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Studies on the Mechanism of Enzymatic Decarboxylation[1a]

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A number of bacterial decarboxylases (aspartic acid, glutamic acid, tyrosine, malonic acid) were investigated in an attempt to determine the role of the carboxyl group in decarboxylation. Decarboxylations were carried out in the presence of H_2O^{18} and the evolved carbon dioxide was examined for oxygen-18 enrichment. There was very little enrichment and this was attributable to exchange reactions occurring even in the absence of decarboxylation. The level of enrichment was in any case too low to support any mechanism in which the water oxygen is stoichiometrically involved. The carbon dioxide evolved appears to be derived exclusively from the substrate with no labilization of the oxygen atoms. Similar results were found in the non-enzymatic metal catalyzed decarboxylation of oxalacetic acid. The results are compatible with the mechanism of Metzler, Ikawa and Snell[40] which proposed a non-hydrolytic cleavage of carbon–carbon bond with the release of the carboxyl group intact. The linkage of the carboxyl group to the enzyme surface may be electrostatic or possibly in the form of an ester, but an acyl intermediate is ruled out.

The mechanism of enzymatic amino acid decarboxylation has been the subject of a number of studies.[2–5] It appears to be well established that pyridoxal phosphate is an obligatory coenzyme for most, if not all, amino acid decarboxylases and that this coenzyme is tightly bound to the enzyme surface.[6–9] The decarboxylases are stereospecific[10] and one of the enzyme–substrate points of attachment is accounted for by the formation of a Schiff base between the α-amino group of the substrate and the aldehyde group of the bound coenzyme, as proposed originally by Werle and coworkers.[2a] More recent studies by Mandeles, Koppleman and Hanke,[3,5] showing that only one atom of deuterium from D_2O becomes incorporated into the product amine, led to a revision (proposed with F. Westheimer) of the Werle scheme with respect to the nature of the intermediate forms but the involvement of a Schiff base intermediate was confirmed.

In none of the studies to date has the possible involvement of the carboxyl group in enzyme–substrate complex formation been specifically investigated. Substrate specificity studies indicate that the carboxyl group must be free for scission of the C_1–C_2 bond and there is thus, presumably at least, an electrostatic binding of the carboxyl group to the enzyme. The present studies were undertaken in an attempt to further define the mechanism of decarboxylation by determining to what extent the oxygen atoms of the carboxyl group are labilized during the reaction and to what extent oxygen atoms of water in the medium participate in the CO_2 formation. Four bacterial amino acid decarboxylases and the malonic acid decarboxylase of *Pseudomonas fluorescens* were investigated. In addition, the metal catalyzed decarboxylation of oxalacetic acid was examined as a model of non-enzymatic decarboxylation.

Materials and Methods

Enzyme Preparation and Enzyme Assays.—*Clostridium welchii S.R. 12.* ATCC #8009 was grown and lyophilized as described by Meister and co-workers.[11] Reference is always to the dried cells of *Clostridium welchii* unless otherwise specified.[12] *Escherichia coli* ATCC #11246 was grown, harvested and an acetone powder prepared as described by Najjar and Fisher.[13]

Streptococcus faecalis R. cultured by the method of Gale[14] was used as an acetone dried powder.[12]

Pseudomonas fluorescens 23 was prepared as described by Hayaishi and Stanier[15] and purified as described by Hayaishi and Fraser.[16]

Enzyme activities were determined manometrically in the Warburg apparatus at 37° according to standard procedures.[17]

Partial Purification of Glutamic Acid Decarboxylase from *Clostridium welchii*.—Dried cells were ground with three aliquots of Alumina (Alcoa A-301) in a mortar. The material was centrifuged 20 minutes at 18,000 × *g* in a Servall angle-head centrifuge. The supernatant was brought to 80% saturation by the addition of solid ammonium sulfate. The sediment was separated and redissolved in water. This was divided into three crude fractions by ammonium sulfate precipitation—a fraction precipitated at 40% saturation (f_1), a fraction precipitated between 40 and 55% saturation (f_2), and the fraction soluble at 55% saturation (S_2). All fractions were dialyzed 18 hours against distilled water. The fractionations and dialyses were carried out at about 4°.

