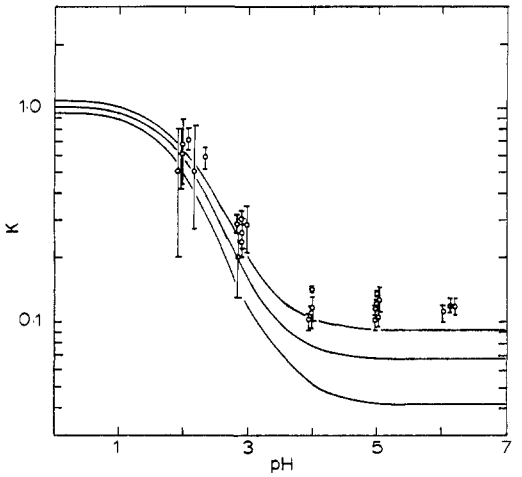


Figure 4. Apparent equilibrium constant for the hydration equilibrium experiments in H_2O at 30°, μ = 0.3 M (KCl). Points are derived from approach to equilibrium experiments; the central line is based on the equilibrium constants from the nmr experiments; the two lines on either side show the magnitude of the estimated standard deviations in K based on the standard deviations of the equilibrium constants from the nmr experiments.

(K_1^{app}, K_2^{app}, and K_3^{app} are defined by eq 1, 2, and 3.) Rate constants calculated from eq 6 and 7 are given in Table IV; for this purpose K_2^{app} and $K_3^{app}/(1 + K_1^{app})$ were calculated using the parameters derived from the nmr experiments.

The kinetics of hydration and dehydration of pyruvic acid have been studied in other laboratories. Eigen, *et al.*,[13] reported, for the dehydration of 2,2-dihydroxypropionic acid (pyruvic acid hydrate) at 25°, an uncatalyzed path with rate constant 0.22 sec^{-1} and an acid-catalyzed path with rate constant 1.25 M^{-1} sec^{-1}. Pocker and Meany[14] reported a rate constant of 0.00092 sec^{-1} for the uncatalyzed dehydration of 2,2-dihydroxypropionate ion at 0°. Allowing a factor of about 10 for the 30° temperature difference between these experiments and the present work, this corresponds to a rate of dehydration of about 0.01 sec^{-1}.

These values may be compared with the rates of dehydration of the "hydrate" of acetopyruvic acid. The rate constant for the anion, taken as the value of k_{-2} at pH 5 and 6, is 0.011 sec^{-1}; although the low pH results from this study are of low precision, it appears that by pH 2, where the hydrate (pK_a = 3.2) is mainly present in the acid form, the rate constant for hydration (k_{-2}) has leveled off at about 0.06 sec^{-1}.[15] Thus, the rate constants for acetopyruvate are quite similar to those for pyruvate.

This study was undertaken as a foundation for studies of the reactions of acetopyruvate with amines, since it was found that these reactions gave complicated kinetic behavior. These studies are reported in the accompanying paper.[4] The results reported above define the equilibria established for acetopyruvate in dilute aqueous solution in the pH range 0–7, and the rate constants for processes taking more than 10 sec to come to equilibrium.

Acknowledgment. The author gratefully acknowledges critical discussions, advice, and encouragement provided by Professor F. H. Westheimer, who originally suggested this problem. I would like to thank Professor K. L. Williamson, Mt. Holyoke College, who kindly ran the 100-MHz nmr spectra.

(13) M. Eigen, K. Kustin, and H. Strehlow, *Z. Phys. Chem.*, 31, 140 (1962).

(14) Y. Pocker and J. E. Meany, *J. Phys. Chem.*, 74, 1486 (1970).

(15) The k_{obsd} values for hydration of 0.2 and 0.4 sec^{-1} obtained from the last stages of the reactions at pH 1 or 0, respectively, indicate that there *is* an acid-catalyzed reaction.

Acetopyruvate. Enamine Formation with Aminoacetonitrile. Models for Acetoacetate Decarboxylase[1]

J. Peter Guthrie*

Contribution from the James Bryant Conant Laboratory of the Department of Chemistry, Harvard University, Cambridge Massachusetts 02138.
Received September 14, 1971

Abstract: The reaction of aminoacetonitrile with acetopyruvic acid (2,4-dioxovaleric acid) leads to the production of two isomeric products, which have been identified as the enamines formed by reaction at the α or γ keto function. The two enamines form at closely similar rates and the equilibrium constants are similar, so that the kinetics of the reaction are complicated. Analysis has led to rate constants for the two enamine forming reactions which may be compared with the rate constant for the reaction of acetopyruvate with acetoacetate decarboxylase, for which it is a powerful inhibitor.

Acetoacetate decarboxylase reacts rapidly and reversibly to form a stable compound with acetopyruvate.[2] This compound, which is believed to be an enamine on the basis of its uv spectrum, is apparently formed by reaction of the acetopyruvate with the ϵ amino group of a lysine residue in the active site.[2c] It

* Address correspondence to the Department of Chemistry, University of Western Ontario, London, Canada.

(1) Abstracted in part from the Ph.D. Thesis of J. P. G. (1968). Supported by Grant No. GM 04712 from the Institute of General Medical Sciences of the National Institutes of Health, and by a National Research Council of Canada Special Scholarship.

