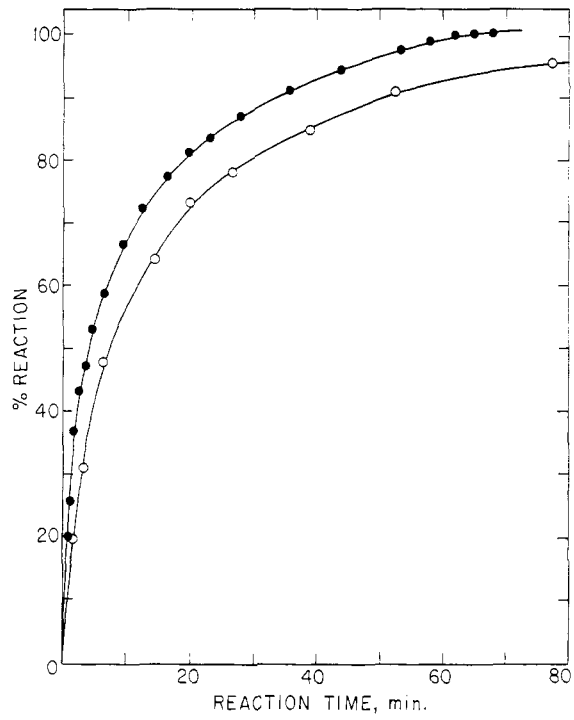

FIGURE 3: The rate of reaction between 4.8×10^{-6} M enzyme (subunits) and 2.5×10^{-6} M acetopyruvate at 1° in 0.05 M phosphate buffer (pH 5.95). (●) From the change of A_{325} and (○) from inhibition; "100% reaction" for the spectrophotometric experiment represents the maximum change observed in the presence of a large excess of acetopyruvate, as in Figure 2; for the assay, it represents total loss of enzyme's activity.

Reaction Rates. The rate of reaction between acetopyruvate and acetoacetate decarboxylase can be measured spectrophotometrically, or by the loss of enzymic activity with time. Both of these methods have been employed; the data shown in Figure 3 refer to concentrations of enzyme subunits and acetopyruvate of 4.6 and 2.5×10^{-6} M, at pH 5.95 and 1°. The rate is apparently slightly faster when measured spectrophotometrically than when measured by loss of enzymic activity; part of the difference may arise from the partial dissociation of the enzyme–inhibitor complex during enzymic assay. A crude estimate of the second-order rate constant for the reaction at 1° is then 350 M^{-1} sec^{-1}. The rate constant at 30° is probably at least ten times as great.

Furthermore, with the assumption that the dissociation constant for the enamine is about 2×10^{-7} M, one can calculate that at 30° the rate of dissociation of the enamine is less than 10^{-3} sec^{-1}, or that the half-time for dissociation is around 10 min. Measurements of the rate of dissociation of the compound (*i.e.*, the measurements of the recovery of activity) confirm this estimate (Figure 4). The rate of dissociation of the enzyme–substrate compound is sufficiently slow that an assay that takes only 90 sec is not accompanied by a large dissociation of the enamine, although of course some does occur. The low rate also explains how it has proved possible to prepare

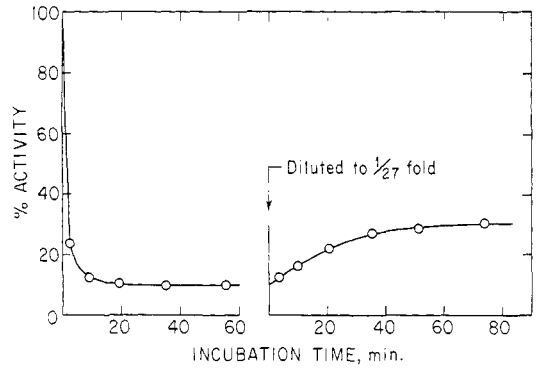

FIGURE 4: Enzyme activity as a function of time of incubation at 30° in 0.05 M phosphate buffer (pH 5.95), showing the rate of dissociation of the enamine when a solution is diluted for assay. Concentrations: enzyme (subunits) 2.3×10^{-5} M; acetopyruvate, 1.1×10^{-5} M. The solutions were diluted 30-fold in pH 6 phosphate buffer immediately prior to assay.

