Histidine Decarboxylase of *Lactobacillus* 30a. VI. Mechanism of Action and Kinetic Properties*

Paul A. Recsei and Esmond E. Snell

ABSTRACT: When histidine decarboxylase from *Lactobacillus* 30a is reduced at 0° with $NaBH_4$ in the presence of [^{14}C]histidine, the label is fixed to protein. Following acid hydrolysis and column chromatography, N^2-(1-carboxyethyl)[^{14}C]histidine and larger amounts of N^1-(1-carboxyethyl)[^{14}C]histamine were isolated and identified by comparison with synthetic unlabeled standards. Only the latter compound was isolated when the enzyme was similarly reduced with [^{14}C]histamine. The result demonstrates that the essential pyruvoyl residue of this enzyme undergoes Schiff base formation with both the substrate and product of the enzyme, and thus supports a Schiff base mechanism for the decarboxylation reaction.

The enzyme exhibits an almost constant V_{max} between pH 2.9 and 7.6, indicating that no groups titratable within this range are important to its catalytic action. Its affinity for histidine decreases markedly at both the acidic and the basic ends of this range in a way that indicates that ionized groupings with pK values of about 4.2 (probably a single carboxyl group of the enzyme) and 6.5 (probably an imidazole group(s)) are important for binding of histidine. Michaelis–Menten kinetics are followed between pH 2.9 and 7.0; sigmoidal kinetics are observed and become increasingly pronounced with increasing pH above pH 7.0.

Histidine decarboxylase (histidine carboxy-lyase, EC 4.1.1.22) from *Lactobacillus* 30a appears to be a decamer containing (per molecule) five subunits of molecular weight 29,700 and five subunits of molecular weight 9000 (Riley and Snell, 1970). The larger of the two subunits contains a functionally essential pyruvoyl residue bound through an amide linkage to the amino group of a phenylalanyl residue (Riley and Snell, 1968, 1970); no pyridoxal phosphate is present (Rosenthaler *et al.*, 1965; Chang and Snell, 1968). On the assumption that this enzyme acts by a mechanism analogous to that of pyridoxal phosphate containing amino acid decarboxylases, Riley and Snell (1968) postulated that decarboxylation requires formation of an imine between the pyruvoyl residue and histidine (Figure 1). Following decarboxylation, hydrolysis of a second imine intermediate would yield histamine and regenerate the free enzyme. In this report we demonstrate formation of these imine intermediates by borohydride reduction of reaction mixtures, followed by hydrolysis and isolation of the predicted secondary amines. The effect of pH on the kinetic parameters of the enzyme also is described.

Methods

Histidine decarboxylase was purified to homogeneity (specific activity at pH 4.8 is 81 μmoles of CO_2 min^{-1} mg^{-1}) as described by Riley and Snell (1968). A partially purified preparation was also studied to determine whether heat treatment altered kinetic properties of the enzyme. For this purpose, 40 g of cells was suspended in enough 0.2 M ammonium acetate buffer (pH 4.8) to bring the final volume to 100 ml, then subjected to sonic oscillation for 25 min at 15° and 80 W in a Branson Sonifier. After dilution to 320 ml with buffer, cell debris was removed by centrifugation. Finely divided ammonium sulfate was added in small portions to the supernatant solution at 0°, and the fraction soluble at 42% but insoluble at 70% of saturation was collected, dissolved in 20 ml, of water, and dialyzed for 5 hr against several changes of 0.025 M KCl. The specific activity of this preparation at pH 4.8 was 11.0.