(2) (a) R. Davies, *Biochem. J.*, 37, 230 (1943); (b) R. Colman, Ph.D. Thesis, Radcliffe College, Cambridge, Mass., 1962; (c) W. Tagaki, J. P. Guthrie, and F. H. Westheimer, *Biochemistry*, 7, 905 (1968).

had earlier been shown that one of the lysine residues of the enzyme forms an imine with the substrate, acetoacetate, as an essential step in the mechanism of decarboxylation.[3]

If indeed acetopyruvate reacts with the same lysine ϵ NH_2 groups as does the substrate, acetoacetate, comparison of the kinetic behavior of acetoacetate and acetopyruvate should shed light on the nature of the enzymic reaction. However, it was necessary for this comparison to have as a reference point relative rates for reactions of acetoacetate and acetopyruvate with a simple amine. The compound chosen was aminoacetonitrile, AAN, which had earlier been used as a model for the reaction of the enzyme with acetoacetate, because its pK_a (5.34[4a]) was optimal for catalysis of decarboxylation.[4b] The essential lysine amino group has since been shown to have a pK_a of 5.9.[5]

Although the kinetics of enamine formation for this "simple" model case were complicated, they have been analyzed, and rate constants for reactions of aminoacetonitrile with the α- and γ-carbonyl groups have been evaluated in the pH range of interest for comparison with the enzyme.

Experimental Section

Materials and Methods. Aminoacetonitrile sulfate was obtained from "aminoacetonitrile bisulfate" (Aldrich, mp 124–129°) by recrystallization from aqueous ethanol, mp 174–177° dec (lit.[6] 160°).[7] The purity of the recrystallized salt was checked by titration with sodium hydroxide. Aminoacetonitrile was prepared from the "bisulfate," mp 124–129°, by neutralization with sodium methoxide in methanol.[8] The amine had bp 76–77° (11 mm) (lit.[8] 65° (8 mm)), and was stored under nitrogen in sealed glass ampoules in a freezer. Acetopyruvic acid, APY, and its lithium salt, LiAPY, were prepared as described previously.[9] Reagent grade chemicals were used for buffers without further purification. Nmr spectra were recorded on a Varian A-60 spectrometer; pH was measured using a Radiometer pH Meter 4. Least-squares caluclations were carried out on an IBM 1620, using a program based on the rigorous least-squares procedure described by Wentworth.[10]

***S*-Benzylthiouronium Salts of the Enamines from APY and AAN (3 and 4).** APY (0.02 mol), AAN (0.03 mol), and 4-picoline (0.002 mol, to serve as buffer), each dissolved in water and adjusted to pH 6, were mixed in a final volume of 55 ml of water. After 30–45 min, the solution was poured into a solution of *S*-benzylthiouronium chloride (0.022 mol) in warm ethanol and the mixture stirred in an ice bath for 15 min. The precipitate was filtered and then dried *in vacuo* to a powdery solid (5.12 g, 77%). The crude salt was added to boiling acetonitrile (45–50 ml/g of solid), and refluxed for 5 min, protected with a drying tube. The hot solution was filtered and cooled to give **3**, which was recrystallized from acetonitrile: mp 130–132°; nmr (DMSO-d_6) δ 1.83 (s, CH_3CO), 4.36 (m, $NCCH_2N$, $PhCH_2S$), 5.34 (s, $CH{=}C$), 7.27 (s, $PhCH_2S$).

Anal. Calcd for $C_{15}H_{18}N_4O_3S$: C, 53.89; H, 5.43; N, 16.76; S, 9.59. Found: C, 54.10; H, 5.35; N, 16.51; S, 9.76.

The insoluble residue was extracted with portions of boiling methanol which on cooling deposited crystals of **4**. This, after recrystallization from methanol–ethanol, had mp 162–163°: nmr (DMSO-d_6) δ 1.98 (s, $CH_3C(NHCH_2CN){=}C$), 4.35 (br, $PhCH_2$, $NCCH_2N$), 5.57 (s, $CH{=}C$), 7.29 (s, $PhCH_2S$).

Anal. Calcd for $C_{15}H_{18}N_4O_3S$: C, 53.89; H, 5.43; N, 16.76; S, 9.59. Found: C, 54.00; H, 5.51; N, 16.21; S, 9.27.

Reduction of 3 with Borohydride. **3** showed no more than 3% change in absorbance at its λ_{max} after 3 hr in 1:1 glacial acetic acid–absolute ethanol. **3** (0.23 g), dissolved in 20 ml of 1:1 ethanol–acetic acid was treated with two 5-ml portions of a saturated solution of sodium borohydride in ethanol. The reaction mixture was poured into 100 ml of water, the pH was adjusted to *ca.* 1 with concentrated HCl, and the solution was evaporated. The residue was taken up in 0.1 *N* HCl and evaporated. The residue was dissolved in ethanol, filtered to remove insoluble material, and evaporated: nmr (D_2O) δ 2.08 (s, CH_3CO), 3.25 (d, J = 5 Hz, $COCH_2$), 3.90 (s, $NCCH_2ND$), 4.17 (s, $PhCH_2S$), 4.20 (d, J = 5 Hz, CH_2CH), 7.20 (s, $PhCH_2S$).