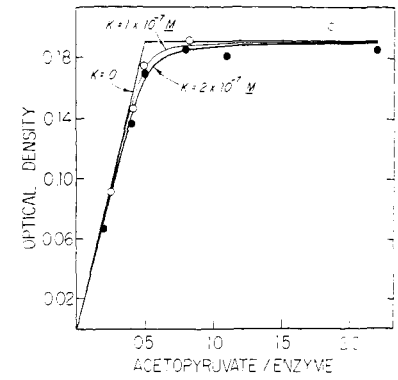

FIGURE 5: Spectrophotometric titration of enzyme with acetopyruvate at 325 mμ in 0 05 M (pH 5.92) phosphate buffer at 30°. (○) (enzyme) = 1.40×10^{-5} M in subunits and (●) (enzyme) = 8.67×10^{-5} M in subunits.

the enzyme–inhibitor compound. The precipitation procedure yields the enamine of the enzyme and acetopyruvate, and this material is then dissolved in buffer and reprecipitated. Presumably, while the compound is out of solution and present as a precipitate in ammonium sulfate suspension, dissociation does not occur; the periods of time when the enzyme is in solution are short and at all times the temperature was 0–4°. The recovery of enzymic activity only amounts to about 5% during the precipitation–reprecipitation procedure; the enamine therefore is largely intact, as would be predicted from the rate data.

Spectrophotometric "Titration" of the Enzyme. The formation of an enamine from acetopyruvate and enzyme can be followed spectrophotometrically by observing the increase in the optical density at 325 mμ. The dissociation constant can be calculated from the data shown in Figure 5 on the assumption that one molecule of acetopyruvate reacts with two subunits, or more precisely that it reacts with about 60,000 mol wt units of enzyme. (If the reaction actually occurs with each subunit, but some subunits are blocked and unreactive, the dissociation constant here determined will not be affected.) The final values of the extinction coefficient correspond to that of the complex. The equilibrium constant, K, is $(E_0 - 2C)(A_0 - C)/2C$, where E_0 and A_0 are the initial concentrations of subunits of the enzyme and of acetopyruvate, and C is the concentration of the complex (*i.e.*, the enamine) calculated from the spectrophotometric data. The calculation assumes that only negligible quantities of other species are present, such as carbinolamine, Schiff base, or products from the reactions of more than 1 mole of inhibitor with two subunits.

The intersecting straight lines in Figure 5 represent a titration, assuming that the reaction between acetopyruvate and enzyme is completely irreversible, *i.e.*, that the dissociation constant of the compound is zero. The straight lines intersect at a ratio of acetopyruvate to enzyme of 0.5. Although the experimental points fall somewhat below the line for an irreversible reaction, they are consistent with a dissociation contant for the enzyme–inhibitor compound of between 1 and 2×10^{-7} M, and provide for an easy and accurate titration of the enzyme.

"Titration" of the Enzyme by Measurements of Inhibition. The rate of formation of the enzyme–inhibitor complex and the equilibrium constant for its dissociation can also be approximated from studies of inhibition. Such studies were first made for the decarboxylase from *Clostridium madisonii* by Colman (1962). Although the enamine partially dissociates during the 90-sec assay, the process is sufficiently slow to allow a reasonable determination of activity. The rate of formation of the enamine can be estimated from the results of inhibition studies, and these data are comparable to those from spectrophotometric titrations (Figure 3). The comparison of the results by these two methods shows the substantial agreement between them but also illustrates the extent of error.

Dissociation Constant of the Enamine. The equilibrium constant for the dissociation of the enamine can also be estimated from activity measurements, as shown in Figure 6. The straight lines represent simply a titration, with 1 mole of acetopyruvate reacting with two subunits. Again, the intersecting straight lines in Figure 6 represent formation of enamine with a dissociation constant of zero; the minor deviation of the experimental points from these lines indicates that the dissociation constant is small. In view of the partial dissociation of the enamine during assay, more accurate results could scarcely be anticipated.

