2 *N* sodium hydroxide solution. The crystalline product which separated was collected, washed with water, dried (280 mg.) and recrystallized from benzene and hexane, m.p. 135–136°. The melting point of a mixture of this hydrogenation product and the starting material was 105–124°.

Anal. Calcd. for $C_{14}H_{16}N_2$: C, 79.21; H, 7.60; N, 13.20. Found: C, 79.42; H, 7.82; N, 13.20.

Fraction C was distilled at 120–140° (0.2 mm.) and weighed 5.6 g. Half of this oily mixture was chromatographed on 120 g. of aluminum oxide (Woelm, basic, activity 3). Elution with a mixture of hexane and benzene (2:3) gave a crystalline compound, which on twice recrystallizing from benzene and pentane melted at 77.5–78.5° and was 1,2-bis-(4-pyridyl)-2-methyl-1-propanone (VII). Its infrared spectrum showed one carbonyl peak at 1685 cm.$^{-1}$.

Anal. Calcd. for $C_{14}H_{14}N_2O$: C, 74.30; H, 6.25; N, 12.37. Found: C, 74.00; H, 6.17; N, 12.51.

Further elution of the aluminum oxide column with benzene and a benzene–ether (9:1) mixture gave 400 g. of an additional crystalline compound. For analysis it was twice recrystallized from ether and pentane and subsequently distilled. It had one carbonyl peak at 1705 cm.$^{-1}$ and was the structural isomer, 3,3-bis-(4-pyridyl)-2-butanone (VIII), m.p. 76–77°.

Anal. Calcd. for $C_{14}H_{14}N_2O$: C, 74.30; H, 6.25. Found: C, 74.03; H, 6.04.

Rearrangement of the Pinacol 2,3-Bis-(2-pyridyl)-2,3-butanediol.—The pinacol was rearranged as described under the general method. Temperature and reaction time were 160° and 9 hours, respectively. In another experiment the rearrangement was carried out at 160° for 2 hours and then the temperature was raised to 230° for 2 hours. The oily mixture, obtained by evaporation of the ethyl acetate extract, turned dark at room temperature; storage at −8° prevented discoloration. In the experiment carried out at 160°, from 5.5 g. of pinacol 2.2 g. of distilled oily product (b.p. 100–140° (0.03 mm.)) was obtained. In the second experiment at 230°, from 33 g. of pinacol only 6.02 g. of distilled yellow product was isolated. Five grams of this yellow, viscous liquid was chromatographed on 200 g. of aluminum oxide (Woelm, basic, activity 3). Hexane–benzene mixtures 4:1 and 3:1 eluted a crystalline compound. This was recrystallized several times from ether and pentane and hexane alone and then melted at 88–90°. It had a carbonyl peak at 1690 cm.$^{-1}$, and was 1,2-bis-(2-pyridyl)-2-methyl-1-propanone (X).

Anal. Calcd. for $C_{14}H_{14}N_2O$: C, 74.30; H, 6.25. Found: C, 74.78; H, 6.19.

A hexane–benzene mixture 1:1 eluted a compound which was distilled at 120–130° (0.03 mm.) and recrystallized twice from benzene and pentane. It melted at 110–112° and had an ultraviolet max. at 249–250 mμ (ε 3,900). No structure has been assigned to this compound.

Anal. Calcd. for $C_{13}H_{10}N_2$: C, 80.38; H, 5.19; N, 14.42. Found: C, 80.62; H, 5.11; N, 14.43.

A benzene–ether mixture 9:1 eluted another crystalline compound from the above column, which was recrystallized twice from ether and pentane and distilled at 110–112° (0.03 mm.). It melted at 66–67°, had an ultraviolet max. at 312–313 mμ (ε 24,690) (ethanol) and the structure 6-(2-pyridyl)-7-methyl-5H-1-pyridine (XII) is proposed for it.

Anal. Calcd. for $C_{14}H_{12}N_2$: C, 80.74; H, 5.81; N, 13.45. Found: C, 80.72; H, 5.71; N, 13.58.

A benzene–ether mixture 1:1 eluted a third compound from the above column which distilled at 120–130° (0.03 mm.) and which was recrystallized from benzene. It formed yellow prisms melting at 86–87.5° and was 2-pyridyl 2-picolyl ketone (XIII).

Anal. Calcd. for $C_{12}H_{10}N_2O$: C, 72.71; H, 5.09; N, 14.12. Found: C, 73.04; H, 4.87; N, 13.82.

This compound was synthesized according to the procedure given by Goldberg, *et al.*,[9] and the two compounds were found to be identical. Their infrared absorption spectra were identical and a mixture of the two samples melted undepressed at 86–88°.

From the rearrangement experiment carried out in sulfuric acid at 160° for 9 hr. a 920-mg. sample of the distilled reaction product was chromatographed as above. Benzene alone eluted 140 mg. of a compound which on recrystallization from a mixture of benzene and hexane melted at 65–66°. The ultraviolet spectrum showed maximal absorption at 231–233 mμ (ε 17,410). The analytical data correspond with structure XI, 2,3-bis-(2-pyridyl)-1,3-butadiene.

Anal. Calcd. for $C_{14}H_{12}N_2$: C, 80.74; H, 5.81. Found: C, 80.43; H, 5.86.

SUMMIT, N. J.