Enzyme activity was measured either manometrically (Chang and Snell, 1968) or, for kinetic experiments, by release of [^{14}C]CO_2 from carboxyl-labeled L-histidine. The latter assay was similar[1] to that described for ornithine decarboxylase by Russell and Snyder (1968) except that the reaction was carried out in stoppered 5-ml test tubes and [^{14}C]CO_2 was absorbed onto accordion-pleated strips of Whatman No. 1 paper suspended in the neck of the test tubes and spotted with 0.02 ml of a 1:2 mixture of ethanolamine and methyl Cellosolve. Incubation mixtures contained varying amounts of L-histidine (including 0.03 μCi of L-[*carboxyl*-^{14}C]histidine (Calbiochem), specific activity 9.1 mCi/mmole) dissolved in 0.35 ml of buffer. Citrate–phosphate buffers, prepared by mixing 0.1 M citric acid and 0.2 M dibasic potassium phosphate, were used at pH 7.2 and below; at pH 7.6, 0.2 M potassium phosphate buffer was used. After 15 min at 37°, 0.05 ml of enzyme solution was pipetted into the reaction mixture and the test tube was stoppered immediately. After 5 to 15 min with gentle shaking at 37°, the reaction was stopped by injecting 0.3 ml of 6 N H_2SO_4 through the rubber stopper and past the paper strip. After shaking an additional

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[1] We are indebted to Dr. D. R. Morris, University of Washington, for details of his modified assay procedure for ornithine decarboxylase, which we have applied almost without change to assay of histidine decarboxylase.

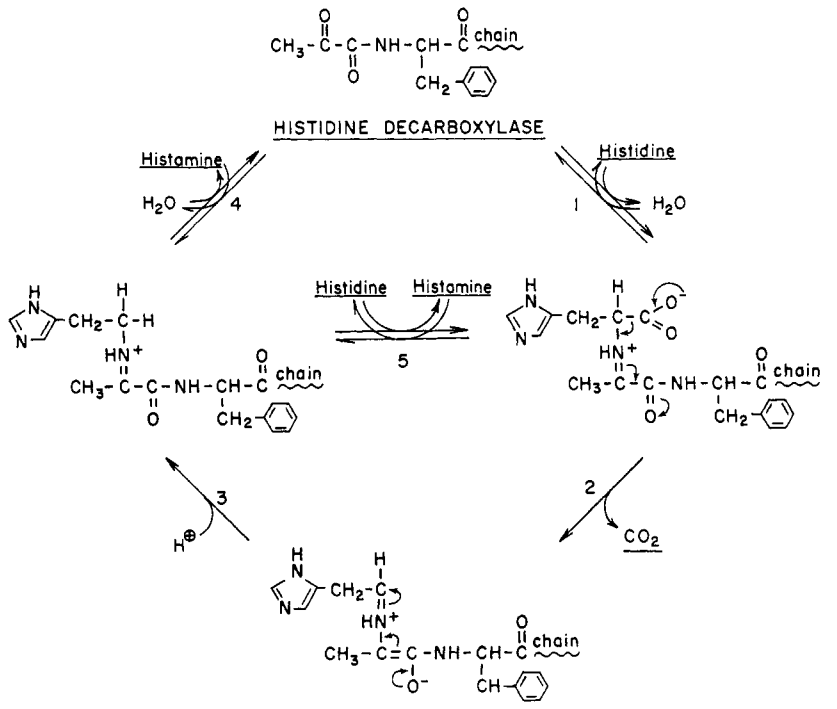

FIGURE 1: Suggested reaction mechanism for the participation of the pyruvoyl residue of histidine decarboxylase in the decarboxylation of histidine (modified from Riley and Snell, 1968).

20 min at 37°, the paper strip was removed and counted in 15 ml of toluene–phosphor (Russell and Snyder, 1968). Under our conditions counts measured on the paper were reproducibly 54% of those released as [^{14}C]CO_2 from carboxyl-labeled histidine. Specific activities obtained by use of this correction factor agreed with those determined manometrically with larger amounts of the same enzyme preparations. Blanks lacking enzyme showed negligible radioactivity. Enzyme activity was linear for the duration of the incubation period and was directly proportional to the amount of enzyme used.

Proton magnetic resonance measurements were made in D_2O solutions at 60 Mc in the Varian A-60 nuclear magnetic resonance spectrometer.