Reduction of the Sodium Salt of 3 and Reaction of the Reduced Material with Semicarbazide. **3**, as its *S*-benzylthiouronium salt (2 g), was dissolved in a small volume of methanol and put onto a column containing 100 ml of Dowex-50 (sodium form) in methanol. Elution of the sodium salt of **3** was followed by uv spectrophotometry. Fractions with **3** were pooled and evaporated. The sodium salt was reduced with borohydride using the same procedure as for the *S*-benzylthiouronium salt. After evaporating the reduced material from 0.1 *M* HCl, the residue was dissolved in ethanol (80 ml) and filtered. To this solution was added 1.35 g of semicarbazide hydrochloride in the minimum of water, and 1.7 ml of pyridine. The solution was stirred for 2 hr at 55°, and then evaporated to dryness, giving a yellow oil which crystallized on adding 6 ml of water. Filtration gave 0.38 g of solid. The mother liquor was adjusted to pH 1 by adding concentrated HCl, and a further 0.07 g was obtained; total yield, 45%. After four recrystallizations from water, material of mp 228–230° was obtained: nmr (DMSO-d_6) δ 1.83 (s, $CH_3C{=}N$), 5.99, 7.05 (AB q, J = 16 Hz, $CH{=}CH$). The literature value[11] for the melting point (218°) of the semicarbazone of acetoacrylic acid was lower by 12°. An authentic sample was prepared from acetoacrylic acid (mp 123.5–125°; lit.[13] mp 125°; prepared[12,13] from levulinic acid), mp 225–227°; mixture melting point with the semicarbazone derived from **3** showed no depression. The nmr spectra of the two samples of the semicarbazone showed superimposable methyl and olefinic signals.

Anal. Calcd for the semicarbazone of acetoacrylic acid, $C_6H_9N_3O_3$: C, 42.10; H, 5.30; N, 24.55. Found: C, 42.14; H, 5.32; N, 25.01.

Reduction of APY. APY (2.00 g) dissolved in 200 ml of 1:1 ethanol–acetic acid was treated at 5-min intervals with 50-ml portions of a solution of 1.3 g of sodium borohydride in 300 ml of absolute ethanol: (after work-up as for the reduction of **3**) nmr (D_2O) δ 2.72 (s, CH_3CO), 4.57 (d, J = 5 Hz, $COCH_2$), 5.10 (t, J = 5 Hz, CH_2CHOD; uncertain assignment because this is very close to the solvent peak). The crude reduced material was placed in a sublimer and heated for 1 hr, 120°, 0.1 mm. The product, 0.24 g, was acetoacrylic acid. After two crystallizations from methylene chloride (mp 119–122°; mixture melting point with an authentic sample showed no depression) the ir and nmr spectra were identical with those of an authentic sample.

Reduction of Enamine 4. **4** did not react with sodium borohydride either in ethanol or in acetic acid–ethanol, but did react, though extremely slowly, with trimethylamineborane. **4** (250 mg) as its sodium salt reacted with trimethylamineborane (71.4 mg) in glacial acetic acid (25 ml) for 20 days. After work-up, the nmr spectrum (D_2O) showed at least four times as much absorption at *ca.* δ 1.4 as at *ca.* δ 2.1 so that the major product had a methyl group attached to a saturated carbon, but this high-field absorption was a broad hash with no discernible pattern. The most that could be learned was that by the time the enamine chromophore had disappeared, the major products had undergone reduction at C-4. The product of the reduction was an intractable gum from which no pure compounds could be isolated.

Kinetics Procedure. For the enamine formation kinetics, freshly prepared solutions of AAN in buffer (with ionic strength adjusted to 0.3 with KCl) were used. Reaction was initiated by adding 100 μl of a solution of APY in aqueous buffer at the pH of the experiments. The reaction was followed at 310 nm using a Cary-15.

For the kinetics of enamine hydrolysis, stock solutions of **4** in methanol and **3** in acetonitrile were prepared. Reaction was initiated by adding 20–100 μl of enamine solution to 3.0 ml of

(3) S. Warren, B. Zerner, and F. H. Westheimer, *Biochemistry*, **5**, 817 (1966).

(4) (a) G. W. Stevenson and D. Williamson, *J. Amer. Chem. Soc.*, **80**, 5943 (1958); (b) J. P. Guthrie and F. H. Westheimer, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **26**, 562 (1967).

(5) D. E. Schmidt, Jr., and F. H. Westheimer, *Biochemistry*, **10**, 1249 (1971).

(6) A. Klages, *Ber.*, **36**, 1506 (1903).

(7) The literature values for the melting points of the bisulfate and sulfate of aminoacetonitrile are 101[6] and 160°,[6] respectively.

(8) A. H. Cook, I. Heilbron, and A. L. Levy, *J. Chem. Soc.*, 205 (1948).

(9) J. P. Guthrie, *J. Amer. Chem. Soc.*, **94**, 7020 (1972).

(10) W. E. Wentworth, *J. Chem. Educ.*, **42**, 96, 162 (1965).

(11) H. Stetter and C. W. Schellhammer, *Justus Liebigs Ann. Chem.*, **605**, 58 (1957).