CO_2 Collection Technique.—A major technical problem was the elimination or minimizing of non-enzymatic exchange between CO_2 and H_2O^{18} during and subsequent to decarboxylation. A number of experimental arrangements were tried and discarded because of the considerable and variable non-enzymatic exchange observed. The extent of such exchange in closed vessels of various sizes and shapes

(1) (a) Based on a thesis submitted to the Graduate faculty of Georgetown University by Simon Rothberg in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Presented in part before the Division of Biological Chemistry, A.C.S., Atlantic City, Sept. 1956. (b) Dermatology Service, General Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md.

(2) (a) E. Werle and K. Heitzer, *Biochem. Z.*, **299**, 420 (1938); (b) E. Werle and W. Koch, *ibid.*, **319**, 305 (1949).

(3) R. Koppleman, S. Mandeles and M. E. Hanke, *Federation Proc.*, **11**, 242 (1952).

(4) H. Wieland and A. Bergel, *Ann.*, **439**, 196 (1924).

(5) S. Mandeles, R. Koppleman and M. E. Hanke, *J. Biol. Chem.*, **209**, 327 (1954).

(6) H. M. R. Epps, *Biochem. J.*, **38**, 242 (1944).

(7) B. M. Guirard and E. E. Snell, THIS JOURNAL, **76**, 4745 (1954).

(8) P. Holtz, A. Engelhardt and G. Thielecke, *Naturwiss.*, **39**, 256 (1952).

(9) S. Udenfriend, C. T. Clark and E. Titus, THIS JOURNAL, **75**, 501 (1953).

(10) E. F. Gale, *Biochem. J.*, **39**, 46 (1945).

(11) A. Meister, H. A. Sober and S. F. Tice, *J. Biol. Chem.*, **189**, 577 (1951).

(12) We are indebted to Dr. Alton Meister for the *Clostridium welchii* preparations used in some of these experiments, and to Dr. Sidney Udenfriend for the *Streptococcus faecalis R.*

(13) V. A. Najjar and J. Fisher, *ibid.*, **206**, 215 (1954).

(14) E. F. Gale, *Biochem. J.*, **34**, 846 (1940).

(15) O. Hayaishi and R. Y. Stanier, *J. Bact.*, **62**, 691 (1951).

(16) O. Hayaishi and P. Fraser, *J. Biol. Chem.*, **215**, 125 (1955).

(17) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Tissue Metabolism," Burgess Publ. Co., Minneapolis, Minn., 1951.

is indicated in Table I.[18] It will be noted that the extent of exchange was a direct function of the surface area of the liquid phase and an inverse function of the over-all volume of the vessel. From these studies it was clear that it would be necessary to remove the CO_2 from the H_2O^{18}-containing reaction vessel as rapidly as formed to reduce non-enzymatic enrichment. A system in which N_2 was constantly bubbled through the reaction vessel and into a CO_2 trap was tried but proved unsuitable. The experimental conditions finally settled on and used in all the experiments reported here virtually eliminated non-enzymatic enrichment by (1) removing the gas rapidly from the reaction vessel into a large collection bulb, (2) shortening the reaction time to a minimum necessary to obtain enough CO_2 for mass spectrometry and (3) running the decarboxylations at 25° instead of 37°.

TABLE I

NON-ENZYMATIC H_2O^{18}–CO_2 EXCHANGE AT 37°

Expt.	Vessel[a]	Vol. of vessel cc.	Vol. H_2O, cc.	Mole ratio H_2O/CO_2	% Enrichment at 15 min.	30 min.	45 min.	60 min.
18	A	7.685	1.0	844	10.2	16.9	28.0	38.6
29	B	9.848	1.0	944	26.5	50.2	66.3	..
30	B	9.848	1.0	1125	28.6	53.8	69.7	..
41	C	24.04	1.0	4320	7.4	13.2	18.4	..
42	C[1]	24.04	1.0	3550	12.1	25.2	36.8	..
44	D	236	2.0	8460	3.7	8.1	11.8	15.8

[a] The shapes and dimensions of the vessels used were as follows: A, simple equilibration tube—10 mm. × 135 mm.; B, simple equilibration tube—10 mm. × 135 mm. with bottom blown to diameter of 15 mm.; C, modified double-arm Sprinson-Rittenberg tube—1.0 cc. of H_2O^{18} all in one arm; C[1], same tube as in C, but 0.5 ml. H_2O^{18} in each arm; D, modified Sprinson–Rittenberg tube with 200-cc. bulb separating vacuum stopcock and arms.