Miscellaneous Methods. High-voltage electrophoresis was performed on Whatman No. 1 paper on a flat-bed apparatus designed by Crestfield and Allen (1955). Radioactive spots on thin-layer chromatograms (Eastman silica gel layers on a plastic backing) and paper electropherograms were detected by autoradiography with No-Screen X-Ray film. Autoradiograms were reproduced by contact photography on Fotorite paper. Scintillation counting of materials in solution was done in Bray's (1960) solution; counting efficiency was determined by using an internal standard of [^{14}C]toluene.

Results

Reduction of the Enzyme in the Presence of L-[*ring*-2-^{14}C]-*Histidine.* Table I shows that one or more intermediates formed between L-[*ring*-2-^{14}C]histidine and histidine decarboxylase at 0° become stable to dialysis after reduction with sodium borohydride. Such fixation of label to protein would be expected if the mechanism for histidine decarboxylation shown in Figure 1 is valid; reduction of the imine linkages between histidine or histamine and the pyruvoyl residue of the enzyme would prevent hydrolysis of these complexes during dialysis. On acid hydrolysis the corresponding secondary amines [N^2-(1-carboxyethyl)histidine and N^1-(1-carboxyethyl)-histamine] containing both the pyruvate residue and a substituted imidazole residue should be formed. These compounds have not been described previously; synthesis of reference samples was therefore undertaken.

Synthesis of N^2-(1-Carboxyethyl)histidine. L-Histidine hydrochloride monohydrate (0.45 g) was dissolved with 0.6 g

TABLE I: Fixation of Label to Histidine Decarboxylase by Reduction in the Presence of Ring-Labeled Histidine.[a]

Sample	dpm/0.01 μmole of Active Site
Enzyme + labeled L-histidine	270
Enzyme + labeled L-histidine + $NaBH_4$	6350

[a] To a stirred solution of crystalline histidine decarboxylase (0.38 mg, 0.002 μmole) in 0.06 ml of 2 M ammonium acetate buffer (pH 5) was added at 0° 0.04 ml of a solution containing 0.062 μmole (1.0 μCi) of L-[*ring*-2-^{14}C]histidine (Nuclear-Chicago) and 20 μmoles of $NaBH_4$ (Metal Hydrides, Inc.) in 0.005 M NaOH. After 30 sec 0.02 ml of a solution containing 20 μmoles of $NaBH_4$ in 0.005 M NaOH was added. The final pH was 5.3. After 5 min at 0° the solution was dialyzed exhaustively against distilled water before measuring the radioactivity. For the control, $NaBH_4$ was omitted.

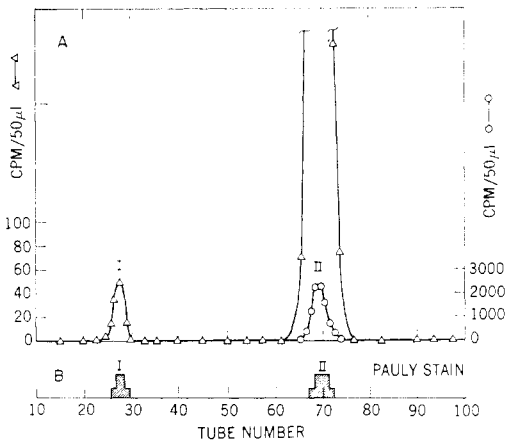

FIGURE 2: Elution profiles. (A) Elution profile of the acid hydrolysate of histidine decarboxylase labeled by reduction in the presence of [^{14}C]histidine. Histidine decarboxylase (0.08 μmole) in 0.3 ml of 2 M ammonium acetate buffer (pH 5) was mixed with 0.3 ml of 1.3 M $NaBH_4$ containing 2.7 μmoles (20 μCi) of L-[*ring*-2-^{14}C]histidine in 0.005M NaOH. The final pH was 5.7. After exhaustive dialysis against water, the solution was dried and the residue was hydrolyzed in 2 ml of 6 N HCl at 105° for 21 hr in sealed, evacuated tubes. The dried hydrolysate was dissolved in 1 ml of water, acidified with concentrated HCl, then applied to a column (35 × 1 cm) of Dowex 50W-X8 which had been equilibrated with pyridine (0.1 M)–acetate buffer (pH 2.8). The column was eluted with a gradient prepared from 150 ml of this buffer and 300 ml of pyridine (2 M)–acetate buffer (pH 5.6). (B) Elution profile of standard N^2-(1-carboxyethyl)-histidine (I) and N^1-(1-carboxyethyl)histamine (II) under the conditions described in A.