(12) E. D. Hughes and H. B. Watson, *J. Chem. Soc.*, 1929 (1953).

(13) L. Wolff, *Ann.*, **264**, 246 (1891).

Table I. Observed Rate Constants for Enamine Formation[a,b]

pH	$[AAN]_t$, M	λ_2, sec^{-1}	λ_3, sec^{-1}	$\lambda_2 + \lambda_3$, sec^{-1}	$\sigma_{\lambda_2+\lambda_3}$,[f] sec^{-1}	k_f,[g] M^{-1} sec^{-1}	k_r,[h] sec^{-1}
3.96[c]	0.0874	0.0147	0.00169	0.0164	0.00079		
3.96[c]	0.0175	0.00770	0.00985	0.00869	0.00041		
3.96[c]	0.00874	0.00729	0.00106	0.00835	0.0012		
3.96[c]	0.0623	0.0109	0.00148	0.0124	0.00018		
3.95[c]	0.0399	0.00865	0.00113	0.0107	0.00033		
3.96[c]						0.088 ± 0.011	0.0071 ± 0.0006
4.93[d]	0.0840	0.00791	0.000497	0.00841	0.00031		
4.96[d]	0.0168	0.00215	0.000378	0.00253	0.00014		
4.98[d]	0.00841	0.00139	0.000204	0.00159	0.000054		
4.94[d]	0.0760	0.00620	0.00166	0.00785	0.00060		
4.94[d]	0.0399	0.00396	0.00470	0.00443	0.00023		
4.94[d]						0.0908 ± 0.0011	0.000847 ± 0.00006
5.82[e]	0.0445	0.00217[i]					
5.81[e]	0.0249	0.00128[i]					
5.80[e]	0.0178	0.000912[i]					
5.80[e]	0.0142	0.000743[i]					
5.80[e]	0.0107	0.000577[i]					
5.80[e]	0.0107	0.000577[i]				0.0478 ± 0.00073	0.000066 ± 0.000012

[a] In H_2O, 30.0 ± 0.1°, μ = 0.3 M (KCl). [b] Initial APY concentration was 1.07–2.14 × 10^{-4} M. [c] Acetate buffer, 0.140 M. [d] Acetate buffer, 0.0722 M. [e] Phosphate buffer, 0.0815 M. [f] Error limits calculated from the covariance matrices evaluated by the least-squares program. [g] Slope of a graph of $\lambda_2 + \lambda_3$ or k_{obsd} *vs.* $[AAN]_t$. [h] Intercept of a graph of $\lambda_2 + \lambda_3$ or k_{obsd} *vs.* $[AAN]_t$. [i] k_{obsd} instead of $\lambda_2 + \lambda_3$; simple first-order kinetic plots were obtained.

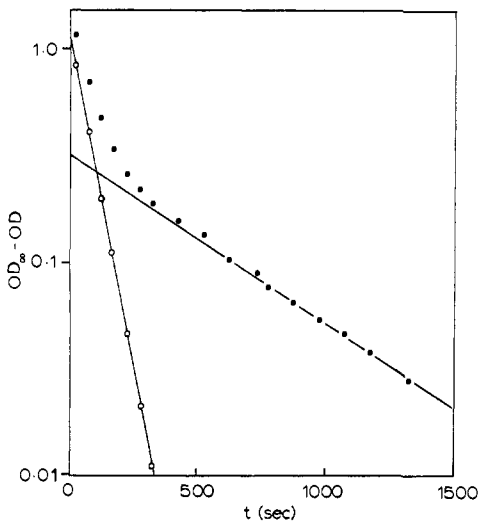


Figure 1. First-order plot for enamine formation at pH 5; [AAN] = 0.0874 M, 30°, μ = 0.3 M: (●) observed values of OD − OD_∞ plotted on semilog paper; (○) difference between observed values of OD − OD_∞ and the line determined by data taken after 500 sec plotted on semilog paper.

buffer, previously equilibrated for 15 min at 30°. The reaction was followed at 310 or 315 nm using a Zeiss PMQII.

Determination of the Ratio of Equilibrium Constants (r_1). To an nmr tube containing about 25 mg of LiAPY was added 0.5 ml of a 1 M solution of $(AAN)_2H_2SO_4$, adjusted to the desired pH with 1 M K_2HPO_4. The sample was mixed thoroughly and let stand at room temperature for at least 3 hr (pH 5 or lower) or at least 24 hr (pH near 6). The spectrum was then recorded, using a sweep width of 50 Hz. The areas of the signals from the methyl groups of **3** and **4** were measured using a planimeter, and the ratio was calculated: r_1 = [**3**]/[**4**]. Correction for APY absorption was made using spectra of APY alone at a similar pH. The values so obtained were (pH, r_1): 2.85, 0.38; 2.90, 0.49; 3.39, 0.55; 3.33, 0.56; 3.33, 0.56; 3.36, 0.62; 3.35, 0.58; 4.01, 0.80; 4.17, 0.81; 4.72, 0.80; 5.30, 0.78; 5.41, 0.90; 5.39, 0.98; 6.13, 0.73; 6.13, 0.71; 6.13, 0.85.