[CONTRIBUTION FROM THE MALLINCKRODT LABORATORIES OF HARVARD UNIVERSITY]

Isotope Effects in the Enzymatic Decarboxylation of Oxalacetic Acid

BY STANLEY SELTZER,[1a] GORDON A. HAMILTON[1b] AND F. H. WESTHEIMER

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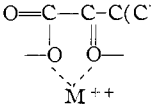
$HO_2C-CO-CH_2-C^{18}O_2H$ has been prepared and decarboxylated in the presence of metal ions and in the presence of the partially purified enzyme from *Micrococcus lysodeikticus*. The reaction catalyzed by Mn^{++} at 10° showed an isotope effect, k^{12}/k^{13}, of nearly 1.06, wheras the ratio for the enzymatic reaction was 1.00. Further, the enzymatic decarboxylation proceeds less rapidly in D_2O than in H_2O, whereas the rate for the metal ion-promoted reaction is unaffected by this change in solvent. These facts show that the carbon–carbon bond scission is the rate-controlling process for the non-enzymatic reaction but not for that promoted by the enzyme. The actual rates make it probable that the carbon–carbon bond cleavage for the enzymatic reaction exceeds that for the non-enzymatic reaction by a factor of the order of 10^8.

In 1941, Krampitz and Werkman[2] isolated from the aerobic bacteria, *Micrococcus lysodeikticus*, a thermolabile enzyme which catalyzes the decarboxylation of oxalacetic acid

$$HO_2C-CO-CH_2-CO_2H \longrightarrow HO_2C-CO-CH_3 + CO_2 \quad (1)$$

A metal ion, preferably Mn^{++} or Mg^{++}, is required[2] as cofactor. Later, Krebs[3] observed that various cations (*e.g.*, Mn^{++}, Cu^{++}, Al^{+++}) catalyze the decarboxylation in the absence of enzyme. Subsequently, the mechanism of the metal ion-catalyzed decarboxylation of dimethyloxalacetic acid was shown[4] to proceed by the steps

$$HO_2C-CO-C(CH_3)_2-CO_2H + M^{++} \rightleftharpoons$$



$$+ 2H^+ \quad (2)$$

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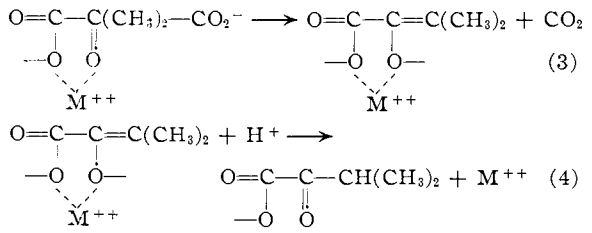
(2) L. O. Krampitz and C. H. Werkman, *Biochem. J.*, **35**, 595 (1941).

(3) H. Krebs, *ibid.*, **36**, 303 (1942).

(4) R. Steinberger and F. H. Westheimer, THIS JOURNAL, **71**, 4158 (1949); **73**, 429 (1951).



Presumably the non-enzymatic decarboxylation of oxalacetic acid proceeds similarly from a chelate of the ketoacid to the chelate of the enol of the product, which in this case is the enol of pyruvic acid. The final step in the process is then the ketonization of this enol. The work of Tchen and Vennesland[5] suggests that the same general mechanism also obtains in an enzymatic carboxylation process.

In the present work, oxalacetic acid was prepared[6] with about 10% C^{13} in the carboxyl group which is lost on decarboxylation. Then the isotope effects in the enzymatic and non-enzymatic decarboxylations were observed. Similarly, the reaction was carried out in D_2O and in H_2O as solvents and the rates measured. The implications of these measurements for the mechanism of the decarboxylation are discussed.

Experimental

Materials. Oxalacetic Acid-1-C^{13}.—A Grignard reagent was prepared from 28.5 g. of methyl iodide in 100 cc. of ether. A mixture of 3.87 g. of barium carbonate (61.7% excess C^{13}; Eastman Kodak Co.) and 10.5 g. of reagent grade sodium carbonate was treated with sulfuric acid, and the carbon dioxide so generated passed into the solution of the Grignard reagent. The contents of the Grignard flask were decomposed with ice, 40 cc. of concentrated sulfuric acid and 50 g. of silver sulfate. The ether was then distilled off, and the acetic acid-1-C^{13} was steam distilled. The distillate was titrated with 1.8 *N* NaOH and the water removed with a Rinco rotary evaporator. The sodium acetate-1-C^{13} was dried *in vacuo* at 110°. The yield was 8.80 g., or 91% of theory.

Redistilled triethyl phosphate (31 cc.; Eastman Kodak Co.) was poured over the dried sodium acetate and the mixture heated.[7] A stream of dry nitrogen helped carry the ethyl acetate-1-C^{13} into a Dry Ice trap. A second distillation provided 8.0 g. of ethyl acetate. The ethyl acetate was condensed with ethyl oxalate by means of sodium ethoxide in ethanol solution according to the procedure of Wislicenus.[8] The yield of crude diester was 9.7 g. Forty cc. of concentrated hydrochloric acid[9] was added to this ester and the solution placed in the refrigerator for 48 hours. The crystals of oxalacetic acid which formed were filtered on a sintered glass funnel and dried over NaOH *in vacuo;* the yield was 1.75 g. The material after recrystallization from acetone–benzene melted at 152.0–152.8°, with decomposition.

Extraction of the Enzyme.—*Micrococcus lysodeikticus* (American Type Culture Collection No. 4698) was grown in submerged cultures.[10] Cells from the original slant were first transferred to 250-cc. erlenmeyer flasks containing 50 cc. of medium and shaken for 24 hr. at 32°. Ten-cc. aliquots of cell suspension were then transferred to 2-l. flasks. Finally carboys, containing 12–14 liters of medium, were inoculated with 1 liter of cell suspension. These carboys were both stirred at 1550 r.p.m. and aerated (1 liter of air per minute) for about 36 hours; Dow-Corning Antifoam A Spray was added to control foaming. The growth medium[10] contained 1% glucose, 0.4% proteose peptone, 0.4% yeast extract, 1% sodium bicarbonate, 1% dibasic potassium phosphate and enough sodium hydroxide to bring the *p*H to 8. The bulk of the medium was tap water.