The Inhibition Constant for Acetopyruvate. An accurate value for the inhibition constant for acetopyruvate at pH 5.92 and 30° can be obtained from kinetic measurements. Since the reaction between enzyme and acetopyruvate is not instantaneous, the components must be incubated to establish equilibrium before the acetoacetate is added. The resulting data are plotted by the standard Lineweaver–Burk method to obtain the inhibitor constant. The actual data require two comments. (a) The Michaelis constant for the enzyme is large, so large in fact that its accurate

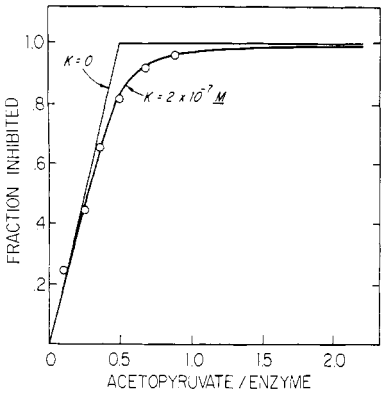

FIGURE 6: Activity titration of enzyme with acetopyruvate in 0.05 M phosphate buffer at pH 5.92. The measurements of activity were made by diluting the mixture 30-fold in pH 6 phosphate buffer immediately prior to assay. (○) (enzyme) = 1.13×10^{-5} M in subunits.

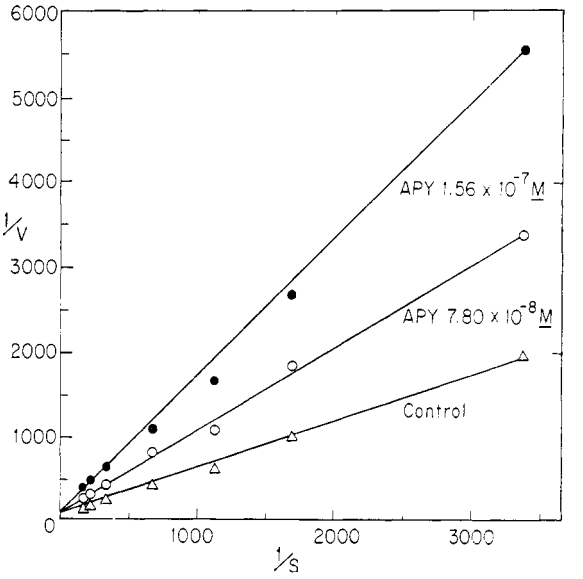

FIGURE 7: Lineweaver–Burk plot for the inhibition of acetoacetate decarboxylase by acetopyruvate. Because K_M is so large (about 10^{-2} M) the plots of $1/V$ *vs.* $1/(S)$ appear to go nearly through the origin. The enzyme solutions were 8.32×10^{-8} M in subunits, mol wt 29,000.

determination is difficult. This difficulty is apparent in Figure 7, where the plots of $1/V$ *vs.* $1/S$ pass nearly through the origin. Fortunately, the determination of an inhibition constant is independent of the value of the Michaelis constant, and (b) since the inhibitor–enzyme reaction is relatively slow at least at the very low concentrations here used, the determination of the inhibition constant is somewhat atypical. The value of about 10^{-7} M for the dissociation constant, determined from the data of Figure 7, nevertheless demonstrated how tightly the inhibitor is bound.

Rates of Enamine Formation from Aminoacetonitrile. The rates of formation of enamines from pivaloylpyruvate and from acetopyruvate with aminoacetonitrile were followed spectrophotometrically. At pH near 6 and 30°, both compounds showed a rate constant around 0.03 M^{-1} sec^{-1}.

Attempts to determine the rate maximum in the pH–rate profile for the reaction between aminoacetonitrile and acetopyruvate have so far proved abortive; the kinetics are so complex that they have not yet been completely analyzed. Probably however the maximum rate constant for the reaction at 30° is less than 1 M^{-1} sec^{-1}. Aminoacetonitrile was chosen for these studies because it is the best "model" (*i.e.*, best chemical catalyst) for the nonenzymic decarboxylation of acetoacetic acid so far discovered (Guthrie and Westheimer, 1967).

Discussion

The Enamine. Acetopyruvate reacts with acetoacetate decarboxylase to form the enamine II according to eq 1. The evidence for this statement is as follows.

A. MODEL SYSTEMS. Aminoacetonitrile reacts with ethyl acetopyruvate to form an enamine that has been crystallized and identified. The compound has the correct analysis and molecular weight (by mass spectrum). Its structure as an enamine is unequivocally demonstrated by its nmr spectrum. In particular, the singlet at δ 6.0 shows the integrated intensity and chemical shift for a single vinyl proton and thus clearly distinguishes the compound from the isomeric Schiff base, which has no vinyl protons at all. The nuclear magnetic resonance and mass spectral data do not however distinguish among various possible enamines, and in particular do not distinguish between $CH_3COCH{=}C(CO_2C_2H_5)NHCH_2CN$ (III) and $CH_3(NCCH_2NH)C{=}CH{-}COCO_2C_2H_5$ (IV) or between the *cis* and *trans* forms of either III or IV.