Apparatus.—The *reaction vessel* used was a spherical 25-ml. flask containing the enzyme, H_2O^{18} and a magnetic stirrer. An *addition tube*, fitted to the reaction vessel with a standard taper joint, contained substrate, buffer and H_2O^{18}. The reaction vessel was connected through an overflow trap and a manifold system to a series of 500-ml. *collection bulbs* fitted with vacuum stopcocks. The entire system, including the collection bulbs, was degassed and at time zero the contents of the *addition tube* were dumped into the *reaction vessel*. The CO_2 liberated during successive time intervals was collected in separate 500-ml. bulbs. When the collection bulb stopcock was closed at the end of a collection period Dry Ice was brought up beneath the bulb to condense water in an effort to minimize any continuing non-enzymatic exchange. Storing the gas in the collection bulb for as much as four hours under these conditions did not increase the enrichment. The special glassware used was fabricated at the glass shop, Instrument Section, National Institutes of Health.

Isolation and Purification of Products.—The decarboxylation was halted by immersion of the reactor tube in Dry Ice–alcohol. The reaction mixture was then thawed, centrifuged at 2° for 15 minutes at 3000 r.p.m. and the supernatant was immediately removed, frozen and lyophilized. Aliquots were streaked on specially washed[19] Whatman #3 paper and chromatographed ascending in 70% propanol: water in a tank 12″ high by 9″ in diameter. The partition was allowed to go for about seven hours and the paper was dried in a circulating oven at 65° for 15 minutes. The amino acid fractions eluted by water were lyophilized and the dried material was stored in a desiccator over $CaSO_4$.

Preparation of O^{18}-Organic Compounds for Mass Spectrometry.—The O^{18}-content of substrates and products was in every case determined by analysis of CO_2 derived from them. Two different pyrolysis techniques were employed for converting the oxygen of organic compounds quantitatively to carbon dioxide. The first, that of Doering and Dorfman,[20] was used only in the early part of the work. The second, that of Rittenberg and Pontocorvo,[21] was used for most of the studies reported here. The purified, dry compounds were pyrolyzed at 350° for 30 minutes with dried $HgCl_2$ as a catalyst.

The O^{18}-content of water[22] was determined by equilibration with carbon dioxide as described by Cohn and Urey.[23]

Mass Spectrometry.[24]—The O^{18}-content of the carbon dioxide liberated in decarboxylation experiments and that obtained in equilibration experiments was measured by obtaining the ratio of mass 46 to mass 44.[25,26]

Tank carbon dioxide[27] was obtained by passing the gas through a Dry Ice–alcohol trap and collecting in a bulb previously evacuated to better than 10^{-4} mm. Although ten micromoles of gas was necessary for optimal operational conditions, an accurate ratio could be determined with as little as one micromole of gas. The reproducibility of a ratio on the same sample was ±0.5%. Thus for a sample with 0.2 atom % excess O^{18} the maximum error was of the order of 1%.

In the manifold collection system, the gas in the 500-cc. bulb was passed through two traps, the first immersed in Dry Ice–alcohol and the second in liquid nitrogen. The carbon dioxide admitted to the inlet system passed back again through the first trap.

A special breakoff leading to the inlet system of the mass spectrometer[28] accommodated the tube used in the Rittenberg–Pontocorvo pyrolysis procedure.[21] The Dry Ice–alcohol non-condensable gases were passed into a quinoline trap and allowed to react so as to effect complete removal of HCl. The CO_2 was passed through a Dry Ice–alcohol trap and admitted to the inlet system of the spectrometer.