of sodium pyruvate in 10 ml of water. The pH was adjusted to about 10.5 with 0.5 M NaOH; after 30 min at room temperature, solid $NaBH_4$ (1 g) was added in several portions over a 1-hr period. The mixture was acidified with HCl, then applied to a column (50 × 2 cm) of Dowex 50W-X8 which had been equilibrated with pyridine (0.1 M)–acetate buffer (pH 2.8). The column was eluted with a linear gradient prepared from 500 ml of pyridine (0.1 M)–acetate buffer (pH 2.8) and 500 ml of pyridine (1 M)–acetate buffer (pH 4.8). The desired product was detected in the effluent fractions by its positive reaction with the Pauly reagent and differentiated from histidine by its very different migratory characteristics on paper electrophoresis at pH 5.6 and 9.0. Fractions containing it were combined, evaporated to dryness, then redissolved in water, and again dried. After several such cycles of dissolution and drying, the free base was precipitated from the concentrated solution by addition of acetone and dried under vacuum at 78°. The yield was 0.2 g (40%). The nuclear magnetic resonance spectrum at 60 Mc of the product in deuterium oxide showed one methyl group at δ 1.5 (doublet), one methylene group at 3.4 (doublet), two methine protons at 4.0 (multiplets), and two protons on the imidazole ring at δ 7.5 and 8.7 (singlets). *Anal:* Calcd for $C_9H_{13}N_3O_4$: C, 47.58; H, 5.73; N, 18.50. Found: C, 47.40; H, 5.81; N, 18.26.

Synthesis of N^1-(1-Carboxyethyl)histamine. This compound was prepared and purified in the same way as the corresponding histidine derivative. The procedure was modified by replacing histidine in the reaction mixture with histamine dihydrochloride (0.6 g), and the Dowex column was eluted with a linear gradient established between 500 ml of pyridine (0.1 M)–acetate buffer (pH 2.8) and 500 ml of pyridine (2 M)–acetate buffer (pH 5.6). Fractions were monitored as described earlier; those containing the desired product were combined and dried. To remove sodium hydroxide, which was eluted with the product, the residue was dissolved in 10 ml water, applied to a column (50 × 2 cm) of Dowex 50W-X8 in the H^+ form, and eluted with 0.5 M NH_4OH. Fractions containing the product were pooled and dried under vacuum at 78°. The yield was 0.11 g (18%). The nuclear magnetic resonance spectrum of the product showed one methyl group at δ 1.5 (doublet), two methylene groups at δ 3.3 (multiplets), one methine proton at δ 3.7 (quartet), and two protons on the imidazole ring at δ 7.1 and 8.0 (singlets). *Anal:* Calcd for $C_8H_{13}N_3O_2$: C, 52.46; H, 7.10; N, 22.95. Found: C, 52.12; H, 7.38; N, 22.82.

TABLE II: Cocrystallization of N^2-(1-Carboxyethyl)histidine with Radioactive Material from Peak I (Figure 2) to Constant Specific Radioactivity.

No. of Crystallizations	Yield[a] (%)	dpm/10 mg
1	(100)	468
2	94	485
3	87	496
4	82	495
5	60	540
6	48	510

[a] Data are calculated in terms of 70 mg of crystals obtained from crystallization 1, corrected for aliquots taken for counting.