Reaction of APY + AAN, Followed by Nmr. Spectra of solutions containing LiAPY and AAN (prepared as for r_1 determinations) were recorded immediately after mixing and then repeatedly at suitable intervals.

Results

Kinetics of Enamine Formation. For the reaction of APY and AAN at pH values at or below 5, it was quite apparent that simple first-order kinetics were not being observed. Figure 1 shows the results of a first-order plot of the data obtained at pH 4, with 0.0874 M AAN and 0.140 M buffer. After 500 sec the points fall on a straight line; if the line defined by the points taken after 500 sec is extrapolated to earlier times, one can calculate the difference between the observed and extrapolated values and plot this difference against time, obtaining a good straight line relationship. This behavior corresponds to an integrated rate law of the form

$$A = a + be^{-\lambda_2 t} + ce^{-\lambda_3 t} \quad (1)$$

The data were fitted to this five-parameter equation by the method of least squares. As will be discussed below, λ_2 and λ_3 are complicated functions of rate constants; the useful quantity for further calculations is $\lambda_2 + \lambda_3$. At pH 5.8 the system showed what appeared to be simple first-order behavior; reactions were followed to 8–10 half-lives. The data for pH 5.8 were fitted to the equation

$$A = a + be^{-\lambda t} \quad (2)$$

by least squares. The parameter a in eq 1 and 2 corresponds to the infinity value of absorbance which is here treated as an adjustable parameter; in no case did the value of a differ from the final observed value by more than 0.004. The "rate constants" evaluated in this way are listed in Table I. In other experiments[14a] it was shown that $\lambda_2 + \lambda_3$ increased with increasing buffer concentration; this behavior was consistent with the catalysis observed for hydrolysis, but the effect was small. Consideration of the propagation of errors during the calculation of microscopic rate constants showed that, although extrapolation to zero buffer con-

(14) (a) J. P. Guthrie, Ph.D. Thesis, Harvard University. (b) In separate experiments the hydrolysis reaction was shown to be subject to general acid catalysis.

centration would be desirable, it would entail a prohibitive loss in precision. Consequently, rate constants were evaluated at finite buffer concentration.

Enamine Hydrolysis. In order to analyze the kinetics of enamine formation it proved necessary to have values for the rate constants for hydrolysis of the two enamines under the conditions used for the kinetics of the forward reaction. The rate constants for hydrolysis of **3** and **4** are given in Table II.[14a,b]

Table II. Rate Constants for Hydrolysis of **4** Enamines[a]

Enamine	pH	$k_{obsd} \times 10^4$, sec^{-1}	Concn of org solvent, *M*
4	4.00[b]	8.14	0.797[g]
4	3.98[b]	8.58	0.487[g]
4	3.96[b]	8.87	0.246[g]
4	3.96[b]	9.22 ± 0.12[f]	0[f]
4	4.97[c]	1.24	0.797[g]
4	4.95[c]	1.29	0.487[g]
4	4.94[c]	1.31	0.246[g]
4	4.93[c,e]	1.35 ± 0.02[f]	0[f]
4	5.87[d]	0.243	0.797[g]
4	5.85[d]	0.260	0.487[g]
4	5.82[d]	0.265	0.246[g]
4	5.80[d,e]	0.270 ± 0.005[f]	0[f]
3	4.02[b]	46.8	0.617[h]
3	3.98[b]	49.1	0.376[h]
3	3.97[b]	54.4	0.190[h]
3	3.96[b,e]	59.7 ± 2.4[f]	0[f]
3	4.92[c]	7.23	0.127[h]
3	4.94[c]	7.26	0.314[h]
3	4.93[c]	7.45	0.190[h]
3	4.91[c,e]	7.31 ± 0.12[f]	0[f]
3	5.85[d]	1.44	0.376[h]
3	5.83[d]	1.51	0.254[h]
3	5.81[d]	1.55	0.127[h]
3	5.81[d,e]	1.60 ± 0.02[f]	0[f]

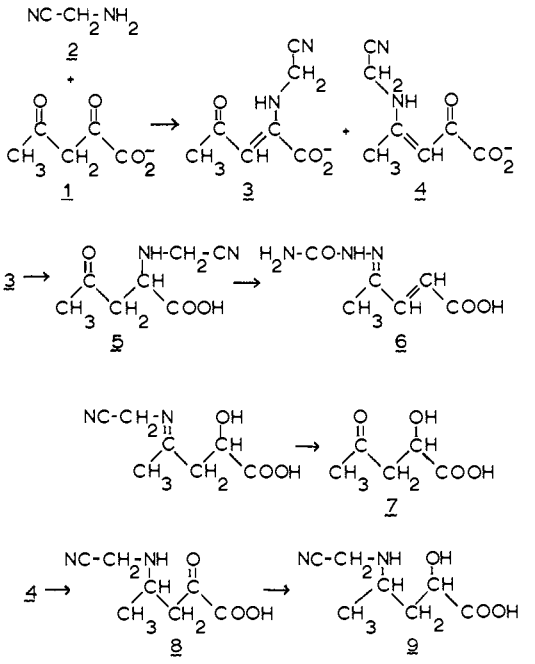
[a] The temperature was 30.0 ± 0.1°; ionic strength was 0.3 *M* (KCl). [b] Acetate buffer, 0.140 *M*. [c] Acetate buffer, 0.0722 *M*. [d] Phosphate buffer, 0.0815 *M*. [e] pH of buffer containing no organic solvent. [f] Extrapolated values. [g] Methanol. [h] Acetonitrile.