Results and Discussion

Control Experiments.—The degree of O^{18}-enrichment in CO_2 expected on the basis of factors other than the decarboxylation process itself was determined in control studies like those shown in Table II. Tank CO_2 was incubated with an approximately equimolar amount of product (γ-aminobutyric acid) under conditions otherwise paralleling those used in subsequent decarboxylation experiments. A sample of CO_2 taken at six minutes showed 4.8% enrichment. Omitting enzyme from the incubation did not decrease the rate of enrichment, showing that it was not attributable to a catalytic activity of *Cl. welchii*, but rather to non-enzymatic H_2O^{18}–CO_2 exchange. The somewhat greater enrichment observed in the second experiment probably is due to the use of a smaller quantity of CO_2.

In these experiments CO_2 was present in the gas phase *above* the medium throughout the incubation whereas in the decarboxylation experiments the gas is continuously formed *within* the H_2O^{18} medium during the incubation. As a control more nearly approximating the actual experimental conditions

(18) Throughout this paper the degree if O^{18} enrichment of substrates and products is expressed as a percentage of that expected if equilibration with the H_2O^{18} of the medium employed were complete:

$$\% \text{ enrichment} = \frac{\text{atoms } \% \text{ excess } O^{18} \text{ in compd.}}{\text{atoms } \% \text{ excess } O^{18} \text{ in } H_2O^{18} \text{ of medium}} \times 100.$$

(19) The Whatman #3 paper was prepared by percolation in water for four days and then in alcohol for four additional days. This was found to be necessary because of water-soluble oxygen-containing impurities in the paper as supplied.

(20) W. von E. Doering and E. Dorfman, THIS JOURNAL, **75**, 5595 (1953).

(21) D. Rittenberg and L. Pontocorvo, *Intern. J. Appl. Radiation and Isotopes*, **4**, 210 (1956).

(22) H_2O^{18} was obtained from the Stuart Oxygen Company, San Francisco, Calif., on allocation of the Atomic Energy Commission.

(23) M. Cohn and H. C. Urey, THIS JOURNAL, **60**, 679 (1938).

(24) G. P. Barnard, "Modern Mass Spectrometry," Institute of Physics, London, Chapter V, 1953.

(25) The Consolidated-Nier Mass Spectrometer, Model 21-201, was used for all the stable isotope measurements.

(26) Consolidated Engineering Corporation, "Mass Spectrometer, Model 21-201," Operation and Maintenance Manual, Consolidated Eng. Co., Pasadena, California, 1948.

(27) "Bone dry" carbon dioxide obtained from the Matheson Company, Inc., East Rutherford, N. J.

(28) F. Highhouse and J. White, *Rev. Sci. Inst.*, **21**, 101 (1950).

TABLE II

% ENRICHMENT OF CARBON DIOXIDE IN CONTROL EXPERIMENTS

System: reaction vessel—106 mg. *Clostridium welchii*, 12.8 mg. γ-aminobutyric acid, 130 μmoles tank carbon dioxide. Addition tube—1.8 cc. H_2O^{18} (1.276 atoms % excess—final), 0.2 cc. 3 *M* acetate buffer (*p*H 4.9)

Time (min.)	6[a]	9[a]
% enrichment	4.8	7.8
% enrichment/min.	0.80	0.86
μmoles gas in sample	116	14

System: reaction vessel—13 mg. γ-aminobutyric acid, 65μ moles tank carbon dioxide. Addition tube—1.8 cc. H_2O^{18} (1.276 atoms % excess—final), 0.2 cc. 3 *M* acetate buffer (*p*H 4.9)

Time (min.)	6[a]	9[a]
% enrichment	5.7	11.4
% enrichment/min.	0.94	1.27
μmoles gas in sample	58	7

[a] CO_2 samples were withdrawn from the reaction flask at the end of the sixth and ninth minute of reaction.

the enrichment of CO_2 formed by addition of H_2O^{18}-containing acetate buffer (*p*H 4.9) to sodium bicarbonate was studied. The CO_2 derived in this manner had less than 0.5% enrichment.

