When the crystal structures of the analogs ara-s^{4}U, 4-thiouridine,[16] the 1-methyl-4-thiouracil–9-methyladenine complex,[29] and 3′-*O*-acetyl-4-thiothymidine[30] are compared with each other it becomes evident that the hydrogen bonding of the heterocycles in these structures is largely determined by the packing arrangement: in 4-thiouridine and in the base–pair complex where the heterocycles are markedly stacked, O(2) and N(3) but not S are involved in hydrogen bonding, while in 3′-*O*-acetyl-4-thiothymidine, S but not O(2) and N(3) forms a hydrogen bond. In ara-s^{4}U, however, O(2), N(3), and S do have a hydrogen-bonded partner.

The computations were carried out at the Aerodynamische Versuchsanstalt Göttingen (IBM 7040) and at the Gesellschaft für wissenschaftliche Datenverarbeitung mbH., Göttingen (UNIVAC 1108). The thermal ellipsoids plot was performed at Deutsches Rechenzentrum Darmstadt (IBM 7094).

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Acetoacetate Decarboxylase. Identification of the Rate-Determining Step in the Primary Amine Catalyzed Reaction and in the Enzymic Reaction[1,2]

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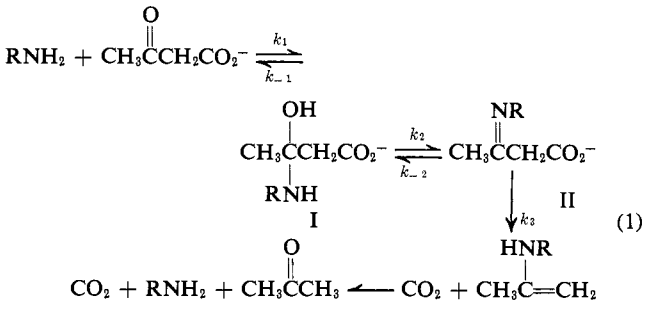
Abstract: The decarboxylation of acetoacetic acid in aqueous solution at 30° catalyzed by aminoacetonitrile shows carboxyl carbon isotope effects (k^{12}/k^{13}) of 1.031 at pH 3.6, 1.032 at pH 4.1, and 1.036 at pH 5.0. The magnitude of the isotope effect and the variation of the isotope effect with pH indicate that decarboxylation is partially rate determining. Quantitative evaluation of all of the rate constants in the mechanism is possible by assuming a value for the isotope effect on the decarboxylation step and calculating individual rate ratios from the pH dependence of the isotope effect. The decarboxylation catalyzed by acetoacetate decarboxylase shows isotope effects of 1.019 at pH 5.3, 1.017 at pH 6.0, and 1.019 at pH 7.2. As in the case of the amine-catalyzed reaction, decarboxylation is partially rate determining, but no single step is entirely rate determining.

The amine-catalyzed decarboxylation of acetoacetic acid proceeds according to the general mechanism[3-6] of eq 1. The first two steps in the reaction,



(1)

formation of the carbinolamine (I) and of the Schiff base (II), are well known, and have been studied in detail in many cases not involving decarboxylation.[7] However, in spite of several recent studies,[4-6] the details of the mechanism of decarboxylation are unclear. In particular, little is known about the relative rates of the various steps in eq 1.

The decarboxylation of acetoacetic acid catalyzed by acetoacetate decarboxylase from *Clostridium acetobutylicum* also proceeds according to the general mechanism[8] of eq 1. Decarboxylation occurs *via* a Schiff base between acetoacetate and the ϵ-amino group of a lysine residue of the enzyme.[9] This particular amino group is abnormal in that it is the only amino group acetylated by acetic anhydride[10] at pH 6, and kinetic studies[11] indicate that it has an unusually

(1) A preliminary communication has been published: M. H. O'Leary and R. L. Baughn, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **30**, 1240 (1971).

(2) This research was supported by National Institutes of Health Grant No. NS 07657 and by a grant from the Research Committee of the University of Wisconsin Graduate School.

(3) K. J. Pedersen, *J. Amer. Chem. Soc.*, **51**, 2098 (1929); H. von Euler and A. Ölander, *Z. Anorg. Chem.*, **147**, 295 (1925); E. Widmark and C. A. Jeppsson, *Skand. Arch. Physiol.*, **42**, 43 (1922); E. Widmark and C. A. Jeppsson, *Chem. Z.*, **93**, 526 (1922); K. J. Pedersen, *J. Phys. Chem.*, **38**, 559 (1934); K. J. Pedersen, *J. Amer. Chem. Soc.*, 60, 595 (1938).