Kinetics and Equilibrium. Nmr Studies. The reaction of APY with AAN could be studied in concentrated solution by nmr spectroscopy. The solutions used were 0.37 *M* in APY, 1 *M* in AAN, and up to 1 *M* in phosphate. Thus, the solutions were at a very high ionic strength, and even so were not well buffered. Consequently only the qualitative kinetic pattern was observable. By the time the first spectrum was recorded, the three peaks of acetopyruvate were largely replaced by a new peak at δ 2.59. Then, more gradually another new peak at δ 2.72 appeared at the expense of the initial product. The two isolated enamines, as their sodium salts, have nmr absorptions at δ 2.67 (enamine **4**) and 2.57 (enamine **3**). By careful integration (using a planimeter) of spectra taken after reaction had gone to completion, the ratios of the concentrations of the two enamines were obtained; the ratio was independent of pH for pH ≥4, but decreased at lower pH. The average of all the values for pH ≥4 is r_1 = 0.82 ± 0.08.

Discussion

Structure Proof. The analytical data for both compounds **3** and **4** show that each has formed by condensation of one molecule of AAN and one of APY. Under the gentle conditions used to form these compounds, the only reasonable mode of reaction is attack by the amine on the ketone carbonyl groups (see Scheme I). This would give a β-ketoimine, which should be

Scheme I



almost completely converted to the tautomeric conjugated enamine. The spectral properties of both these compounds are in accord with this intuitive expectation; in particular both have an intense uv absorption at *ca.* 310 nm and show a one-proton signal in the nmr spectrum in the region where a vinyl proton is expected to absorb.

The structure of **3** was established by means of its reduction by sodium borohydride. An nmr spectrum of the immediate product of the reaction showed that 80% of the product was a compound with a singlet at δ 2.08 and a doublet at 3.25. This spectrum and particularly the singlet at δ 2.08, attributed to CH_3CO, require that the product of reduction be one where reaction has occurred at the α and not the γ carbon. The simplest interpretation of this reaction is that it has given compound **5**. It proved impossible to obtain **5** in crystalline form; however, a crystalline derivative could be obtained in 45% yield based on **3** by reaction with semicarbazide. The analytical and spectral data for this derivative showed clearly that elimination had occurred during semicarbazone formation, to give **6**. An authentic sample of the semicarbazone of acetoacrylic acid was prepared and was identical with the material derived from **3**. It is conceivable that if **3** were the γ-enamine, borohydride reduction might occur at the α-keto group, rather than at the γ-imine, to give α-hydroxylevulinic acid **7**, which should have an nmr spectrum similar to that of **5**, so that it was necessary to prepare it (or at least an equivalent material) to rule out this possibility.

Reduction of APY with borohydride in acetic acid–ethanol, followed by the same aqueous work-up that was used for the reduction of **3**, gave a material whose nmr showed that the major product (at least 80%) had the spectrum expected for **7**. Although α-hydroxy-

Table III. Microscopic Rate Constants for Enamine Formation[a]

pH	k_{21}', M^{-1} sec^{-1} [b]	k_{23}', M^{-1} sec^{-1} [c]	K_1, M^{-1} [d]	K_3, M^{-1} [e]
4	0.0744 ± 0.0090	0.0140 ± 0.0021	12 ± 2	15 ± 2
5	0.0742 ± 0.0023	0.0160 ± 0.0018	101 ± 9	124 ± 11
6	0.0396 ± 0.00091	0.00815 ± 0.00099	248 ± 7	301 ± 26

[a] In water at 30.0 ± 0.10; μ = 0.3 M (KCl). [b] Rate constant for formation of the α-enamine. [c] Rate constant for formation of the γ-enamine. [d] Equilibrium constant for formation of the α-enamine. [e] Equilibrium constant for formation of the γ-enamine.

levulinic acid could not be isolated from the crude reaction product, pyrolysis gave acetoacrylic acid.

The spectra of the products of reduction of APY and **3** are unquestionably different. Thus, the major product from the reduction of **3** with sodium borohydride is unambiguously assigned the structure **5**. This in turn leads to the α-enamine structure for **3**.

It would be satisfying if **4** could be reduced to give **8** which would show a doublet in the nmr at higher field than the singlet of **5**. However, **4** was very resistant to reduction, and when it was reduced at all, it apparently underwent double reduction perhaps to **9** or a product derived from it. This is not surprising, if **4** is the γ-enamine, since reduction of the imine tautomer of **4** would uncover the α-carbonyl, previously present in the unreactive ketoenamine function. Borohydride reduction of **3** or **4** requires that the reaction of the protonated imine (present as a minute fraction of the total concentration) compete successfully with acid-catalyzed decomposition of borohydride. Apparently this condition holds for **3** but not for **4**.

It seems extremely improbable that two enamines which differed only by geometrical isomerism would not be rapidly interconverted in acidic solution; since **3** and **4** show distinctive reactivity differences and preserve their identity over a period of hours in acidic media, they must be positional isomers, and since **3** is the α-enamine, **4** must be the γ-enamine.