Sterile techniques were used throughout. Before each inoculation samples of the cell suspensions were withdrawn, the cells stained with a gram-positive stain[11] and examined under 2000 × magnification. If any contaminating bacteria were found in the inoculums, they were discarded. After some experience was gained in the growth of these bacteria, no contamination was found after complete growth. The method of preparation of the enzyme was essentially that of Herbert,[12] but the purification was discontinued after the first ammonium sulfate precipitation. The enzyme preparation so obtained had been purified about 500-fold over the original cell paste, but was still only about a twelfth as active as Herbert's most highly purified preparation. The specific activity of the material used was 72 μmoles of CO_2/min.-mg. of protein. Some preliminary experiments, with a much less highly purified preparation (2 μmoles CO_2/min.-mg. protein), gave much the same results as those here recorded.

Glutarate Buffer.—Glutarate buffer was prepared from recrystallized glutaric acid, m.p. 97.1–97.9°, neutralized with carbonate-free sodium hydroxide to obtain the stated *p*H.

Determination of the Isotope Effect.—The decarboxylations were carried out at 10.0° in the apparatus shown in Fig. 1. Prepurified nitrogen was scrubbed by the Ascarite in A, by 1 *M* sodium hydroxide solution in B and by water

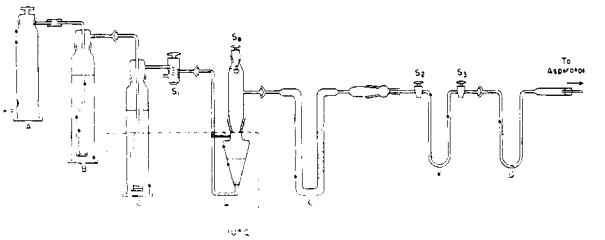


Fig. 1.—Apparatus for determining the isotope effect in decarboxylation.

in C. The gas then was bubbled through the reaction mixture in D and passed through the three traps, E, F and G, cooled by Dry Ice–acetone, liquid nitrogen and liquid nitrogen, respectively. The last trap was connected to an aspirator through an Ascarite tube. The system was swept with nitrogen for 90 minutes before beginning an experiment. Then the stopcocks S_1 and S_3 were closed, and the reaction initiated by dropping enzyme and substrate into a solution of 0.1 *M* buffer plus 0.001 *M* Mn^{++}. The two solids were held in a small glass bucket (with a platinum wire handle) hung on the stopper S_4, and so arranged that turning the stopper allowed the bucket to fall into the reaction mixture. At the completion of the reaction, the stopcocks S_1 and S_3 were opened, and nitrogen passed through the solution at a very rapid rate. Then stopcocks S_2 and S_3 were closed. The carbon dioxide, frozen in trap F, was purified by a series of bulb-to-bulb sublimations and finally transferred to sample tube for mass-spectrometric analysis.

The instrument used for the analysis was a Consolidated-Nier Isotope-Ratio mass spectrometer, model No. 21-201. Each gas sample was introduced into the mass spectrometer about three times, and usually five measurements were made on each entry; all the readings were then averaged. The successive samples showed the same isotopic ratio and showed that memory effects were not large. In general, the accelerating voltage was 1175 volts, and the counter setting on the magnetic field was around 4000. The ratios obtained experimentally were always compared with that obtained on the same day from measurements on a standard sample of CO_2 from a commercial cylinder. At one time during this work, the mass spectrometer was dismantled for repairs; when it was returned to working condition, the

(5) T. T. Tchen and B. Vennesland, *J. Biol. Chem.*, **213**, 533 (1955).

(6) In principle, the natural abundance of C^{13} (about 1%) should be sufficient label. Unfortunately, experimental difficulties were such that clean results could be obtained only with heavier labeling.

(7) G. A. Ropp, THIS JOURNAL, **72**, 2299 (1950).

(8) W. Wislicenus, *Ann.*, **246**, 306 (1888).

(9) L. J. Simon, *Compt. rend.*, **137**, 855 (1903).

(10) R. F. Beers, Jr., *Science*, **122**, 1016 (1955).

(11) K. Thimann, "The Life of Bacteria," The Macmillan Co., New York, N. Y., 1955, p. 42.

(12) D. Herbert, in "Methods in Enzymology," Vol. I (Colowick and Kaplan, eds.), Academic Press, Inc., New York, N. Y., 1955, p. 753.

ratio for the cylinder CO_2 had been appreciably altered, and even the relative ratio for the decarboxylation mixture had shifted. The results here recorded are only those of a single, self-consistent set of observations, obtained after the machine had been repaired.

The isotopic fraction factor k^{12}/k^{13} for the enzymatic process was almost precisely unity in the earlier set of measurements (as with those in Table V and with those obtained with the less highly purified enzyme preparation). However, the value of this ratio found for Mn^{++} catalysis was decidedly lower than those shown in Table V, and was closer to a 3% than to a 6% fractionation. Although the results here reported, which were obtained after the mass spectrometer had been reconditioned, are considered more reliable, the qualitative conclusions are unaffected by the differences between the two sets of measurements.

Rate Measurements.—The rate measurements were carried out in Warburg flasks by the usual technique. Each enzymatic decarboxylation was controlled by a corresponding reaction mixture with Mn^{++} but without enzyme.

Results

Michaelis Constants.—The Michaelis constants K_M for the enzymatic decarboxylation of oxalacetic acid and the equilibrium constants K_E for the enzyme–Mn^{++} complex are presented in Table I.

