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Histidine Decarboxylase of *Lactobacillus* 30a. II. Purification, Substrate Specificity, and Stereospecificity*

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ABSTRACT: An inexpensive culture medium and a modified purification procedure are described which permit the preparation of crystalline histidine decarboxylase from *Lactobacillus* 30a on a comparatively large scale. The decarboxylase does not contain significant amounts of phosphorus, carbohydrate, or metal ions; neither metal ions nor any known coenzyme is required for its action.

The enzyme is highly specific for L-histidine as substrate, but at very high concentrations several histidine analogs (1-methylhistidine, 3-methylhistidine, 2-thiolhistidine, β-(pyridyl-2)-alanine, β-(thiazole-2)-alanine, and β-(1,2,4-triazole-3)-alanine) serve as poor substrates, in part because of their very low affinity for the enzyme. Pyridine is a good competitive inhibitor. On decarboxylation of L-histidine in D_2O a single deuterium atom is introduced on the α-carbon atom to give an optically active monodeuteriohistamine, apparently without inversion of the asymmetric center. No protons on the carbon skeleton of histidine are labilized during the decarboxylation, and there is no evidence for the exchange of any of the protons of the carbon skeleton of histamine with those of the solvent on incubation with the enzyme.

Among the bacterial amino acid decarboxylases so far studied, histidine decarboxylase (histidine carboxylyase, EC 4.1.1.22) is unique in that it does not require pyridoxal phosphate as a coenzyme (Rosenthaler *et al.*, 1965; Mardashev *et al.*, 1965). Despite this fact, it is readily inhibited by cyanide (Rosenthaler *et al.*, 1965) and other carbonyl reagents (Mardashev *et al.*, 1965; Taylor and Gale, 1945), although the concentrations of these agents required for its inhibition are substantially higher than those necessary for inhibition of pyridoxal phosphate enzymes. Although they have been studied only in a very impure state, the specific mammalian histidine decarboxylases appear to require pyridoxal phosphate as a coenzyme (Håkanson, 1963; Shayer, 1963).

This unusual difference in properties lends considerable interest to comparative studies of these enzymes, and particularly to the mechanism of the decarboxylation of histidine by a pyridoxal phosphate free protein. We have described the preparation and some of the properties of crystalline histidine decarboxylase from *Lactobacillus* 30a (Rosenthaler *et al.*, 1965). We describe herein a modified procedure which permits its preparation on a larger scale, together with additional studies of its substrate specificity, and information concerning the stereochemical course of the reaction. A subsequent paper (Chang and Snell, 1968) describes the composition and subunit structure of this enzyme.

Materials and Methods

Special Chemicals and Procedures. Deuterium oxide, L-histidyl-L-histidine, α-methyl-DL-histidine, 1-methyl-L-histidine, 3-methyl-L-histidine, 2-thiol-L-histidine, urocanic acid, and 2-thiazole-DL-alanine were obtained from commercial sources. (1,2,4-Triazole-3-)-DL-alanine and β-(2-pyridyl)-DL-alanine were gifts from Dr. R. Jones and Dr. W. Shive, respectively. For use in metal ion studies, glassware was soaked overnight in 8 N nitric

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acid and rinsed with tap water, distilled water, and finally distilled water which had been extracted with dithizone in carbon tetrachloride; dialysis tubing was heated at 60° for 3 hr in three changes of a 1% solution of sodium bicarbonate, soaked for 3 hr at 60° in three changes of 0.01 M disodium EDTA, and finally rinsed for several hours in several changes of distilled water. Unless otherwise stated, all work was done in 0.2 M ammonium acetate buffer (pH 4.8).