(4) R. W. Hay, *Austr. J. Chem.*, **18**, 337 (1965).

(5) J. P. Guthrie and F. H. Westheimer, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **26**, 562 (1967).

(6) J. P. Guthrie, Ph.D. Dissertation, Harvard University, Cambridge, Mass., 1968.

(7) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill Book Co., New York, N. Y., 1969, Chapter 10; T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Benjamin, New York, N. Y., 1966, Vol. 2, Chapter 8.

(8) F. H. Westheimer, *Proc. Chem. Soc.*, 253 (1963); G. A. Hamilton and F. H. Westheimer, *J. Amer. Chem. Soc.*, **81**, 6332 (1959); I. Fridovich and F. H. Westheimer, *ibid.*, **84**, 3208 (1962).

(9) S. Warren, B. Zerner, and F. H. Westheimer, *Biochemistry*, **5**, 817 (1966); R. A. Laursen and F. H. Westheimer, *J. Amer. Chem. Soc.*, **88**, 3426 (1966).

(10) M. H. O'Leary and F. H. Westheimer, *Biochemistry*, 7, 913 (1968).

(11) (a) S. M. Coutts, Ph.D. Dissertation, Harvard University, Cambridge, Mass., 1967; (b) D. E. Schmidt and F. H. Westheimer, *Biochemistry*, 10, 1249 (1971).

low pK_a. As in the case of the amine-catalyzed reaction, it is not clear what the relative rates of the various steps in the reaction sequence are. Although enzyme and amine operate by similar mechanisms, the overall rates of the two differ by more than 1000-fold,[5] and it is not clear to what extent the relative rates of the various reaction steps are the same in the two cases.

This study is an attempt to obtain more information about these decarboxylation mechanisms by use of carbon isotope effects. We have previously studied carbon isotope effects in enzymatic decarboxylations[12,13] and have shown that clear decisions as to the rate-determining step can frequently be made. The variation of isotope effect with pH is often useful, and we will show that many aspects of the decarboxylation of acetoacetic acid can be clarified by this procedure.

Results

Carboxyl carbon isotope effects on the decarboxylation of acetoacetic acid catalyzed by aminoacetonitrile and catalyzed by acetoacetate decarboxylase have been measured by comparison of the isotopic composition of a CO_2 sample isolated after about 10% reaction with that of a sample isolated after complete decarboxylation. All measurements were made with only the natural abundance of carbon-13. The general method has been described previously.[12]

In Table I are given the isotope effects on the decarboxylation catalyzed by aminoacetonitrile at 30°. The isotope ratios given have been corrected to a constant value of the CO_2 tank standard (this adjusts for electronic drift in the ratio measuring system of the mass spectrometer) and have been corrected for the presence of oxygen-17 by subtracting the value 0.000800 from the measured ratio. The influence of this correction on the isotope effect is quite small, and the correction should not vary with time because the oxygen of the CO_2 being measured is always in equilibrium with water. The isotope effects have been corrected for per cent reaction by the method of Bigeleisen and Wolfsberg.[14]

Table I. Carbon Isotope Effects on the Decarboxylation of Acetoacetic Acid Catalyzed by Aminoacetonitrile at 30° in Aqueous Solution

pH	% reaction	Isotope ratios[a] × 10⁶ Low conversion	100% conversion	k^{12}/k^{13}
3.58	16.8	13621	14010	1.0315
3.59	18.0	13627	14003	1.0306
		Mean		1.0310
4.07	15.5	13589	13988	1.0321
4.07	14.4	13592	14000[b]	1.0325
4.07	13.9	13597	13994	1.0316
4.07	14.1	13588	13990	1.0320
		Mean		1.0320
				±0.0007
5.01	13.7	13553	14009	1.0363
5.03	26.0	13587	14000[b]	1.0355
5.05	13.3	13539	14005	1.0371
5.04	13.1	13549	14003	1.0360
		Mean		1.0362
				±0.0007

[a] Decade settings for the ratio *m/e* 45/44, corrected to tank standard = 14150 and corrected for the presence of oxygen-17. [b] Sample lost. Taken as the mean of the measured 100% reaction samples.