Characterization of Radioactive Adducts Formed by Reduction of Histidine Decarboxylase in the Presence of Labeled Histidine. Histidine decarboxylase was reduced at 0° by adding a solution of sodium borohydride containing L-histidine, as described in Figure 2. After exhaustive dialysis the solution was hydrolyzed with 6 N HCl and fractionated over a Dowex column. Essentially all of the radioactivity was eluted in two peaks (Figure 2A); peak I contained 1.2% and peak II 94% of the counts applied to the column. Neither peak contained sufficient product to give a positive reaction with Pauly reagent. However, when synthetic standards were tested on these same columns the Pauly-positive N^2-(1-carboxyethyl)histidine was eluted in the same position as peak I and N^1-(1-carboxyethyl)histamine was eluted in the same position as peak II (Figure 2B).

To confirm the identity of the radioactive material in peak I of Figure 2A the counts were pooled and added to 90 mg of unlabeled synthetic N^2-(1-carboxyethyl)histidine. Upon repeated crystallization of the mixture from acetone–water, the specific radioactivity remained constant (Table II).

N^1-(1-Carboxyethyl)histamine was not readily crystallized. To confirm its identity with the compound in peak II, the latter material was pooled, evaporated to a small volume, and chromatographed on silica gel in four different solvent systems. In each case a single radioactive zone was obtained that migrated identically with the synthetic standard (Figure 3). Similar samples were subjected to high-voltage paper

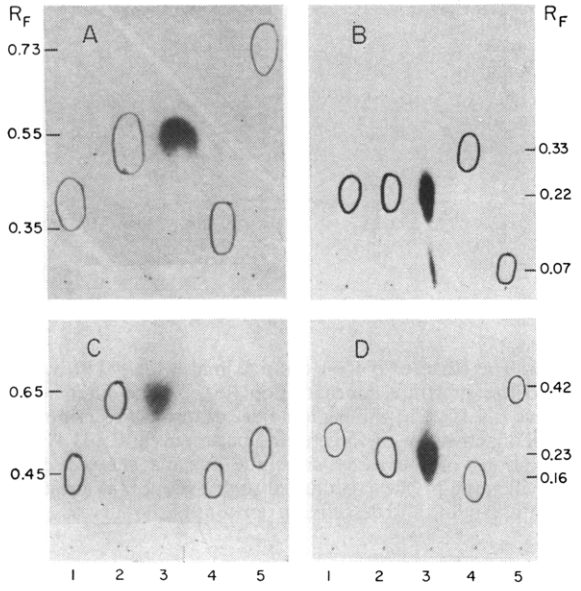

FIGURE 3: Chromatographic comparison of the radioactive material from peak II (Figure 1) with N^1-(1-carboxyethyl)histamine. L-Histidine, N^1-(1-carboxyethyl)histamine, radioactive material from peak II, N^2-(1-carboxyethyl)histidine, and histamine were applied to the silica gel at points 1–5, respectively. The developing solvent systems were: (A) 1-butanol–water–ammonia (2:1:1); (B) 1-propanol–water (7:3); (C) ethyl acetate–methanol–ammonia (3:4:1); and (D) 1-butanol–acetic acid–pyridine–water (4:1:1:2). Chromatograms A, C, and D were developed, dried, and redeveloped without change in solvent. Nonradioactive compounds were detected with Pauly reagent. The radioactive spot was detected by autoradiography.

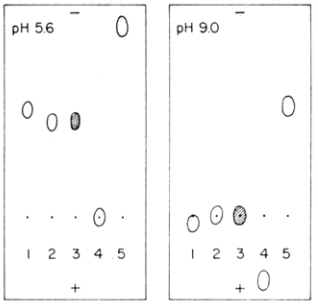

FIGURE 4: Comparative migration pf peak II and N^1-(1-carboxyethyl) histamine on paper electropherograms. L-Histidine, N^1-(1-carboxyethyl)histamine, radioactive material from peak II, N^2-(1-carboxyethyl)histidine, and histamine were applied at points 1–5, respectively. The buffers were 0.1 M acetate–0.3 M pyridine (pH 5.6) and 0.05 M sodium borate (pH 9). Electrophoresis was conducted at 1000 V for 1 hr. Nonradioactive compounds were detected with Pauly reagent. The radioactive spot was detected by autoradiography.

electrophoresis and migrated identically with the synthetic standard at two different pH values (Figure 4).