Decarboxylase activity was determined manometrically in Warburg vessels at 37°. In routine assays the side arm of the flask contained 50 μg of bovine serum albumin and a sample of enzyme protein in 0.05–0.5 ml of 0.2 M ammonium acetate buffer (pH 4.8). The serum albumin was found to protect the enzyme from partial inactivation during thermal equilibration and assay. The main compartment contained 5 mg (24 μmoles) of L-histidine monohydrochloride monohydrate in enough ammonium acetate buffer to bring the total liquid contents of the flask to 3.0 ml. After 15 min at 37° the contents of the side arm and main compartment were mixed and evolution of CO_2 was recorded at 2.5-min intervals for 5–20 min, depending upon its rate, care being taken to avoid significant substrate depletion during the assay period. Activities are expressed in terms of micromoles of CO_2 released per milligram of protein per minute.

Protein concentrations in impure enzyme preparations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard; the purified enzyme was determined by its absorbance at 280 mμ. Samples of histidine decarboxylase of known optical density were exhaustively dialyzed against 0.1 N KCl and then against H_2O, and then dried to constant weight at 104°. The absorbance of a 1% solution of enzyme in 0.2 M ammonium acetate (pH 4.8) in a 1-cm cell was found to be 17.3.[1]

Results

Growth of Lactobacillus 30a. A crude medium (Table I) was used for growing large batches of bacteria because it was cheaper and more convenient to prepare than the semidefined medium of Guirard and Snell (1964) used in previous work (Rosenthaler *et al.*, 1965). Quantities (15 l.) of medium in 20-l. bottles were inoculated with 500 ml of a culture of *Lactobacillus* 30a grown in the same medium, then incubated for 16–24 hr at 37° with no aeration. The cells were then harvested in a refrigerated Sharples centrifuge.

Because of the acidity of the medium, the large inocula used, and the rapid growth of the bacterium, it was not necessary to sterilize the medium in the 20-l. bottles. Nevertheless, the medium was normally sterilized for 15 min at 121° so it could be kept for a few days prior to inoculation without fear of contamination.

Purification Procedure. The procedure of Rosenthaler *et al.* (1965) was modified as follows to permit purification of histidine decarboxylase in quantity from cells grown on large (40–100 l.) batches of crude medium. A protocol of a typical preparation is shown in Table II.

TABLE I: Crude Medium for *Lactobacillus* 30a.

Component	Amt/l. of Double-Strength Medium[a]
Yeast extract (Basamine)	20 g
Sucrose	20 g
Casein hydrolysate	15 g
Histidine hydrochloride·H_2O	2 g
Potassium acetate	3 g
Ascorbic acid	1 g
Pyridoxamine hydrochloride	33 μg
Salts A[b]	20 ml
Salts B[b]	5 ml

[a] Adjust to pH 5.4 with acetic acid. [b] Salts A contain 165 g of $KH_2PO_4 \cdot H_2O$ and 165 g of $K_2HPO_4 \cdot 3H_2O$ in 1 l. of solution. Salts B contain 80 g of $MgSO_4 \cdot 7H_2O$, 4 g of NaCl, 4 g of $FeSO_4 \cdot 7H_2O$, 4 g of $MnSO_4 \cdot H_2O$, and 1 ml of concentrated HCl in 1 l. of solution.

1. PREPARATION OF ACETONE-DRIED CELLS. Harvested cells (300–500 g wet weight) of *Lactobacillus* 30a were mixed with a volume of water equal to the wet cell volume. The resulting slurry was added slowly with rapid stirring to five to ten times its volume of cold (−20°) acetone. The cells were collected in a large Büchner funnel, washed with a volume of cold acetone twice that of the original cell slurry, and then with a similar quantity of cold, anhydrous, peroxide-free ether. They were then spread on sheets of filter paper to dry at room temperature. The acetone-dried cells can be stored at least 1 year at 5° with no loss in decarboxylase activity.

2. PREPARATION OF CELL EXTRACTS. Acetone-dried cells (200–300 g) from step 1 were stirred with 1 l. of ammonium acetate buffer (pH 4.8) at 37° for 5 hr. Cell debris was removed by centrifugation and reextracted with 700-ml portions of the same buffer until the last extract contained only about 5% of the total activity extracted from the cells.