Analysis of the Kinetics of Enamine Formation from APY. The complex kinetic behavior observed at pH 3, 4, and 5 could be described by an integrated rate equation containing two exponential terms. This situation could arise either from a two-stage process with rate constants of similar magnitude or from two parallel processes. Since the reaction proceeds to equilibrium, each process is reversible. Thus, four rate constants are required, as a minimum, to describe the observed behavior. Two products were isolated from the reaction of APY and AAN, both of which were enamines, but which hydrolyzed at rates differing by a factor of at least 5 or 6. Furthermore, nmr spectra of reacting mixtures of APY and AAN also showed peaks assignable to these two products; one formed rapidly, the other formed more slowly and to some extent at the expense of the more rapidly formed product. These observations lead to the conclusion that one is observing two parallel reactions of acetopyruvate.

Formation of either enamine must involve a carbinolamine and then an imine as intermediates; however, these are expected[15a,b] to be present in very low concentration. In fact no spectroscopic evidence for their accumulation was observed, and the kinetics could be analyzed on the assumption that they are only present at steady-state concentrations. Imine–enamine tautomerism is normally more rapid than keto–enol tautomerism;[15b,c] in view of the rapidity of keto–enol tautomerism in APY[9] it seems unlikely that the imine–enamine conversion is the rate-determining step for enamine formation. Studies of imine formation from simple ketones[15d] lead to the expectation that at high pH the rate-determining step will be acid-catalyzed carbinolamine dehydration, changing to rate-determining carbinolamine formation at low pH.

The kinetic system is then

$$3 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{APY} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} 4$$

where **3** and **4** are the two isomeric enamines, k_{21},k_{23} are the pseudo-first-order rate constants for enamine formation, and k_{12},k_{32} are the first-order rate constants for enamine hydrolysis.

An analytical expression for the integrated rate equation may be derived following standard procedures,[16] and leads to an equation of the form $A = a + be^{-\lambda_2 t} + ce^{-\lambda_3 t}$, where $\lambda_2 = (p + q)/2$, $\lambda_3 = (p - q)/2$, $p = k_{12} + k_{21} + k_{23} + k_{32}$, and $q = [p^2 - 4(k_{12}k_{23} + k_{21}k_{32} + k_{12}k_{32})]^{1/2}$. The analytical equation for absorbance as a function of time is far too complex to be of any use directly. However, these expressions for λ_2 and λ_3 serve as a basis for solving the system.

Rate constants for hydrolysis of the two enamines have been accurately determined. The ratio of the equilibrium constants for α- and γ-enamine formation has been determined by nmr spectroscopy. From the expressions for λ_2 and λ_3, $\lambda_2 + \lambda_3 = k_{12} + k_{21} + k_{23} + k_{32}$.

Replacing the pseudo-first-order rate constants k_{21} and k_{23} by k_{21}'[AAN] and k_{23}'[AAN], where k_{21}' and k_{23}' are second-order rate constants, $\lambda_2 + \lambda_3 = (k_{12} + k_{32}) + (k_{21}' + k_{23}')$[AAN] = $k_r + k_f$[AAN]. This is in fact what was observed; k_f and k_r, the slope and intercept, respectively, of plots of $\lambda_2 + \lambda_3$ (or k_{obsd}) against total AAN concentration are found in Table I. It is useful to define two ratios: $r_1 = K_1/K_3$, where K_1 = [**3**]/[AAN][APY] and K_3 = [**4**]/[AAN][APY], and $r_2 = k_{12}/k_{32}$. Then $k_{21}' = k_f/[1 + (1/r_1r_2)]$ and $k_{23}' = k_f/[1 + r_1r_2)]$. This allows the calculation of the rate constants describing the system, collected in Table III.

A small but nonetheless appreciable fraction of APY is present as the hydrate formed by addition of water to the α-carbonyl group. The rates of addition and loss of water have been measured as a function of pH.[9]

Under the conditions of most of the enamine forming reactions the rate of hydration is comparable to the observed rate of enamine formation, and only at pH 5.8 or higher is hydration more than tenfold faster than

(15) (a) J. Hine, C. Y. Yeh, and F. C. Schmalstieg, *J. Org. Chem.*, **35**, 340 (1970); (b) M. L. Bender and A. Williams, *J. Amer. Chem. Soc.*, **88**, 2502 (1966); (c) J. Hine, B. C. Menon, J. H. Jensen, and J. Mulders, *ibid.*, **88**, 3367 (1966); (d) W. P. Jencks, *Progr. Phys. Org. Chem.*, **2**, 63 (1964).

(16) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," 2nd ed, Wiley, New York, N. Y., 1961, pp 173–177.

enamine formation. However, numerical calculations[17] using the rate constants for enamine formation calculated above and the previously measured[9] rate constants for hydration and dehydration of APY showed that hydration–dehydration was not kinetically significant under any of the experimental conditions used.