TABLE I

MICHAELIS AND EQUILIBRIUM CONSTANTS

Buffer	Temp., °C.	K_M Substrate, M	K_E Manganous ion, M
0.1 M acetate[12]	30.0	2×10^{-3}	1.9×10^{-4}
.1 M acetate	30.2	4.5×10^{-3}	
.1 M glutarate	30.2	1.9×10^{-2}	2.0×10^{-4}
.15 M glutarate	30.2	4.2×10^{-2}	
.10 M glutarate	10.0	6.6×10^{-3}	3.8×10^{-4}
.10 M glutarate	10.0	5.3×10^{-3}	

The Michaelis constant determined in this work differs by a factor of two from that presented (for the more highly purified enzyme) by Herbert.[12] The data show that glutaric acid acts as an inhibitor for the reaction; the Michaelis constant is greater for glutarate than for acetate, and the constant increases with increasing glutarate concentration. However, even traces of acetic acid were extremely deleterious to the mass-spectrometric measurements, since one of the fragments from acetic acid has mass 45 and therefore coincides, in the instrument, with $C^{13}O_2$. A non-volatile buffer was therefore essential to the success of the measurements of isotopic ratios.

The equilibrium constant determined for the reaction of Mn^{++} with the enzyme in the presence of glutarate agrees with that found by Herbert in acetate buffers.

Inhibition by CO_2.—The rate of the enzymatic and of the Mn^{++}-catalyzed decarboxylations were measured with an atmosphere of carbon dioxide in the Warburg apparatus; the results are contrasted with those obtained when air was above the solutions. The results are shown in Table II.

The results show that added CO_2 does not appreciably inhibit the metal ion-promoted reaction. The inhibition of the enzymatic reaction is relatively small—only about 40% with one atmosphere of CO_2—but nevertheless, the effect is real. The added CO_2 did not measurably affect the pH of the buffered system.

Rates in D_2O.—The substrate and enzyme solutions were made up in buffered solutions in distilled water, or in 99.75% D_2O, on the same day on which the kinetics were measured. The enzyme for the experiments reported in Table IIIA was a lyophillized preparation which had been kept for about a year at 4°; its activity was substantially the same after this period as when it was freshly prepared, and the results reported for the isotope effect are in qualitative agreement with some preliminary measurements made when the enzyme was first prepared.

TABLE II

RATE OF DECARBOXYLATION OF OXALACETIC ACID IN THE PRESENCE OF CO_2

Substrate, M	Gas phase	Initial rates (arbitrary units) Enzymatic[a]	Mn^{++}
1.91×10^{-2}	Air	10	5.1
1.91×10^{-2}	CO_2	7.2	4.9
3.92×10^{-2}	Air	14	6.6
3.93×10^{-2}	CO_2	8.0	6.8
3.92×10^{-2}[b]	Air	15	6.3
3.92×10^{-2}[b]	CO_2	9.0	5.5

[a] Enzymatic rates are obtained by subtracting the rate for the Mn^{++}-catalyzed reaction from the gross enzymatic rate. The concentration of Mn^{++} was 0.001 M, and the solution was kept at a pH of 5.49 with a 0.1 M acetate buffer. [b] Carried out in the presence of 7.7×10^{-3} M sodium pyruvate.

The kinetic experiments were performed in a manner similar to that for the other determinations of rate. A control experiment, without enzyme, was always conducted in parallel with the enzymatic one, so that the non-enzymatic, Mn^{++}-catalyzed rate could be estimated, and this non-enzymatic rate deducted from that caused by the enzyme. These corrections were sizable, and amounted to 20–50% of the total. The procedure for an experiment was to equilibrate the solutions in the thermostat for 15–20 minutes, add the substrate to the enzyme solution and allow the reaction to proceed for about five minutes before the manometer of the Warburg apparatus was closed. Readings were then taken every two minutes for the next 15 minutes. An amount of enzyme needed to give maximum manometer readings was used; thus the quantity of enzyme was about 50% greater for D_2O than for H_2O. The rates decreased 10–20% during the experiments, but this effect was noted in both solvents. The enzyme is largely but not completely saturated; however, higher substrate concentrations, with more nearly complete saturation, did not change the results appreciably. The buffer solutions were sufficiently concentrated that the pH (or "pD") usually did not change, during an experiment, by more than 0.1 unit. The data, which are reproducible to ± 10% or better, are shown in Tables IIIA and IIIB and Fig. 2.

The ionization constants[13] of acids in D_2O are less than those in H_2O by a factor of about 3. The pH values were measured with a Beckman model G pH meter, using glass and calomel electrodes. When D_2O was used as solvent, the pD was calculated by adding 0.4 unit to the observed meter reading.[14] This empirical correction takes care of the difference in ionization constants[13] for an acid in D_2O as compared to that in H_2O.

(13) C. K. Rule and V. K. LaMer, THIS JOURNAL, **60**, 1974 (1938).

(14) R. Lumry, E. L. Smith and R. R. Glantz, *ibid.*, **73**, 4330 (1951).