3. AMMONIUM SULFATE FRACTIONATION OF THE CELL EXTRACT. The pooled cell extract from 2 was cooled to 0° and finely divided ammonium sulfate was added in small portions with stirring to 45% of saturation. The small precipitate was discarded. Additional ammonium sulfate was then added to 70% saturation. The active precipitate was collected by centrifugation, suspended in 200 ml of water, and dialyzed at room temperature against several changes (1 l. each) of 0.025 N potassium chloride for a total of at least 3 hr.

4. HEAT TREATMENT. The dialyzed protein from step 3 was heated by swirling 200-ml portions in a 500-ml

[1] Two subsequent determinations give a value for $E_{1\%}^{1\,cm}$ of 16.1; this value also agrees with a refractometric determination of protein concentration in the synthetic boundary cell of the analytical ultracentrifuge (D. Riley and E. E. Snell, unpublished data).

TABLE II: Activity of Histidine Decarboxylase at Various Stages of the Modified Purification Procedure.

Active Fraction	Vol (ml)	Total Protein (mg)	Sp Act.	Over-all Yield of Act. (%)
Dried cells (260 g)			0.164[a]	
Extract	1,900	26,400	2.07	(100)
Ammonium sulfate fractionation	200	9,200	4.92	91
Heat treatment	191	1,530	29.6	91
Acetone fractionation	80	895	43.4	77
Sephadex filtration and crystallization	230	422	69.5	58

[a] Expressed in terms of dried cells instead of protein. This batch of cells was grown in crude medium (Table I) containing glucose in place of sucrose and had only half of the specific activity obtainable with the medium of Table I.

erlenmeyer flask in a water bath maintained at 75° until the temperature reached 70°. It was maintained at 70° for 2 min, then cooled in running water. The precipitate was centrifuged out and discarded.

5. ACETONE FRACTIONATION. The supernatant solution from step 4 was cooled to 0° in an ice–acetone bath and nine-tenths volume of cold (−20°) acetone per volume of supernatant was added slowly. Precipitated material was centrifuged and discarded. Additional acetone (five-tenths volume per volume of supernatant) was added and the precipitate was collected by centrifugation. Finally, more acetone (five-tenths volume per volume of supernatant) was added and the precipitate was again collected by centrifugation. The second and third acetone precipitates were saved or discarded, depending upon their specific activity and the amount of histidine decarboxylase they contained. The final acetone supernatant was discarded.

6. SEPHADEX FILTRATION AND CRYSTALLIZATION. The active protein fraction from step 5 was dissolved in 75 ml of ammonium acetate buffer (pH 4.8) and passed at room temperature over a 4 × 50 cm column of Sephadex G-200 equilibrated with the same buffer. The flow rate was about 50 ml/hr. Fractions were collected for 15 min each, and the active fractions were pooled and chilled to 0°. Aqueous ammonia was added to pH 6–8, then finely ground ammonium sulfate was added to bring the solution to 50% saturation at 0°. If the solution became turbid it was centrifuged and the insoluble matter was discarded. No attempt was made to maintain the pH of the solution after the addition of ammonium sulfate. The enzyme was crystallized from the supernatant solution by adding finely ground ammonium sulfate to 70% of saturation at 0°. The crystals obtained after a few hours at 0° were harvested by centrifugation, dissolved in 75 ml of water, and rerun over the Sephadex column. If the elution profile showed a poor separation between the enzyme peak and a smaller peak of inactive protein with a lower molecular weight, the active fractions were pooled, crystallized, and again passed over the column. The active fractions from this final Sephadex column were pooled, crystallized, dissolved in a small amount of water, and recrystallized a number of times. The crystals are normally stored in a 50% saturated ammonium sulfate solution at −10° and are stable indefinitely.