The non-enzymatic, metal-catalyzed decarboxylation of oxalacetic acid was studied, again under conditions similar to those used in the subsequent enzymatic experiments and with a rate of decarboxylation approximately the same as that in the enzymatic experiments. The enrichment observed in the CO_2 was less than 5% and probably can be attributed to CO_2–H_2O^{18} exchange occurring *subsequent* to the decarboxylation process.

Glutamic Acid Decarboxylation.—The glutamic acid decarboxylase of *E. coli* gave CO_2 in which the % enrichment was less than 5% (Table III),

TABLE III

% ENRICHMENT OF CARBON DIOXIDE OBTAINED FROM L-GLUTAMIC ACID IN ENZYMATIC DECARBOXYLATION WITH *E. coli*.

Expt.	Temp., °C.	Time, min.	% Enrichment
120	25	1	2.7
121	25	0.5	4.2

as in the control experiments just described. The % enrichment in CO_2 formed by the action of the glutamic acid decarboxylase of *Cl. welchii* was somewhat higher and varied considerably from experiment to experiment, ranging from 5.9 to 12.8 (Table IV).

TABLE IV

% ENRICHMENT OF CARBON DIOXIDE OBTAINED FROM L-GLUTAMIC ACID IN ENZYMATIC DECARBOXYLATION WITH *Cl. welchii*.

Expt.	Temp., °C.	Time, min.	% Enrichment
60	25	1	7.7
64	25	1	5.9
76	25	5	12.8
83	25	3	11.5
92	25	1	7.4

While the enrichment observed in these experiments was well below that anticipated for any stoichiometric involvement of water oxygens in the decarboxylation process, as discussed later, it was nevertheless well above that anticipated from the control experiments on non-enzymatic CO_2–H_2O^{18} exchange.

The possibility of accelerated CO_2–H_2O^{18} exchange due to the presence of carbonic anhydrase in the preparation was considered. However, repetition of the experiments in the presence of 0.05 *M* Diamox, an effective inhibitor of carbonic anhydrase,[29] did not decrease the observed enrichment.

Non-enzymatic enrichment of unreacted substrate was ruled out by control incubations of glutamic acid and H_2O^{18}-containing medium under several different conditions. After 30 minutes incubation at 25° glutamic acid was reisolated from the medium by paper chromatography and O^{18}-enrichment determined after pyrolysis. At *p*H 3.75 there was no measurable enrichment but at *p*H 1.5 there was 3.9% enrichment. Under the conditions used in the decarboxylation studies (*p*H 4.9), omitting only the enzyme, the glutamic acid substrate showed only 0.8% enrichment.

In the next experiments unreacted substrate and reaction product were isolated after 6 minutes incubation with *Cl. welchii* or with partially purified enzyme under the usual conditions. Both glutamic acid and γ-aminobutyric acid were found to be enriched in O^{18}. The relative enrichment in glutamic acid was 24.2 and 33.8% in two incubations. Because of the presence of endogenous γ-aminobutyric acid in the enzyme preparation, enrichment in the product could not be readily quantitated. However, the very high % enrichment in the unreacted glutamic acid under conditions in which the CO_2 liberated by the decarboxylase showed only up to 12.8% enrichment indicates that the γ-carboxyl group must be most heavily enriched. The O^{18} in the CO_2 most probably is due to enrichment in the α-carboxyl group catalyzed by one or more enzymes in the crude *Cl. welchii* preparation.

Incubations of the complete system were carried out in the presence of 0.01 *M* hydroxylamine, a concentration sufficient to inhibit completely the glutamic acid decarboxylase, probably by complexing with the coenzyme.[30] Despite the complete inhibition of the decarboxylation reaction, examination of the unreacted glutamic acid showed that enrichment had proceeded at about the same rate as in the fully active system (enrichment, 26.9%). This strongly suggests that the observed substrate enrichment is not catalyzed by the glutamic acid decarboxylase itself but rather by other enzymes in the *Cl. welchii* preparation. The possibility that the decarboxylase, while incapable of splitting off CO_2 in the presence of hydroxylamine, is still capable of catalyzing an exchange reaction cannot be ruled out, however. The observed substrate enrichment could be due to reversible reactions such as amide formation and splitting; it could also be due to a "virtual reaction" of the type catalyzed by chymotrypsin in the studies of Doherty and Vas-

(29) W. H. Miller, A. M. Dessert and R. O. Roblin, Jr., THIS JOURNAL, **72**, 4893 (1950).