TABLE IIIA

RELATIVE RATES OF THE ENZYMATIC DECARBOXYLATION OF OXALACETIC ACID IN H_2O AND D_2O AT VARIOUS pH'S

Solvent	Buffer	Substrate concn., M	pH or pD	k_{H_2O}/k_{D_2O}	Relative rate
D_2O	0.1 M glutarate	0.0213	5.36	1.11	1.00
H_2O	.1 M glutarate	.0213	4.92		1.11
D_2O	.1 M glutarate	.0213	5.74	1.27	1.76
H_2O	.1 M glutarate	.0213	5.23		2.22
D_2O	.1 M glutarate	.0213	5.87	1.21	1.98
H_2O	.1 M glutarate	.0213	5.35		2.39
D_2O	.1 M glutarate	.0213	6.08	1.54	2.01
H_2O	.1 M glutarate	.0213	5.64		3.10
D_2O	.2 M acetate	.0213	5.97	1.41	1.96
H_2O	.2 M acetate	.0213	5.47		2.76
D_2O	.1 M succinate	.0213	6.00	1.24	1.67
H_2O	.1 M succinate	.0213	5.50		2.07
D_2O	.1 M phosphate	.0213	6.85	2.13	1.20
H_2O	.1 M phosphate	.0213	6.35		2.55
D_2O	.1 M phosphate	.0213	7.19	1.78	1.20
H_2O	.1 M phosphate	.0213	6.72		2.13
D_2O	.1 M lutidine	.0213	7.04	1.66	0.99
H_2O	.1 M lutidine	.0213	6.57		1.65
D_2O	.2 M acetate	.0426	6.08	1.43	2.10
H_2O	.2 M acetate	.0426	5.61		3.00
D_2O	.1 M glutarate	.0426	5.92	1.39	2.22
H_2O	.1 M glutarate	.0426	5.45		3.10
D_2O	.1 M glutarate	.0426	6.21	1.70	2.31
H_2O	.1 M glutarate	.0426	5.75		3.94
D_2O	1 M glutarate	.0213	5.65		1.48
D_2O	.1 M glutarate	.0213	6.10		1.92
H_2O	.1 M glutarate	.0213	5.24		2.31
H_2O	.1 M glutarate	.0213	5.75		3.11

TABLE IIIB

RELATIVE RATES OF THE Mn^{++}-CATALYZED DECARBOXYLATION OF OXALACETIC ACID IN H_2O AND D_2O

Solvent	Catalyst	pH or pD	k_{D_2O}/k_{H_2O}	Relative rate
D_2O	Mn^{++}	5.37	1.02	1.00
H_2O	Mn^{++}	4.91		1.02
D_2O	Mn^{++}	5.85	0.97	0.92
H_2O	Mn^{++}	5.36		0.88

Nevertheless, without a more detailed knowledge of the function of the acidic and basic groups in the enzyme, the magnitude of the D_2O effect is hard to assess. Should the reactions be compared (as in Table IIIA) at the same buffer ratio or should they be compared (as in Fig. 2) at the ratios where pH equals pD? Until the mechanism is thoroughly understood, the safest comparison is probably that between the rates on the two plateaus, at the maximum rates. Here the rate in H_2O exceeds that in D_2O by a factor of about 1.6. This factor, although not very large, is certainly real. Further investigations in more alkaline regions, with phosphate and lutidine buffers were complicated by extraneous effects (*e.g.*, precipitation of manganous salts) but did not suggest different results. Succinate buffers inhibited the reaction, but the ratio of rates in H_2O and D_2O was about the same as that for glutarate buffers.

The C^{13} Isotope Effect.—The data for the isotope effect with labeled oxalacetic acid are shown in Table IV.

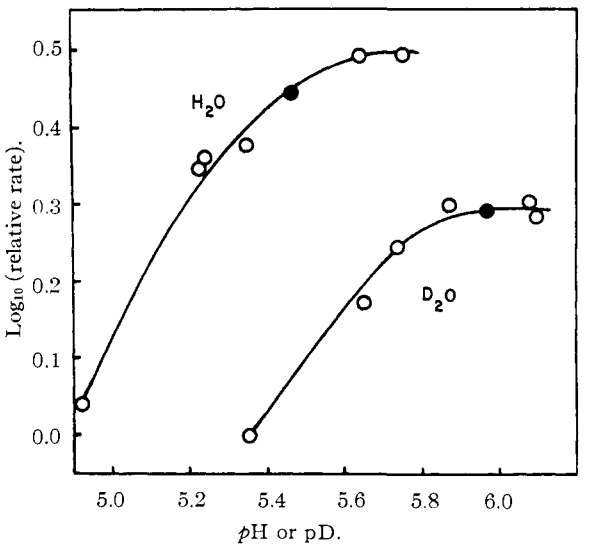


Fig. 2.—Rates of decarboxylation of oxalacetic acid in H_2O and D_2O. Open circles for glutarate buffer; filled circles for acetate buffer.

The isotopic ratio observed for the reaction in the presence of manganous ion or cupric ion is only a first approximation to the k^{12}/k^{13} ratio. For example, in the ninth line of Table IV, the observed ratio of A/B at 12% reaction is 9.874. Since the ratio at complete decarboxylation averages 10.447, the isotopic ratio of the carbon dioxide has been enriched in the light isotope by the factor 1.055.

TABLE IV

C^{13} ISOTOPE EFFECT IN THE DECARBOXYLATION OF OXALACETIC ACID

% Reaction	Catalyst	Obsd. ratio (A)	Standard (B) or (B^*)[a]	A/B	CO_2 from Mn^{++}, %	Cor. A/B
100	Enzyme	0.11870	0.011362	10.447		
100	Cu^{++}	.11946	.011438	10.444		
100	Cu^{++}	.11955	.011438	10.452		
23.8	Enzyme	.11803	.011362	10.388	9.72	10.445
24.0	Enzyme	.11877	.011425	10.396	8.05	10.444
24.6	Enzyme	.11847	.011438	10.358	8.49	10.405
14.1	Mn^{++}	.11328	.011411*	9.938		
15.6	Mn^{++}	.11339	.011414*	9.945		
12.1	Mn^{++}	.11303	.011459*	9.874		
21.8	Cu^{++}	.11352	.011448*	9.927		
22.0	Cu^{++}	.11355	.011447*	9.930		
14.2	Cu^{++}	.11337	.011445*	9.916		

[a] B* refers to a second standard tank of CO_2, enriched 0.10% compared to B.