The reproducibility of the measurements reported in Table I is quite satisfactory. The mean of eight measurements of the composition of the 100% reaction sample is 0.014000 ± 0.000008. Although the results for the low conversion samples at various pH values in Table I are not comparable, the reproducibility of the measurements at each pH is similar to that of the 100% reaction samples. In two of the experiments reported in Table I the 100% reaction sample was lost. In those cases the mean value 0.014000 was used for the 100% reaction sample. The reproducibility of the measured isotope effect at a given pH is quite high, and the variation of the isotope effect with pH is seven times the standard deviation of the measurements at one pH.

The results of measurements of the carbon isotope effect on the enzyme-catalyzed decarboxylation of acetoacetic acid at 30° are given in Table II. The same corrections have been made as were noted above. Again the reproducibility of the 100% reaction samples is quite high, the mean of 18 determinations being 0.013968 ± 0.000011. The reproducibility of the low conversion measurements is not as high as before because of the small size of the sample involved. The variation of the isotope effect with pH is not statistically significant.

Table II. Carbon Isotope Effects on the Decarboxylation of Acetoacetic Acid Catalyzed by Acetoacetate Decarboxylase at 30° in Aqueous Solution

pH	% reaction	Isotope ratios[a] × 10⁶ Low conversion	100% conversion	k^{12}/k^{13}
5.31	11.1	13702	13972	1.0209
5.31	14.3	13749	13979	1.0181
5.31	14.3	13759	13975	1.0170
		Mean		1.0187
				±0.0020
6.00	10.6	13719	13984	1.0204
6.00	10.6	13729	13961	1.0179
6.00	10.6	13746	13952	1.0159
6.00	9.8	13763	13960	1.0151
6.00	10.6	13744	13961	1.0167
6.00	10.6	13768	13968	1.0154
6.00	10.4	13759	13971	1.0163
6.00	21.4	13779	13967	1.0154
		Mean		1.0167
				±0.0018
7.18	7.4	13676	13961	1.0216
7.18	6.9	13697	13992	1.0224
7.18	8.5	13764	13965	1.0152
7.18	7.2	13745	13974	1.0174
		Mean		1.0192
				±0.0035

[a] Decade settings for the ratio *m/e* 45/44, corrected to tank standard = 14150 and corrected for the presence of oxygen-17.

(12) M. H. O'Leary, D. T. Richards, and D. W. Hendrickson, *J. Amer. Chem. Soc.*, **92**, 4435 (1970).

(13) M. H. O'Leary, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **29**, 407 (1970); M. H. O'Leary, *J. Amer. Chem. Soc.*, **91**, 6886 (1969); M. H. O'Leary, *Biochim. Biophys. Acta*, **235**, 14 (1971).

(14) J. Bigeleisen and M. Wolfsberg, *Advan. Chem. Phys.*, 1, 15 (1958).

Discussion

Theory. Carboxyl carbon isotope effects on decarboxylations of the type given by eq 2 have been mea-

$$X{=}C(\text{—})CH_2{-}CO_2^- \longrightarrow CO_2 + X{-}C{=}CH_2 \qquad (2)$$

sured for several cases where X = O, as shown in Table III.

Table III. Isotope Effects on Decarboxylations of the Type

$${-}X{=}C(\text{—})CH_2{-}CO_2^- \longrightarrow CO_2 + {-}X{-}C{=}CH_2$$

Substrate	Catalyst[a]	Temperature, °C	k^{12}/k^{13}	Ref
$HO_2CCOCH_2CO_2H$	Mn^{2+}	10	1.060	*d*
	Cu^{2+}	10	1.056	*d*
	Y^{2+}	25	1.036	*e*
	H^+	25	1.045	*e*
$HO_2CCH_2CO_2H$	None	138	1.037	*f*
	None	137	1.034	*g*
	None[b]	99.1	1.032	*h*
	None[b]	40.3	1.043	*i*
	Quinoline[c]	138	1.031	*j*
$CH_3COCH_2CO_2H$	Aminoacetonitrile	30	1.036	*j*
	Acetoacetate decarboxylase	30	1.017	*k*

[a] In aqueous solution unless otherwise noted. [b] In dioxane. [c] In quinoline. [d] S. Seltzer, G. A. Hamilton, and F. H. Westheimer, *J. Amer. Chem. Soc.*, **81**, 4018 (1959). [e] A. Wood, *Trans. Faraday Soc.*, **60**, 1263 (1964). [f] J. Bigeleisen and L. Friedman, *J. Chem. Phys.*, **17**, 998 (1949). [g] J. G. Lindsay, A. N. Bourns, and H. G. Thode, *Can. J. Chem.*, **30**, 163 (1952). [h] P. E. Yankwich and R. M. Ikeda, *J. Amer. Chem. Soc.*, **81**, 5054 (1959). [i] P. E. Yankwich and R. M. Ikeda, *ibid.*, **82**, 1891 (1960). [j] P. E. Yankwich and R. L. Belford, *ibid.*, **76**, 3067 (1954). [k] This research.