Crystalline *S*-benzylthiuronium salts of two isomeric enamines have been isolated from the reaction between acetopyruvate and aminoacetonitrile and will be described in a later publication. They absorb in the region of 305–318 mμ with extinction coefficients around 15,000.

The ultraviolet absorption of the enamine III has a λ_{max} in alcohol of 335 mμ, and an extinction coefficient of about 14,000. The corresponding data for the enamine prepared in solution from acetopyruvate ion are λ_{max} in water of 315 mμ and an extinction coefficient of about 14,000. These data also confirm the enamine, as opposed to the Schiff base structure of the condensation product. The high extinction and long wavelength of absorption are typical of conjugative enamines (Bowden *et al.*, 1946); Schiff bases, like ketones, are relatively transparent. The product formed from enzyme and acetopyruvate has a λ_{max} of about 325 mμ (midway between the model compounds with ester and anion); the value of the extinction coefficient, although uncertain, is probably somewhere around 19,000, and higher than that for its models. Nevertheless, the similarity is sufficient between model and enzyme compound to substantiate the conclusion that the product is an enamine, although it is uncertain whether the enamine has been formed on the α- or γ-carbonyl group of acetopyruvate. In this connection

we note that the enzyme does not react with pivaloylpyruvate.

B. THE STOICHIOMETRY OF THE ENZYMIC REACTIONS. It has here been demonstrated that one molecule of acetopyruvate inactivates two subunits, and (in an accompanying paper) O'Leary and Westheimer (1968) show that, on acetylation of the enzyme, one acetyl group is sufficient to inactivate two subunits. However, chemical evidence (Lederer *et al.*, 1966; J. Berzofsky and J. Hall, unpublished data) suggest that all the subunits are identical and Neece and Fridovich (1967) have found that, if the enzyme is prepared in the cold, it may be heat activated (*cf.* O'Leary and Westheimer, 1968). Therefore the possibility exists that some of the subunits (perhaps one-half of them) are inactive, and that this fact explains the apparent stoichiometry. Alternatively, perhaps two subunits, even if they are identical, constitute one active site. This question remains for further investigation.

C. DISSOCIATION CONSTANT OF THE ENAMINE OF ACETOACETATE DECARBOXYLASE. The dissociation constant for the enamine formed from the enzyme and acetopyruvate is around 1×10^{-7} M. This is approximately the value of K_I determined from analysis of the Michaelis–Menton kinetics of the inhibition of the enzyme by acetopyruvate, determined with enzyme (subunit) concentration of about 10^{-7} M and acetopyruvate concentration of about $0.8\text{–}1.6 \times 10^{-7}$ M; inhibition was 30–70% complete (Figure 7).

The dissociation constant of the complex can also be determined from the data in Figures 5 and 6. Direct calculations can scarcely be expected to yield accurate results; they involve in each case small differences between large numbers, and the inherent errors (*e.g.*, from dissociation of the enamine during assay) are necessarily magnified. In Figures 5 and 6, the curves are drawn for a dissociation constant of 2×10^{-7} M and fit the data for both spectrophotometric and inhibition data fairly well. Thus the probable dissociation constants for the enamine lies between 1 and 2×10^{-7} M, and the results from studies of the fraction inhibited, from the spectroscopic data, and from analysis of the kinetic data are in reasonable agreement.

D. BOROHYDRIDE REDUCTION. Early in the investigations of acetoacetate decarboxylase, an attempt was made to reduce the enzyme–acetopyruvate compound with borohydride (Colman, 1962). It is now apparent why this attempt failed. Borohydride will reduce carbon to nitrogen double bonds of Schiff bases (Billman and Diesing, 1957) and of Schiff base salts (Schellenberg, 1963). However, only relatively electron-deficient double bonds have so far been reduced by borohydride (Kadin, 1966; Autrey and Tahk, 1967), whereas the double bond in an enamine is electron rich. Therefore, the enamine formed from acetopyruvate cannot be reduced with this reagent, and acetopyruvate can protect the enzyme against the action of substrate and borohydride (Fridovich and Westheimer, 1962). When the borohydride has been decomposed by acid catalysis, the active enzyme can be recovered by prolonged dialysis or by gel filtration to remove acetopyruvate.