However, this is an integrated value, over the first 12% reaction, and the ratio of the rate constants for breaking a C^{12}–C^{12} bond, as compared to that for breaking a C^{12}–C^{13} bond, must be extrapolated to zero per cent, reaction. The correct ratio can be obtained by applying equation 5.[15]

$$N/N_0 = \frac{1 - (1 - f)^{k^{13}/k^{12}}}{f} \qquad (5)$$

where N is the observed isotopic ratio at partial reaction, and N_0 is the isotopic ratio at complete reaction. The parameter "f" is the ratio of the amount of the C^{12} compound present, at the time of sampling, to that initially present. In the work here reported, f was approximated by the fraction of reaction. For the data here shown, (where f

(15) J. Bigeleisen, *Science*, **110**, 14 (1949).

is about 0.15) this approximation causes an error of only 0.6% in f, and a completely negligible error in the isotopic fractionation factor. From the data in Table IV and equation 5, the isotopic rate ratios of Table V were determined.

The results of the experiments catalyzed by enzyme are complicated by the fact that Mn^{++} is needed for enzymatic activity, and an appreciable part of the CO_2 evolved arises from Mn^{++} catalysis. Furthermore (see Table V) the reaction promoted by Mn^{++} shows an isotope effect. Therefore the data have been corrected as follows: The per cent of CO_2 from the reaction with Mn^{++} was calculated from the time and rate constant, and then the isotopic composition of this CO_2 was calculated from the known value of k^{12}/k^{13}, with the aid of equation 5. The observed value of A/B is the weighted mean of the isotopic composition of the CO_2 from the Mn^{++} catalysis and that from the enzymatic reaction; the latter can be calculated and is given as "Corrected A/B" in the last column of Table IV.

Errors.—The errors which may be introduced into this type of calculation have been estimated by Bigeleisen and Allen.[16] Assuming that the error in the isotopic ratio observed is 0.1%, the error in the final computed ratio, k^{12}/k^{13}, is of approximately the same magnitude. The calculation is much less sensitive to errors in the fraction of reaction. Even if the extent of reaction were in error by 5%, the error in the final ratio would only be 0.006.

One source of error which proved especially important was leakage of atmospheric carbon dioxide into the system. The significance of these errors was greatly magnified by the decision to use oxaloacetic acid enriched in C^{13}, rather than material with C^{13} at the level of natural abundance. In very early experiments, some of the connections were rubber, and erratic data were obtained, probably due to leakage of atmospheric CO_2 into the system. After an all-glass apparatus was constructed, almost all runs were consistent, but an occasional experiment led to a result too low by more than twice the normal fractionation; these few experiments were also marked by visible striations in the stopcock. Such runs were discarded; the consistency of the data in Table IV suggests that errors from this source were not large. It has already been noted that an overhauling of the mass-spectrometer changed the absolute values of the fractionation factor; this fact suggests that the numerical value of the fractionation factor here reported may not be precise. A somewhat smaller value would be more nearly consistent with the results of the earlier measurements and with the fractionation factors found in other decarboxylation reactions. However, the qualitative conclusion was the same in all sets of measurements, *i.e.*, the non-enzymatic decarboxylation showed a sizable isotopic fractionation, whereas the enzymatic decarboxylation showed none.

Discussion

The principal data obtained in this research are summarized in Table V.

(16) J. Bigeleisen and T. L. Allen, *J. Chem. Phys.*, **19**, 760 (1951).

TABLE V

ISOTOPIC FRACTIONATION FACTOR

Catalyst	k^{12}/k^{13}	k^{H_2O}/k^{D_2O}	k^{air}/k^{CO_2}
Mn^{++}	1.060	1.0	1.0
Cu^{++}	1.056		
Enzyme	1.002	1.6	0.6

The metal ion-promoted reaction, in common with other such decarboxylations which have previously been observed,[17] shows a sizeable isotope effect; the enzymatic reaction, by contrast, shows none. On the other hand, the metal ion-promoted reaction is unaffected by changing the solvent from H_2O to D_2O, whereas the rate of the enzymatic reaction is somewhat reduced in the deuterated solvent. And the rate of the enzymatic reaction, in contrast to that of the metal ion-promoted reaction, is somewhat inhibited by CO_2.

These data certainly indicate that the rate-controlling step for the enzymatic reaction is not the cleavage of the carbon–carbon bond. Any conclusion about the mechanistic significance of the deuterium isotope effect must be accepted with considerable caution. The effect is real, but rather small, and not much larger than the maximum effects of D_2O found by Robertson and his collaborators[18] for simple displacement reactions where only solvation effects are of importance. Further, especial caution is required in the interpretation of the effect of D_2O in enzymatic reactions, since the change from hydrogen bonding to deuterium bonding might significantly alter the conformation of the coiled protein. However, the change from H_2O to D_2O had essentially no effect upon the non-enzymatic, manganous ion catalyzed reaction. At the very least, the deuterium oxide experiments are consistent with the conclusion that, in the enzymatic process the cleavage of the carbon to carbon bond is not the rate-controlling step of the decarboxylation. The data are consistent with the hypothesis that a proton (or deuteron) is transferred as part of the rate-controlling step, but they by no means may be construed as conclusive (or even as strong) evidence that such is the fact.

The results can then be correlated by several kinetic schemes, of which the simplest is shown below.

SCHEME A

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_1 + CO_2$$

$$ES_1 \xrightarrow{k_3} E + P$$

Here E represents the free enzyme, S represents oxalacetic acid, P represents pyruvic acid, and ES and ES_1 two different enzyme–substrate complexes.

(17) E. Gelles and R. I. Reed, *Nature*, **176**, 1262 (1955); A. A. Bothner-By and J. Bigeleisen, *J. Chem. Phys.*, **19**, 755 (1951); J. Bigeleisen and L. Friedman, *ibid.*, **17**, 998 (1949); J. G. Lindsay, A. N. Bourns and H. G. Thode, *Can. J. Chem.*, **29**, 192 (1951); E. Grovenstein, Jr., and G. A. Ropp, THIS JOURNAL, **78**, 2560 (1956).