Our measurements of the decarboxylation of acetoacetic acid are the first measurements for X = N. The isotope effects observed for the latter case are smaller than those for X = O, presumably because decarboxylation is not entirely rate determining. In this section we will derive the relationship between the observed isotope effect and the mechanism of eq 1, and in subsequent sections we will apply this relationship to the isotope effects which we have measured.

The amine-catalyzed decarboxylation of acetoacetic acid has the same kinetic form as the enzyme-catalyzed decarboxylation. Therefore we will use equations from enzyme kinetics as the starting point for our derivation.

The enzyme-catalyzed reaction of two isotopic species of acetoacetic acid can be considered in terms of an enzyme catalyzing two reactions (in this case, decarboxylations of the two isotopic species) simultaneously. The two substrates are competitive with respect to each other, and the velocity of reaction of substrate a is given[15] by eq 3, where a and b represent

$$v_a = \frac{V_a}{1 + \frac{K_a}{a}\left(1 + \frac{b}{K_b}\right)} \qquad (3)$$

concentrations of the two species, and V_a and K_a are the maximum velocity and the Michaelis constant, respectively. The velocity of reaction of substrate b is given by an analogous expression, and the ratio of the two velocities is given by eq 4, which is the general form

$$\frac{v_a/a}{v_b/b} = \frac{V_a K_b}{V_b K_a} \qquad (4)$$

for the isotope effect on an enzymatic reaction. It remains only to write the steady-state equations for V and K appropriate to the mechanism in question, substitute them into the equation above, and solve.

In the present case a useful simplification results from the introduction of several assumptions at this point. We assume first that there is no isotope effect on any step in eq 1 except the decarboxylation step. Sizable isotope effects for atoms other than hydrogen are observed only when a bond is being broken or formed to the isotopic atom.[12] We also assume that decarboxylation is not reversible under the conditions of our experiments. Our derivation requires that the first-formed "Michaelis complex" in the enzymatic reaction be in equilibrium with free substrate and that the attainment of this equilibrium be rapid compared to k_1. If that is not so, a minor modification is needed in the final equation.

Using the assumptions given in the last paragraph, together with eq 4 and the appropriate steady-state rate equation for a reaction involving two intermediates,[15] the relationship between the observed isotope effect and the mechanism of eq 1 is given by eq 5. Thus the ob-

$$\text{observed}\ \frac{k^{12}}{k^{13}} = \frac{\frac{k_3{}^{12}}{k_3{}^{13}} + \frac{k_3}{k_{-2}}\left(1 + \frac{k_2}{k_{-1}}\right)}{1 + \frac{k_3}{k_{-2}}\left(1 + \frac{k_2}{k_{-1}}\right)} \qquad (5)$$

served isotope effect can be separated into three components—the isotope effect on the decarboxylation step, the partitioning of the carbinolamine intermediate, and the partitioning of the Schiff base intermediate. The observed isotope effect can be no larger than $k_3{}^{12}/k_3{}^{13}$. The larger the ratios k_2/k_{-1} and k_3/k_{-2}, the smaller the observed isotope effect compared to the isotope effect on k_3.

It should be noted that k_2/k_{-1} and k_3/k_{-2} in eq 5 are ratios of rates of various reactions under a specified set of conditions, rather than ratios of rate constants for particular processes. Thus, for example, k_2/k_{-1} represents the ratio of the rate at which carbinolamine I dehydrates to the rate at which it decomposes to free amine and acetoacetate. This fact has several important implications. In the first place, the observed isotope effect will not be altered by draining away of an intermediate due to protonation or deprotonation. Thus, the carbon isotope effect in the mechanism of eq 6 should not vary with pH. Further, if some step in the

$$\begin{array}{ccccccc} E + S & \rightleftharpoons & ES & \rightleftharpoons & ES' & \longrightarrow & EP + CO_2 \\ & & \downarrow\uparrow & & \downarrow\uparrow & & \\ & & ESH & & ES'H & & \end{array} \qquad (6)$$

reaction is subject to acid or base catalysis, then the corresponding rate ratio (and thus the isotope effect) will vary with the concentration of the catalyst. The fact that the rate of a reaction varies with pH does not necessarily imply that the isotope effect on that reaction varies with pH. Variation of an isotope effect with pH requires that one of the three ratios in eq 5 vary with

(15) M. Dixon and E. C. Webb, "Enzymes," 2nd ed, Academic Press, New York, N. Y., Chapter 4.

pH. This variation will be an important point for consideration later.