The Special Nature of the Inhibitor. The reaction between acetopyruvate and acetoacetate decarboxylase is enzymic. The second-order rate constant exceeds 10^3 M^{-1} sec^{-1} at 30°, whereas that for the reaction between aminoacetonitrile and acetopyruvate is less by a factor of perhaps 10^3. The dissociation constant for the enzymic product is about 10^{-7} M, whereas that for acetopyruvate with aminoacetonitrile is about 5×10^{-3} M. Presumably the negative charge on the acetopyruvate attracts the inhibitor to the active site of the enzyme (Fridovich, 1963). The details of the nonenzymic and enzymic reactions have not yet been elucidated; presumably these reactions proceed by way of a carbinolamine (Cordes and Jencks, 1963). But the enzymic nature of the reaction between this special inhibitor and the enzyme is apparent.

Acknowledgments

The authors especially wish to acknowledge the work of Mrs. Roberta Colman who demonstrated the tight binding and measurable reaction rate between acetopyruvate and the acetoacetate decarboxylase from *Clostridium madisonii*. We are also grateful to Mr. Stephen Coutts for the determination of the inhibitor constant by kinetic means.

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Acetoacetate Decarboxylase. Selective Acetylation of the Enzyme*

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ABSTRACT: Acetoacetate decarboxylase can be selectively acetylated with acetic anhydride or with 2,4-dinitrophenyl acetate to introduce one acetyl group for 60,000 mol wt units (two subunits). The acetylated enzyme is completely inactive. The site of the acetylation has been identified as the same lysine residue where a Schiff base with substrate is formed during enzyme decarboxylation. Acetoacetate decarboxylase can be heat activated. If the enzyme is not completely activated prior to acetylation, a partial heat reactivation of the acetylated enzyme can be achieved, but this does not involve loss or rearrangement of acetyl groups. A partial heat reactivation can be carried out for a similar enzyme into which an isopropyl group has been introduced by borohydride reduction of the enzyme–substrate reaction mixture.

The enzyme acetoacetate decarboxylase from *Clostridium acetobutylicum* catalyzes the decarboxylation of acetoacetate by way of a Schiff base intermediate formed between the enzyme and the substrate (Hamilton and Westheimer, 1959). The pathway for the reaction proceeds through the Schiff base of the product (acetone), and this Schiff base can be "trapped" by borohydride reduction, to yield a protein containing a residue of ϵ-*N*-isopropyllysine (Fridovich and Westheimer, 1962; Warren *et al.*, 1966). The protein obtained by this reduction of the Schiff base has been hydrolyzed with trypsin, and the peptide at the active site of the enzyme isolated, purified, and identified (Laursen and Westheimer, 1966); it proved to be Glu-Leu-Ser-Ala-Tyr-Pro-IprLys-Lys.[1] The enzyme is oligomeric (a dodecamer; see Tagaki and Westheimer, 1968) but only one of the lysine residues out of approximately 36 contained in two subunits (Lederer *et al.*, 1966) participates in the enzymic reaction.

Experiments on acetylation of the enzyme now show that the same lysine residue is especially active as a nucleophile; it readily attacks acetic anhydride or 2,4-dinitrophenyl acetate, and is thereby acylated. The resulting acetylated enzyme is essentially inert. The stoichiometry of the process has been established using radiochemically labeled acetic anhydride and by measuring, spectrophotometrically, the amount of 2,4-dinitrophenol formed from the reaction between the enzyme and 2,4-dinitrophenyl acetate. The evidence for the identity of the lysine residue in Schiff base formation and acetylation comes from the isolation and analysis of the peptides obtained by tryptic cleavage of the proteins obtained in these two processes. A complication arose because some samples of the acetylated enzyme can be partially reactivated by heat. Neece and Fridovich (1967) have now discovered that the enzyme itself can be heat activated; acetylation of enzyme that has previously been fully heat activated yields a product that cannot be heat reactivated.

Experimental Section

Materials. Crystalline acetoacetate decarboxylase was generally prepared by a modification of the method

* From the James Bryant Conant Laboratory of the Department of Chemistry, Harvard University, Cambridge, Massachusetts. *Received August 28, 1967.* This work was supported by Grant GM-04712 from the Institute of General Medical Sciences of the National Institutes of Health and the Petroleum Research Fund of the American Chemical Society. A preliminary announcement of this work was made at the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 14, 1966.

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[1] Abbreviation used: IprLys, ϵ-*N*-isopropyllysine.