(18) P. M. Laughton and R. E. Robertson, *Can. J. Chem.*, **34**, 1714 (1956); R. E. Robertson and P. M. Laughton, *ibid.*, **35**, 1319 (1957).

The application of steady-state kinetics to this system leads to equation 6

$$v = \frac{k_1k_2k_3(E_0)(S)}{[k_1k_2 + k_1k_{-2}(CO_2) + k_1k_3](S) + k_{-1}k_{-2}(CO_2) + k_{-1}k_3 + k_2k_3} \quad (6)$$

At high substrate concentration, this expression reduces to (7)

$$v = \frac{k_2k_3(E_0)}{k_2 + k_{-2}(CO_2) + k_3} \quad (7)$$

If the pressure of CO_2 is low, so that $k_{-2}(CO_2)$ is small compared to k_2 or k_3, then at high substrate concentration the relative rates in D_2O and in H_2O are given by (8)

$$\frac{v^{H_2O}}{v^{D_2O}} = \frac{k_3^{H_2O}}{k_3^{D_2O}}\left[\frac{k_2 + k_3^{D_2O}}{k_2 + k_3^{H_2O}}\right] \quad (8)$$

This expression can differ from unity only if k_2 is comparable to or larger than k_3. If k_2 is much larger than k_3, then the rate in water will exceed that in D_2O by the ratio $k_3^{H_2O}/k_3^{D_2O}$.

The experiments with isotopic carbon were competitive; that is to say, the rates were not measured individually for the acids, but rather the change in isotopic composition on partial decarboxylation of a mixture of isotopic acids was observed. Therefore the rate equations 6, 7 and 8 could not be employed, but rather a new steady-state derivation was needed, where the system examined contained all the steps

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES_1 + CO_2$$

$$E + S^* \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES^* \underset{k_{-2}^*}{\overset{k_2^*}{\rightleftarrows}} ES_1 + C^*O_2$$

$$ES_1 \xrightarrow{k_3} E + P$$

Here the compounds substituted with C^{13} are marked with an asterisk; the rate constants which are affected by the isotopic substitution are similarly marked. Solution of this system leads to the kinetic equation 9

$$\frac{d(CO_2)}{d(C^*O_2)} = \frac{k_1k_2[k_{-1}k_3 + k_2^*k_3](S) + k_1k_{-1}k_{-2}^*(C^*O_2)(S) - k_1k_{-1}k_2^*k_{-2}(CO_2)(S^*)}{k_1k_2^*[k_{-1}k_3 + k_2k_3](S^*) + k_1k_{-1}k_2^*k_{-2}(CO_2)(S^*) - k_1k_{-1}k_2k_{-2}^*(C^*O_2)(S)} \quad (9)$$

In the present experiments, it has been observed that $(C^*O_2)/(CO_2) = (S^*)/(S)$. At sufficiently low pressure of carbon dioxide, the last two terms in equation 9 are negligible. Further, since these terms are almost equal, the difference between them at low pressures of carbon dioxide can be neglected. Therefore, under these conditions, (9) reduces to (10)

$$\frac{d(CO_2)}{d(C^*O_2)} = \frac{k_2(k_{-1} + k_2^*)(S)}{k_2^*(k_{-1} + k_2)(S^*)} \quad (10)$$

This expression indicates that there will be no isotopic fractionation if and only if k_2 and k_2^* are very large compared to k_{-1}. That is to say, the "desorption" of the acid from the enzyme must be slow relative to decarboxylation. Qualitatively, this means that the acids are adsorbed at random, without regard for isotopic substitution, and that the decarboxylation is very rapid. Of course, the "adsorption" process may represent a chemical reaction rather than a simple physical process.

The mechanism here outlined is not the only one which is possible, and two others have been examined. These are summarized in the equations.

SCHEME B

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} CO_2\text{-}ES_1 \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} ES_1 + CO_2$$

$$ES_1 \xrightarrow{k_4} E + P$$

SCHEME C

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} CO_2\text{-}ES_1$$

$$CO_2^* + CO_2\text{-}ES_1 \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} CO_2 + CO_2^*\text{-}ES_1$$

$$CO_2\text{-}ES_1 \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} ES_1 + CO_2$$

$$ES_1 \xrightarrow{k_5} E + P$$

In these equations, CO_2–ES_1 represents the enzyme-substrate complex with CO_2 bound to an enzymatic site. Both B and C are capable of representing the data, provided that desorption is less rapid than decarboxylation.

These mechanistic schemes postulate that the decarboxylation step is reversible. However, since desorption is slow, they are consistent with the observed[19] facts that labeled CO_2 is introduced into oxalacetate during its decarboxylation, but the rate of this reverse reaction is small. The schemes are also consistent with the small diminution of rate observed when the decarboxylation is carried out in the presence of an atmosphere of CO_2.