Amine-Catalyzed Decarboxylation. The magnitude of the carbon isotope effect on the amine-catalyzed decarboxylation of acetoacetic acid indicates that decarboxylation is at least partially rate limiting. In order to treat the isotope effect data more quantitatively it is necessary to know the pH dependences of the three ratios in eq 5. Knowing these, we will assume a range of reasonable values for k_3^{12}/k_3^{13} and derive values for the other two ratios. The conclusions which results from this treatment are largely independent of the assumed value of k_3^{12}/k_3^{13} and provide a satisfactory and consistent quantitative picture of the decarboxylation mechanism.

The decarboxylation of acetoacetic acid catalyzed by aminoacetonitrile shows no general acid or general base catalysis.[6] Thus only specific acid or base catalysis can be involved in the decarboxylation mechanism.[16] This is consistent with what is found in mechanistic studies of Schiff base formation using weakly basic amines.[7] Carbinolamine dehydration (k_2 in eq 1) is usually specific acid catalyzed, whereas reversion of the carbinolamine intermediate to starting materials is either uncatalyzed or general acid catalyzed. The lack of observable general catalysis in the present reaction requires that k_{-1} be uncatalyzed. Because k_2 is specific acid catalyzed, k_{-2} must also be specific acid catalyzed. The decarboxylation step, k_3, is also specific acid catalyzed. Thus the following pattern emerges: k_3/k_{-2} is independent of pH, since both reactions are specific acid catalyzed; k_2/k_{-1} is a linear function of the hydrogen ion concentration, increasing by a factor of ten for each decrease of one pH.

The only remaining term in eq 5 is the isotope effect term k_3^{12}/k_3^{13}. The decarboxylation step is specific acid catalyzed, and we expect that there will be only one transition state for decarboxylation. The structure of the transition state will not vary with pH, even if the rate of passage of substrate through this transition state (*i.e.*, the rate of reaction) varies with pH. Thus k_3^{12}/k_3^{13} is independent of pH.[17]

To obtain a more quantitative estimate of the magnitudes of the ratio terms in eq 5 we will estimate the value of k_3^{12}/k_3^{13} and calculate other ratios from this and the pH dependence of the observed isotope effect.[19] Although we do not know the value of k_3^{12}/k_3^{13} in this case, we known that carbon isotope effects on reactions of the type given in eq 2 are in the range from 1.04 to 1.06 at 25°. We will estimate that k_3^{12}/k_3^{13} is in this range and will calculate the value of the factor $(k_3/k_{-2})(1 + k_2/k_{-1})$ from eq 5 for each pH and for each assumed value of k_3^{12}/k_3^{13}. Using the values of this factor at the three pH values and knowing the pH dependencies of the two ratios in the factor, we can calculate the values of the two ratios. The results of these calculations are given in Table IV. Because it is necessary to assume the value for k_3^{12}/k_3^{13} there is still some variation possible in the values of the two derived ratios. However, the conclusions of this study are to a large extent independent of the exact value of this isotope effect.

Table IV. Derived Rate Ratios for the Amine-Catalyzed Decarboxylation of Acetoacetic Acid[a]

Assumed k_3^{12}/k_3^{13}	Calculated k_3/k_{-2}	Calculated k_2/k_{-1} pH 4.07	Calculated k_2/k_{-1} pH 5.04
1.04	0.14	0.45	0.05
1.05	0.42	0.18	0.02
1.06	0.71	0.13	0.01

[a] In order to calculate these ratios, a value of the factor $(k_3/k_{-2})\cdot(1 + k_2/k_{-1})$ was calculated from eq 5 for each observed isotope effect and each assumed value of k_3^{12}/k_3^{13}. A plot of the value of this factor *vs.* hydrogen ion concentration for a given value of k_3^{12}/k_3^{13} then yields values for k_3/k_{-2} and k_2/k_{-1}.