The validity of the unequal distribution of radioactivity between peaks I and II (Figure 2) was verified in two ways. (a) The recovery of N^2-(1-carboxyethyl)[^{14}C]histidine from the reduced, labeled enzyme was not increased by adding the synthetic standard prior to acid hydrolysis. Separate trials also showed that both N^2-(1-carboxyethyl)histidine and N^1-(1-carboxyethyl)histamine were stable under the conditions of hydrolysis. (b) An experiment similar to that described in Figure 2 was carried out in which [*ring*-2-^{14}C]histamine replaced labeled histidine. In this case, only peak II was detected following acid hydrolysis and chromatography on Dowex 50W-X8. Similarly, when [^{14}C]histidine was added to the enzyme solution (instead of to the $NaBH_4$ solution) in experiments similar to that of Figure 2, only N^1-(1-carboxyethyl)[^{14}C]histamine (peak II) was found in the reduced hydrolyzed protein, *i.e.*, decarboxylation of substrate was complete before the reduction could be carried out.

Estimation of Total Binding Sites by Use of [^{14}C]*Histamine and* $NaBH_4$ *Reduction.* Even though a concentration of histidine 14 times its K_M value was added, the amount of label fixed to histidine decarboxylase by borohydride reduction corresponded to only 15% (in Table I) and 6.2% (in Figure 2) of the enzyme-bound pyruvate present (5 equiv/190,000 g of enzyme (Riley and Snell, 1968, 1970)). This low yield is not unexpected, because (a) most of the histidine was apparently decarboxylated before reduction occurred (*cf.* Figure 2), and the K_I value for histamine (a competitive inhibitor of the decarboxylase with K_I = 11 mM) is much higher than the K_M value for histidine (0.36 mM); (b) reduction was only about 50% complete, as judged by residual activity of the reduced enzyme solution; and (c) since borohydride and histidine were added simultaneously to the free enzyme, an undetermined fraction of the enzyme-bound pyruvate may have been reduced to inactive enzyme-bound lactate before the reaction with histidine. To determine the maximum amount of histamine bound the enzyme (0.93 mg, 0.005 μmole) was incubated at room temperature with 25 μmoles (3.6 μCi) of uniformly labeled [^{14}C]histamine in 0.19 ml of 2 M potassium phosphate buffer, pH 6.0. 2-Octanol (0.01 ml) was added to prevent foaming, followed by a total of 260 μmoles of $NaBH_4$ in 0.02 ml of 0.005 N NaOH. The $NaBH_4$ was added in ten 0.002-ml portions, and between each addition 0.001 ml of 6 N HCl was added slowly to the solution. The final pH was 6.8. The solution was dialyzed exhaustively against distilled water before measuring radioactivity. For the control, $NaBH_4$ was omitted. Under these conditions the enzyme was 95% inactivated as determined manometrically. An experimental value of 5.2 moles of bound histamine per mole of enzyme was obtained. This value agrees (within experimental error) with the expected value of 4.6 calculated from the relationship, $[EI] = [E_t][I_t]/(K_I + [I_t])$, for an enzyme containing five pyruvoyl residues per molecule.

Effect of pH on Kinetic Behavior of Histidine Decarboxylase. Between pH 2.9 and 6.5 crystalline histidine decarboxylase exhibits simple Michaelis–Menten kinetics, as shown in Figure 5A,B for the "optimum" pH of 4.8. In these cases, V_{max} and K_m values were obtained from the double-reciprocal plots in the usual way; their values at pH 4.8 (V_{max} = 80 μmoles of CO_2 min^{-1} mg^{-1} of protein; K_m = 0.36 mM) agreed reasonably well with those determined in a different buffer by the much less sensitive manometric procedure (V_{max} = 81 μmoles of CO_2 min^{-1} mg^{-1}; K_m = 0.9 mM (Rosenthaler *et al.*, 1965)).