The data do not, of course, uniquely determine a mechanistic pathway for the enzymatic reaction; in fact (see the paragraph below) they merely serve to point up the major questions involved. However, the absence of a C^{13} isotope effect shows that the cleavage of the carbon-to-carbon bond is not involved in the rate-controlling step. The diminution of rate in D_2O as compared to H_2O is consistent with the hypothesis that a proton transfer is involved in the rate-controlling step; this conclusion, however, must be accepted with the strong reservations discussed above, since the influence of D_2O on the enzyme itself is unknown. If, however, the hypothesis is tentatively accepted that a proton is transferred in the rate-controlling step, then the general mechanism for the enzymatic reaction may parallel that for the non-enzymatic process, presented in equations 2, 3 and 4. When the reaction is catalyzed by metal ions, reaction 3, the actual decarboxylation, is rate controlling; when the reaction is catalyzed by the enzyme from *Micrococcus lysodeikticus*, the slow step is reaction 4,

(19) L. O. Krampitz, H. G. Wood and C. H. Werkman, *J. Biol. Chem.*, **147**, 243 (1943); M. F. Utter and H. G. Wood, *ibid.*, **164**, 455 (1946).

the conversion of the enol of pyruvate to the keto acid.[20]

The rate of the enzymatic decarboxylation greatly exceeds that for the non-enzymatic process. The rate for the reaction of the enzyme–substrate complex is probably about 50,000 times as great as that observed by Graham[21] for the ferric ion–dimethyloxalacetic acid complex and exceeds the rate for the cupric ion-catalyzed decarboxylation of oxalacetic acid by a comparable factor. But the ion which activates the enzyme is Mn^{++}, and in the non-enzymatic process this ion is only $^1/_{250}$ as effective as Cu^{++}. Further, the decarboxylation is not the rate-controlling step in the enzymatic reaction and probably is faster than the rate-controlling step by a factor of at least 10. This suggests that the enzyme may promote the decarboxylation by a factor of the order of 10^8.

(20) No account has here been taken of a possible role for biotin in this decarboxylation process; see H. C. Lichstein, *Federation Proc.*, 16, 211 (1957). G. A. Hamilton and F. H. Westheimer, THIS JOURNAL, in press.

(21) R. Graham, Thesis, University of Chicago, 1953.

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CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE CENTRAL RESEARCH LABORATORIES, ARMOUR AND COMPANY]

Isolation of Proteolytic Enzymes from Solution as Dry Stable Derivatives of Cellulosic Ion Exchangers

BY M. A. MITZ AND R. J. SCHLUETER

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A method of isolating proteins from solution is afforded by the use of cellulosic ion exchangers. The basis of this method is a stable derivative obtained by drying the combination of protein and cellulosic ion exchanger. In this report a general method of preparation which yields an active product with a very small amount of extraneous ions is described. To demonstrate the fundamental process, six proteins and four cellulose ion exchangers are used. The acidic proteins pepsin and acylase, as well as the neutral kidney and spleen tissue proteases, combine with the anion exchanger diethylaminoethylcellulose, whereas the basic proteins, chymotrypsin and trypsin, combine with acidic forms of modified cellulose such as carboxymethylcellulose, cellulose phosphate and cellulose citrate. These cellulosic ion exchangers combined with from 1 to 50% protein by weight retain the physical characteristics of the cellulose and can be freeze dried or air dried. The protein in the dried exchanger derivatives can be solubilized under suitable acidic or basic conditions. Moreover, the desorbed enzyme compares favorably in activity with the starting enzyme.

Introduction

Since 1954, when cellulosic ion exchangers were first applied to protein chromatography,[1] many proteins have been fractionated by this type of exchanger.[2] These same exchangers should be useful in the storage, preservation and utilization of proteins, provided a protein sorbed from solution can be dried without loss in biological activity. In this report, the process of isolating proteins from solution as derivatives of cellulosic ion exchangers is described.

In most studies of protein chromatography on modified celluloses, the proteins have been sorbed from dilute buffers of low ionic strengths. Preliminary studies here have indicated that complete elimination of the buffer increases the amount of protein sorbed; the less buffer and salt in the protein solution, the more sites available for protein sorption. It is possible to form protein–exchanger combinations free of inorganic ions which might be a source of trouble on drying as well as a source of contamination. In this study, therefore, the concentration of inorganic salts has been kept as low as possible.

For several reasons, proteolytic enzymes were chosen as representative proteins for this study. They are readily available in reasonably pure form, are well characterized and can be assayed both by enzyme activity and by ultraviolet absorption. There are also wide variations in the isoelectric points and *p*H requirements for stability among this group of enzymes.

Which cellulosic ion exchanger to use in isolating a specific enzyme depends in part on the acidity or basicity of the enzyme. Most enzymes, except very basic ones like lysozyme, will react with the weakly basic cellulosic anion exchangers,[3] and basic enzymes will also react with cellulosic cation exchangers. For enzymes sorbed by either type, the choice can be based on facilitating characteristics of the enzyme or the product.

The preparations of the following combinations of enzyme and cellulosic ion exchanger were studied; **pepsin** with DEAE-cellulose[4] hydrochloride; **acylase** with DEAE-cellulose; **bovine kidney and spleen proteases** with DEAE-cellulose; **trypsin and chymotrypsin** with CM-cellulose, cellulose citrate and cellulose phosphate.

Experimental

Cellulosic Ion Exchangers.—DEAE-cellulose was prepared by the method of Peterson and Sober[5] from 2-chlorotriethylamine hydrochloride and α-cellulose. It had an exchange capacity of 0.8 meq./g.

Five cellulosic cation exchangers were used: cellulose phosphate (1.15 meq./g.) was prepared by method No. 11 of Jurgens, *et al.*[6]; cellulose citrate (2.01 meq./g.) was a

(1) H. A. Sober and E. A. Peterson, THIS JOURNAL, **76**, 1711 (1954).

(2) H. A. Sober and E. A. Peterson, "Ion Exchangers in Organic and Biochemistry," Interscience Publishing Co., Inc., New York, N. Y., 1957, Chap. 16, p. 327.

(3) M. A. Mitz and S. S. Yanari, THIS JOURNAL, **78**, 2649 (1956).

(4) The abbreviations used are DEAE-cellulose for diethylaminoethylcellulose and CM-cellulose for carboxymethylcellulose.

(5) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(6) J. F. Jurgens, J. D. Reid and J. D. Guthrie, *Textile Research J.*, **18**, 42 (1948).