With all four rate constants required to describe the reaction of APY with AAN in hand, the analysis of the kinetics may be tested by comparing the observed and calculated values for λ_2 and λ_3: see Table IV. The

Table IV. Comparison of Observed and Calculated Values for λ_2 and λ_3[a]

pH	[AAN], M	$10^3\lambda_2$, sec^{-1} Obsd	$10^3\lambda_2$, sec^{-1} Calcd	$10^4\lambda_3$, sec^{-1} Obsd	$10^4\lambda_3$, sec^{-1} Calcd
4	0.0874[b]	14.7	13.2	1.69	1.43
	0.0623[b]	10.9	11.0	1.48	1.36
	0.0399[b]	8.65	9.16	1.13	1.27
	0.0175[b]	7.70	7.32	0.99	1.12
	0.00874[b]	7.29	6.63	1.06	1.03
5	0.0840[c]	7.91	8.24	4.97	2.38
	0.0399[c]	3.96	4.25	4.70	2.32
	0.0168[c]	2.15	2.14	3.78	2.46
	0.00841[c]	1.39	1.42	2.04	2.00
6	0.0445[d]	2.17	2.26		0.49
	0.0249[d]	1.28	1.33		0.48
	0.0178[d]	0.912	0.990		0.47
	0.0142[d]	0.743	0.819		0.46
	0.0107[d]	0.577	0.652		0.46

[a] Reaction of APY with AAN, 30.0 ± 0.1°. [b] Acetate buffer, 0.140 M. [c] Acetate buffer, 0.0722 M. [d] Phosphate buffer, 0.0815 M.

agreement is good, the only serious discrepancy being for λ_3 at pH 5. The most likely explanation is that the reaction was not followed long enough and that the experimental value is in error. (The reactions were followed for about 4000 sec, until there was apparently no further change in optical density, and the data gave good first-order plots for the later data points. However, numerical integration[17] using the rate constants evaluated above showed that these tests are insufficiently stringent.) Fortunately, since λ_3 is smaller than λ_2 and the *sum* was used for the evaluation of rate constants, even large errors in λ_3 have a negligible effect on the values of the rate constants calculated by the procedure used here.

Equilibrium constants for the individual enamine-forming reactions may also be calculated from the rate constants for the forward and reverse reactions; these equilibrium constants are found in Table III.

Implications for the Enzymic Mechanism. This investigation of the rates of enamine formation from acetopyruvate and aminoacetonitrile was undertaken to provide a model for the reaction of acetopyruvate with acetoacetate decarboxylase. Now that values have been obtained for the rate and equilibrium constants needed to describe the model system, they may be compared to those for the enzymic reaction. The rate constant for enamine formation from APY and acetoacetate decarboxylase is 3000 M^{-1} sec^{-1} at pH 6 and 30° [18]; it is not known which carbonyl group of APY reacts. The corresponding rate constant (α position of APY) for AAN is 0.04 M^{-1} sec^{-1}. The rate enhancement of 10^5 is so large that one may confidently say that there is something special about the reaction of acetoacetate decarboxylase with APY. It may be compared with that for the specific reaction of the enzyme with acetoacetate, the second-order rate constant for which can be calculated from the data of Coutts[19]; at pH 5.95 $k_2 = k_{cat}/K_M = 2.4 \times 10^5\ M^{-1}$ sec^{-1}.

At pH 6, the second-order rate constant for AAN-catalyzed decarboxylation is $4.0 \times 10^{-3}\ M^{-1}$ sec^{-1},[4] so that the rate enhancement of the enzymic over the model reaction, calculated using the second-order rate constants, is 10^8.

The rate enhancement for decarboxylation of acetoacetate is 10^3 larger than the rate enhancement for enamine formation from acetopyruvate. Since imine formation is believed to be at least partially rate determining for both aminoacetonitrile and acetoacetate decarboxylase catalyzed decarboxylation,[5,20] this means that the *more* reactive carbonyl group (in acetopyruvate) shows *less* rate enhancement in its reaction with the active site amino group.

This reversal of reactivity probably should not be surprising, in view of the known properties of the enzyme, which is known to bind monovalent anions quite strongly[21]; thus, it is to be expected that when acetoacetate or acetopyruvate are bound noncovalently to the enzyme, the carboxylate groups will occupy the anion binding site. If, as one would certainly expect, the ketone of the acetoacetate is then correctly positioned relative to the reactive amino group, it is apparent that *neither* ketone of acetopyruvate will be correctly positioned. Thus, in order to have a ketone in the reactive position in the active site, the carboxylate must be displaced from the anion binding site. The relative unreactivity of acetopyruvate toward the enzyme is then a consequence of the price in decreased binding energy which must be paid in order to reach the transition state.

Acknowledgment. The author gratefully acknowledges critical discussions, advice, and encouragement provided by Professor F. H. Westheimer, who originally suggested this problem.

(17) A computer program for numerical integration of the equations for complicated kinetic systems, kindly made available by Dr. N. C. Baird, Chemistry Department, University of Western Ontario, was used for these calculations.

(18) P. A. Frey, personal communication.
(19) S. M. Coutts, Ph.D. Thesis, Harvard University, 1967.
(20) M. H. O'Leary and R. L. Baughn, *J. Amer. Chem. Soc.*, **94**, 626 (1972).
(21) I. Fridovich, *J. Biol. Chem.*, **238**, 592 (1963).