Above pH 7.0, the kinetics become sigmoidal, as shown for pH 7.6 in Figure 5C, D. In these cases, values of V_{max} were obtained by extrapolating the concave upward curves on the double-reciprocal plot (*cf.* Figure 5D) to the ordinate. Values for $[S]_{0.9}$, $[S]_{0.5}$, and $[S]_{0.1}$, the substrate concentrations

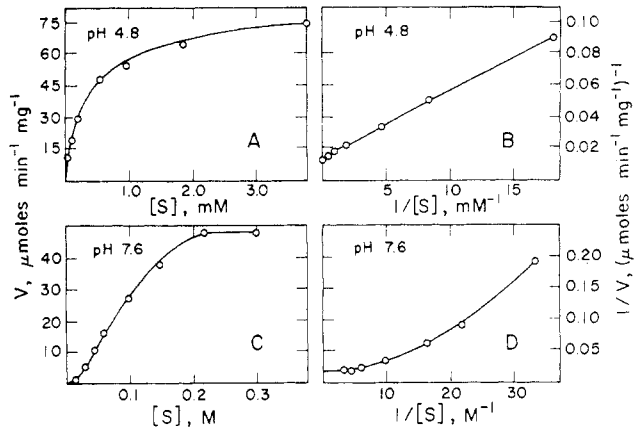

FIGURE 5: Relation of substrate concentration [S] to rate (v) of decarboxylation at pH 4.8 and 7.6. A and C are direct plots; B and D are Lineweaver–Burk plots. A constant amount of enzyme (2.68 μg) was used for each of the rate determinations at pH 7.6. See Methods for assay conditions.

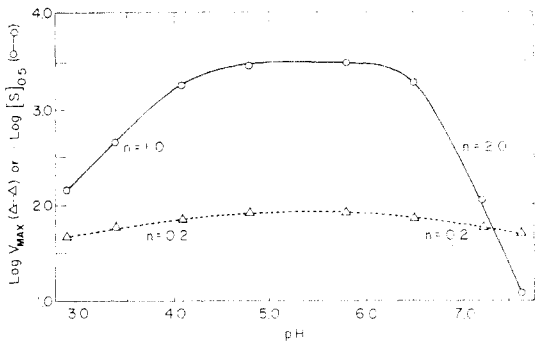

FIGURE 6: The effect of pH on the maximal velocity (V_{max}, dashed line) and the substrate concentration ($[S]_{0.5}$, solid line) necessary to achieve 0.5 V_{max} in the histidine decarboxylase reaction. No irreversible inactivation of the enzyme occurs even at pH 2.9 under the conditions of the assay since the enzyme showed maximal activity when the pH was readjusted to 4.8. Slopes (n) are given for the linear ascending and descending portions of the curves.

at which the initial velocity, v, is 90, 50, and 10%, respectively, of V_{max} were then read from linear plots of substrate concentration, [S], against v. At pH 7.6 the R_s value ($[S]_{0.9}/[S]_{0.1}$; Koshland *et al.*, 1966) is 6, and the slope of the Hill (1913) plot is 2.0. At pH 7.2 the R_s value is 10, and the slope of the Hill plot is 1.7. Both indices reflect the increasingly sigmoidal nature of plots of [S] *vs.* v with increasing pH.