(30) E. Roberts, *J. Biol. Chem.*, **198**, 495 (1952).

low[31] and observed in several other enzymatic systems.[32–35]

Aspartic Acid Decarboxylation.—The CO_2 derived from decarboxylation of aspartic acid by *Cl. welchii* showed considerably higher % enrichment than that derived from the action of glutamic acid decarboxylase (Table V).

TABLE V

% ENRICHMENT OF CARBON DIOXIDE OBTAINED FROM L-ASPARTIC ACID DECARBOXYLATION IN ENZYMATIC DECARBOXYLATION WITH *Cl. welchii*

Expt.	Temp., °C.	Time, min.	% Enrichment
94	25	5	22.7
95	25	1	19.0
96	25	3	17.4

Again, however, the enrichment observed was less than that anticipated for a reaction involving a stoichiometric contribution from water oxygen. Examination of unreacted aspartic acid again revealed considerable substrate enrichment but the product, α-alanine,[11] was free of O^{18}, indicating that the exchange was limited to the β-carboxyl group.

As in the case of the glutamic acid decarboxylase, enrichment of substrate proceeded even in the presence of inhibitory concentrations of hydroxylamine, suggesting that the exchange was catalyzed by an enzyme or enzymes other than the decarboxylase itself. Further evidence for this was obtained by partial purification of the enzyme system. The fraction between 40 and 55% ammonium sulfate saturation (f_2), which was rich in glutamic acid decarboxylase activity, contained little or no aspartic acid decarboxylase activity. This fraction nevertheless was active in catalyzing enrichment of aspartic acid.

Tyrosine Decarboxylation.—CO_2 derived from decarboxylation of tyrosine by *Strep. faecalis* under conditions similar to those used above showed 8 to 12.2% enrichment. Substrate was not examined for possible enrichment.

Malonic Acid Decarboxylation.—This reaction has been studied by Gray[36] and Hayaishi[37] and has been shown to be a coenzyme-A dependent reaction. Wolfe and Rittenberg proposed that a malonyl-di-coenzyme-A complex plays a catalytic role and by a transfer reaction yields a monosubstituted derivative which is the form decarboxylated. Studies by Hayaishi suggest that only one of the carboxyl groups becomes conjugated with coenzyme A.

In the present studies decarboxylation in the presence of H_2O^{18} led to the formation of CO_2 with only 10.1 and 14.2% enrichment. The results are compatible with the different mechanisms proposed by Hayaishi[38] and Wolfe and Rittenberg[39] and indicate that the CO_2 originates from the carboxyl group beta to the coenzyme-A activated group.

(31) D. G. Doherty and F. J. Vaslow, THIS JOURNAL, **74**, 931 (1952).
(32) F. Vaslow, *Biochem. Biophys. Acta*, **16**, 601 (1955).
(33) D. B. Sprinson and D. Rittenberg, *Nature*, **167**, 484 (1951).
(34) R. Bentley and D. Rittenberg, THIS JOURNAL, **76**, 4883 (1954).
(35) S. S. Stein and D. E. Koshland, Jr., *Arch. Biochem. Biophys.*, **39**, 229 (1952).
(36) C. T. Gray, *J. Bact.*, **63**, 813 (1952).
(37) O. Hayaishi, THIS JOURNAL, **75**, 4367 (1953).
(38) O. Hayaishi, *J. Biol. Chem.*, **215**, 125 (1955).

Discussion

There are several mechanisms by which the carboxyl group might be involved in enzyme–substrate complex formation prior to decarboxylation. A number of these, listed below, should be readily distinguishable on the basis of enrichment in the CO_2 when decarboxylation proceeds in the presence of H_2O^{18} (Fig. 1).