The mechanism of decarboxylation of acetoacetic acid catalyzed by aminoacetonitrile can be summarized as follows: At pH 5 and above decarboxylation is rate determining. The Schiff base intermediate hydrates to form carbinolamine slightly more rapidly than it decarboxylates. The carbinolamine intermediate reverts to starting material much more frequently than it dehydrates. From the data in Table IV it is clear that below about pH 4 the rates of the acid-catalyzed steps have increased sufficiently that decarboxylation is no longer rate determining. Instead, attack of the amine on the keto acid is rate determining. The relative rates of decarboxylation and hydration of the Schiff base remain the same as they were at high pH.

Guthrie and Westheimer[5,6] have also studied the decarboxylation of acetoacetic acid catalyzed by aminoacetonitrile. Based on the near identity of the decarboxylation rate and the rate of formation of the Schiff base between ethyl acetoacetate and aminoacetonitrile, they concluded that Schiff base formation is rate determining in both cases. Although our results show that Schiff base formation is not in fact rate determining in the decarboxylation at high pH, decarboxylation is only a few times slower than Schiff base formation and the parallel between the rates of the two reactions is reasonable. In a subsequent study, Jordan and Westheimer[20] have measured individual rate constants for the amine-catalyzed decarboxylation of acetoacetic acid by rapid kinetic methods and have reached the same conclusions that we have reached here.

Enzyme-Catalyzed Decarboxylation. It is not possible to interpret the isotope effects on the enzyme-catalyzed decarboxylation in as much detail as was possible for the amine-catalyzed reaction. We do not know the nature of the catalysis of the various steps in eq 1, and therefore cannot predict whether the isotope

(16) In principle it is possible that some reaction step which is rapid compared to the rate-determining step might be general acid or general base catalyzed. Under those conditions general catalysis would not be seen. However, this possibility is inconsistent with the conclusions of this paper concerning the relative rates of the various steps in the decarboxylation.

(17) There is one necessary qualification for this statement. At low pH the starting state is acetoacetic acid, rather than acetoacetate anion. If there is an isotope effect on the equilibrium between these two species, this will cause a change in isotope effect as the "ground state" changes from the anionic to the neutral form. However, the heavy isotope should concentrate in the neutral acid,[18] causing the observed isotope effect to increase at low pH. This has not been observed in this case, either because the effect is too small to be noticeable or because we have not reached sufficiently low pH in our experiments.

(18) W. Spindel, M. J. Stern, and E. U. Monse, *J. Chem. Phys.*, **52**, 2022 (1970).

(19) In principle it is possible to solve this system without assuming a value for k_3^{12}/k_3^{13}. However, such a procedure requires that the observed isotope effects be extremely precise, because large errors in the derived ratios would result from small errors in the isotope effects. The precision of our isotope effects does not merit such a procedure.

(20) F. H. Westheimer, personal communication, March 1971.

effect should be pH dependent. However, it is noteworthy that within experimental error the observed isotope effect is invariant with pH in a range where the maximum velocity of the decarboxylation increases, goes through a maximum, and decreases again.[11a]

The magnitude of the carbon isotope effect on the enzymatic decarboxylation indicates clearly that decarboxylation is at least partially rate determining. In this case it is possible to estimate the value of k_3^{12}/k_3^{13} by a different method. The rate of carbonyl oxygen exchange of acetoacetic acid catalyzed by acetoacetate decarboxylase has been measured by Hamilton.[21] At pH 6.5 the ratio of the rates of decarboxylation and exchange, k_d/k_e, is approximately unity. This partitioning is related to eq 1 by eq 7. The term on the right side of

$$\frac{k_d}{k_e} = \frac{k_3}{k_{-2}}\left(1 + \frac{k_2}{k_{-1}}\right) \qquad (7)$$

eq 7 appears also in eq 5, and enables us to calculate that k_3^{12}/k_3^{13} is about 1.034. However, the uncertainty in the oxygen exchange measurement is on the order of ± 0.5, so the uncertainty in k_3^{12}/k_3^{13} is large. Thus the isotope effect on the decarboxylation step may be slightly smaller than that observed in the amine-catalyzed decarboxylation.