The effect of pH on V_{max} and $[S]_{0.5}$ is shown in Figure 6, which emphasizes the unusually broad optimal pH range displayed by this enzyme. The apparent affinity of the enzyme for its substrate decreases appreciably in going from pH 4.8 to 2.9. The downward curvature of the slope indicates that this is most likely due to the titration of a single carboxyl group (pK near 4.2) situated on the free enzyme (Dixon and Webb, 1964) and involved in the formation of the enzyme-substrate complex. The acidic titration of this or a similar group also leads to a small decrease in V_{max}. The slow rate of this decrease (n = 0.2; see Figure 6) indicates that the ionized form of the group is not required for catalysis, but may contribute indirectly to it by properly orienting the substrate at the active site. The basic titration, probably of one or two groups on the enzyme–substrate complex with a pK between 6 and 7, *e.g.*, imidazole groups, also produces only a slight decrease in V_{max}, indicating that these groups may also be involved in orienting the substrate at the active site, but cannot play a major catalytic role. The rapid increase in $[S]_{0.5}$ in going from pH 5.8 to 7.6 reflects a major effect of these ionizations on substrate binding but is difficult to interpret in any greater detail because the kinetics become sigmoidal near pH 7. It seems clear from the relatively flat V_{max} curve that ionizable groupings with pK values between 2.9 and 7.6 do not play any major catalytic role in decarboxylation of histidine.

To determine whether any of the procedures used to obtain the crystalline enzyme (in particular the heat step) altered the kinetic properties of histidine decarboxylase, a crude enzyme solution prepared by a procedure not involving a heat step (see Methods) was tested. At pH 4.8 this preparation is kinetically identical, within experimental error, with the crystalline enzyme. It exhibits simple Michaelis–Menten kinetics and has a $[S]_{0.5}$ value of 0.31 mM.

Discussion

The isolation of both N^2-(1-carboxyethyl)histidine and N^1-(1-carboxyethyl)histamine from histidine decarboxylase by borohydride reduction in the presence of histidine, and of N^1-(1-carboxyethyl)histamine when histidine is replaced by histamine, verifies occurrence of steps 1 and 4 of the mechanism postulated for action of histidine decarboxylase (Figure 1) and shows that step 4 is fully reversible. Reversibility of the latter step is consistent with the fact that histamine is an excellent competitive inhibitor of the enzyme. Step 3, however, must be relatively irreversible, since Chang and Snell (1968) were unable to detect labilization of any of the hydrogen atoms of the carbon skeleton of histamine during prolonged incubation with the enzyme.

In pyridoxal phosphate dependent decarboxylases, the carbonyl group is present as an internal aldimine formed with the ε-amino group of a lysine residue (Blethen *et al.*, 1969); Strausbach and Fischer, 1970), and formation of the enzyme–substrate complex proceeds by transimination between this internal aldimine and the substrate. If a similar internal aldimine were present in histidine decarboxylase, we should have isolated N^6-(1-carboxyethyl)lysine in hydrolysates of borohydride-reduced enzyme. We have not found this product under any of several conditions of reduction tried; instead only lactate is formed (*cf.* Riley and Snell, 1968). On this basis, it appears that the carbonyl group of histidine decarboxylase is free in the purified enzyme. Nonetheless, the ease with which relatively large amounts of N^1-(1-carboxyethyl)-histamine are formed during reduction of the enzyme in the presence of histidine suggests the possibility that formation of the enzyme–substrate complex here, too, may occur in part by transimination between the histamine–enzyme imine formed by step 3, Figure 1, and histidine to form histidine–enzyme imine, with displacement of the product, *i.e.*, by step 5 as well as by steps 4 + 1 of Figure 1. This appears unlikely, however, unless two imidazole binding sites are present near the active site of the enzyme.

The physiological significance, if any, as well as the structural basis of the shift from Michaelis–Menten to sigmoidal kinetics at pH values above 7.0 is unknown. The affinity for histidine (as measured by $[S]_{0.5}$ values) is already low at these pH values; the sigmoidicity of the curve lowers the effectiveness of the enzyme even further at low histidine concentrations, even though V_{max} remains high. Similar effects of pH have been observed with the pyridoxal phosphate dependent arginine decarboxylase,[2] and have been extensively studied with liver pyruvate kinase (Rozengurt *et al.*, 1969).

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[2] E. Groman and E. E. Snell, unpublished data.