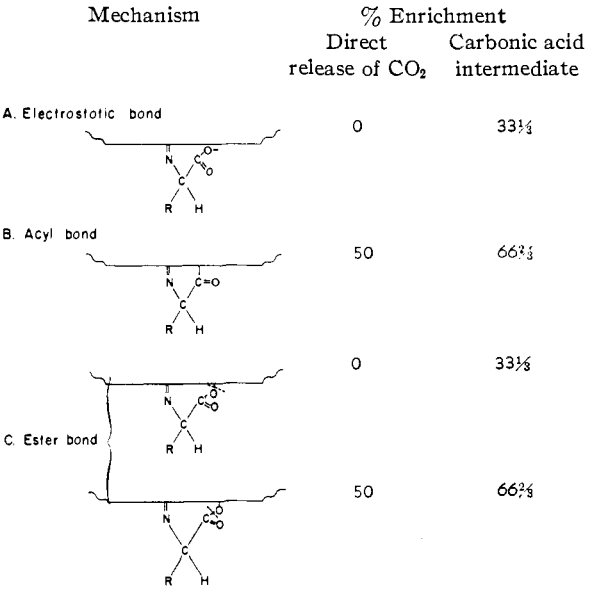

Fig. 1.—Possible modes of bonding between carboxyl group and enzyme surface.

1. Formation of an acyl–enzyme bond. Cleavage should yield CO_2 with one-half the atoms % excess O^{18} of that in the H_2O^{18} of the medium, *i.e.*, relative *per cent. enrichment*, 50.

2. Formation of an ester–enzyme bond. Cleavage on one side of the ester oxygen could yield CO_2 free of O^{18}; cleavage on the other side would yield CO_2 with 50% enrichment.

3. Exclusively electrostatic bonding to the enzyme surface. Elimination of CO_2 by simple carbon–carbon bond scission would result in no enrichment.

4. In each of the above schemes additional enrichment might occur due to hydration either before or after splitting off of CO_2. For example, in case 1 above, hydration of the CO_2 released from enzyme–substrate combination with 50% enrichment would enrich it further so that the CO_2 as collected would show $66^2/_3$% enrichment. The expected enrichment would be the same if hydration of the enzyme–substrate complex occurred prior to CO_2 release.

In none of the five decarboxylation reactions studied did the enrichment of the released CO_2 approach the levels predicted for stoichiometric involvement of water oxygens in the mechanism. The minimal enrichment observed in the case of

(39) J. B. Wolfe and S. C. Rittenberg, *ibid.*, **209**, 885 (1954).

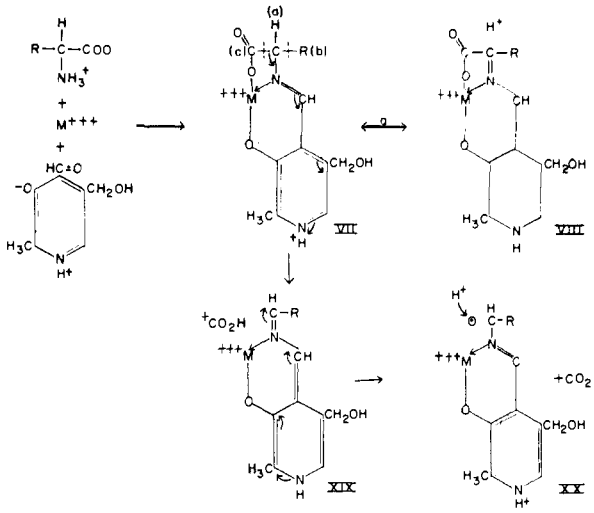

Fig. 2.—Scheme of Metzler, Ikawa and Snell for pyridoxal catalyzed decarboxylation.

some of the studies is almost certainly attributable to non-enzymatic CO_2–H_2O^{18} exchange and to substrate enrichment enzymatically catalyzed by processes independent of the decarboxylation mechanism, as discussed above. It is concluded that in all of the enzymatic reactions studied and in the metal-catalyzed decarboxylation of oxalacetic acid as well, the carboxyl group is released intact by simple cleavage of a carbon–carbon bond. Water does not appear to play a role in the CO_2-release mechanism nor does the CO_2 equilibrate with water in the course of the reaction except at the rate anticipated for non-enzymatic CO_2–H_2O^{18} exchange.