The decarboxylation of acetoacetic acid catalyzed by acetoacetate decarboxylase is more than a 1000-fold faster than that catalyzed by aminoacetonitrile.[5] In both of these reactions the decarboxylation step is partially, but not entirely, rate limiting. The enzyme apparently accelerates the decarboxylation step to very nearly the same extent as it accelerates the other steps. The similarity of the isotope effects obtained in the two cases makes it likely that the transition states have similar structures in the two cases. Since the starting states are the same (free acetoacetate) and therefore have the same energy, it follows that the energy of the transition state for decarboxylation has been changed by a considerable amount without any large change in structure. The means by which this is accomplished is not clear, but one attractive possibility is the polarity effect demonstrated by Crosby, *et al.*, for a closely related decarboxylation.[22]

(21) G. A. Hamilton, Ph.D. Dissertation, Harvard University, Cambridge, Mass., 1959.

Experimental Section

Materials. Acetoacetate decarboxylase isolated from *Cl. acetobutylicum* was kindly provided by Professor F. H. Westheimer and J. V. Connors.[23] Aminoacetonitrile sulfate was recrystallized three times from ethanol–water. Lithium acetoacetate was prepared as described by Hall.[24] Doubly distilled deionized water was used for all buffers. The following buffers were used: pH 3.58, sodium chloroacetate; pH 4.07 and 5.02, sodium acetate; pH 5.31, pyridinium sulfate; pH 6.00, sodium phosphate; pH 7.18, *N*-ethylmorpholine sulfate.

Kinetic Measurements. All kinetic measurements were carried out on a Cary 15 spectrophotometer at 270 nm at 30°. The absorbance change corresponding to 100% reaction was measured by allowing solutions of acetoacetate with either enzyme or amine present to decarboxylate for at least ten half-lives.

Isotope Effect Measurements. These procedures have been described previously.[12] All solutions were degassed by bubbling prepurified CO_2-free N_2 through them for at least 30 min. The enzyme was purified by chromatography on Sephadex G-25 using degassed buffer immediately before use. Reactions were quenched by the addition of sufficient H_2SO_4 to lower the pH to 1.0, and the flasks were immediately frozen to prevent any trace of acid-catalyzed decarboxylation. Approximately 0.01–0.02 *M* acetoacetate was used for all measurements. The aminoacetonitrile concentration was 0.15–0.4 *M*.

The decade settings given in Tables I and II are appreciably different from the actual isotope ratios for *m/e* 45:44 because of the design of the electronics in the isotope-ratio mass spectrometer. As discussed by Nier,[25] the observed ratios are directly proportional to the actual abundances and can thus be used directly in the calculation of isotope effects.

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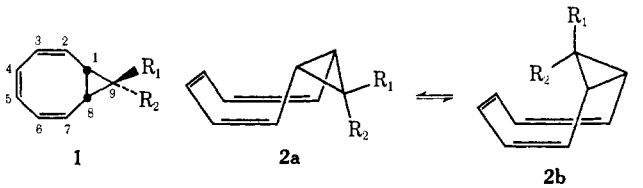
(22) J. Crosby, R. Stone, and G. E. Lienhard, *J. Amer. Chem. Soc.*, **92**, 2891 (1970).
(23) F. H. Westheimer, *Methods Enzymol.*, 14, 231 (1969).
(24) L. M. Hall, *Biochem. Prepn.*, 10, 1 (1963).
(25) A. O. Nier, *Phys. Rev.*, 77, 789 (1950).

Communications to the Editor

The Stereochemistry of Uniparticulate Electrophilic Additions to *cis*-Bicyclo[6.1.0]nonatrienes[1]

Sir:

The *cis*-bicyclo[6.1.0]nonatriene system **1** is an intriguing chemical entity due chiefly to: (a) its possible conformational flexibility which interchanges in a very fundamental way the spatial relationship of the cyclopropane ring to the nonplanar triene unit (*cf.* **2a** and **2b**); (b) the distinctly different alignment of $p\pi$ and cyclopropane orbitals particular to these individual conformations; and (c) the latent potential of **1** for a variety of thermal and photochemical pericyclic changes. The advent of orbital symmetry theory has caused considerable attention to be focused currently on bicyclo-



[6.1.0]nonatriene rearrangements in an attempt to unravel which of the many alternative pathways open to these polyenes is actually operative under a variety of conditions.[2] By contrast, electrophilic additions

(1) Unsaturated Heterocyclic Systems. LXXXVI. For paper LXXXV, see L. A. Paquette, L. B. Anderson, J. F. Hansen, S. A. Lang, Jr., and H. Berk, *J. Amer. Chem. Soc.*, in press.