On the basis of these studies it is possible to rule out any of the mechanisms described above which would result in enrichment of the CO_2. The results are compatible with the mechanism recently proposed by Metzler, Ikawa and Snell on the basis of their studies of pyridoxal phosphate catalyzed reactions.[40] These workers propose that the formation of an amino acid–pyridoxal complex as shown in Fig. 2, with the carboxyl group involved only electrostatically can weaken the carboxyl carbon–α-carbon bond by electromeric displacement of electrons to the heterocyclic nitrogen. The enzyme or an enzyme–metal complex[41] is presumed to be equivalent to the simple metal ion used in the non-enzymatic model experiments. The present results show that splitting of this carbon–carbon bond does not involve water and that the carboxyl group is released intact.

(40) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

(41) B. M. Guirard and E. E. Snell, *ibid.*, **76**, 4745 (1954).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF CALIFORNIA AT LOS ANGELES]

The Role of Neighboring Groups in Replacement Reactions. XXII. Competition between *o*-MeO-5 and Ar_1-3 Participation in Solvolysis of *o*-Methoxyneophyl Toluenesulfonate[1,2]

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Solvolysis of *o*-methoxyneophyl *p*-toluenesulfonate involves two competing anchimerically assisted ionization processes. One of the ionization modes is the Ar_1-3-assisted one; the other involves *o*-MeO-5 participation. From Ar_1-3-assisted ionization, rearranged substitution and elimination products are obtained. From *o*-MeO-5-assisted ionization is obtained mainly 3,3-dimethyl-4,5-benzodihydrofuran. From the composition of the isolated products it is possible to dissect the first-order rate constants of acetolysis and formolysis into component k_Δ^{Ar} and k_Δ^{OMe} values corresponding to each of the anchimerically-assisted ionizations. The ratio, $k_\Delta^{OMe}/k_\Delta^{Ar}$, is quite sensitive to the nature of the solvent, being much smaller in formic than in acetic acid. Ar_1-3-assisted ionization of *o*-methoxyneophyl toluenesulfonate is unusually slow in comparison with the *p*-isomer, while *o*-MeO-5-assisted ionization is approximately as fast as that of δ-methoxybutyl toluenesulfonate.

In solvolysis of 2-*o*-anisylethyl *p*-toluenesulfonate (I), there is no indication[3] of substantial competition of *o*-methoxyl participation, in the sense of I → II, with Ar_1-3[4] participation to yield III. On the other hand, with *o*-methoxyneophyl toluenesulfonate (VII), *o*-MeO-assisted ionization competes very successfully with the Ar_1-3-assisted process. The results obtained in a study of this solvolysis are now reported and discussed.

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(2) Some of the material of this paper has been reported in summary by S. Winstein at the Symposium on Dynamic Stereochemistry of the Chemical Society, Manchester, England, March 31, 1954 [*Chemistry & Industry*, 562 (1954)].

(3) S. Winstein, C. R. Lindegren, H. Marshall and L. L. Ingraham, THIS JOURNAL, **75**, 147 (1953).

(4) (a) S. Winstein, R. Heck, S. Lapporte and R. Baird, *Experientia*, **12**, 138 (1956); (b) R. Heck and S. Winstein, THIS JOURNAL, **79**, 3105 (1957).

Results

***o*-Methoxyneophyl *p*-Toluenesulfonate.**—The *o*-methoxyneophyl alcohol was obtained from the sequence of reactions previously employed for neophyl alcohol[5,6] itself. This sequence involves formation of the neophyl chloride from methallyl chloride and anisole, followed by oxidation of the Grignard reagent of the chloride. Originally, the sequence of reactions was employed with the intention of preparing *p*-methoxyneophyl alcohol.[7] However, alkylation of anisole with methallyl

(5) F. C. Whitmore, C. A. Weisgerber and A. C. Shabica, Jr., *ibid.*, **65**, 1469 (1943).

(6) S. Winstein, B. K. Morse, E. Grunwald, K. C. Schreiber and J. Corse, *ibid.*, **74**, 1113 (1952).

(7) S. Winstein and R. Heck, *ibid.*, **78**, 4801 (1956).