The specific activity of the enzyme is not increased significantly by recrystallization. The crystalline enzyme sediments as a single component in sucrose density gradients (Rosenthaler *et al.*, 1965) and in the analytical ultracentrifuge (Chang and Snell, 1968). It shows only traces of contaminating protein on starch gel or disc electrophoresis, has an isoelectric point at pH 4.4 and a broad pH optimum between pH 4.5 and 6.0, and contains no pyridoxal phosphate as indicated by spectrum of the protein or by microbiological assay (Rosenthaler *et al.*, 1965).

Phosphorus and Carbohydrate Analyses. A lyophilized sample (20 mg) of crystalline enzyme, analyzed by the Microanalytical Laboratory, Department of Chemistry, University of California, contained less than 0.01% of phosphorus, corresponding to less than 0.7 g-atom/mole (190,000 g; Chang and Snell, 1968) of native protein. This rules out the possibility that any phosphorus-containing coenzymes, including pyridoxal phosphate, are present in the fully active enzyme. The carbohydrate content of histidine decarboxylase, determined by the phenol–sulfuric acid method against a D-mannose standard (Dubois *et al.*, 1956), was less than 0.03%, corresponding to less than 0.25 hexose residue/mole.

Analyses for Metal Ions. Lyophilized samples of histidine decarboxylase from various stages of the purification procedure were kindly analyzed for metal ions by emission spectrography by Drs. K. Fuwa and Bert L. Vallee. None of the ions determined is present in amounts stoichiometric to the enzyme (Table III). Appreciable iron is present in the crystalline sample, but in the material from the acetone fractionation, which is 88% pure, there are only 0.34 and 0.56 g-atom of iron and aluminum, respectively, per 190,000 g of protein, corresponding to a maximum of 0.39 and 0.64 g-atom per mole of enzyme; the aluminum content of the heat-treated sample provides a maximum of only 0.23 g-atom of aluminum/mole of enzyme. Extra aluminum was added to the lyophilized sample of crystalline enzyme prior to analysis, accounting for its high content of this

TABLE III: Metal Ion Content of Purified Fractions Containing Histidine Decarboxylase.[a]

Purification Step	Purity[b] (%)	g-atoms/190,000 g of Protein						
		Al	Mn	Sr	Ca	Fe	Ba	Zn
Heat step	71	0.20		0.11	1.8	0.82		*c*
Acetone precipitate								
I	66	1.2	0.15	*c*	3.4	0.76	0.040	4.4
II	88	0.56	0.087	0.069	1.5	0.34	0.015	
Sephadex eluate	98	>2.1	0.080	0.069	1.8	0.42	0.012	1.0
Crystalline sample	100	>6.0	0.17	*c*	*c*	0.78	0.031	

[a] Samples containing 20–50 mg of protein from each of the various steps in the purification procedure were dialyzed against several changes of 0.01 M EDTA (pH 4.6) for a total of 12 hr, then against several changes of 0.04 M potassium chloride equilibrated with dithizone in carbon tetrachloride for a total of 12 hr, and finally against several changes of water equilibrated with dithizone in carbon tetrachloride for a total of 24 hr. They were then lyophilized in acid-washed glassware and sent to Drs. K. Fuwa and B. Vallee, who kindly determined the metal content. Only the results from the last steps of purification are presented in the table. Cd, Co, Li, Mo, Ni, or Sn was not detected in any sample; Mg and Cr were present only in traces (<0.01 g-atom/190,000 g of protein). [b] Expressed as per cent of the specific activity of the crystalline sample. [c] Trace.

ion. The high zinc content of the almost pure material from the Sephadex column also is spurious, since the heat-treated sample contains only traces of this ion.

Before lyophilization, these samples were assayed with precautions to exclude, as far as possible, all added metal ions. All solutions had been exhaustively extracted with dithizone in carbon tetrachloride; all glassware used had been soaked in 8 N nitric acid and rinsed with dithizone-extracted water. The samples were fully active. These results indicate that neither bound nor free metal ions are essential for action of this enzyme.

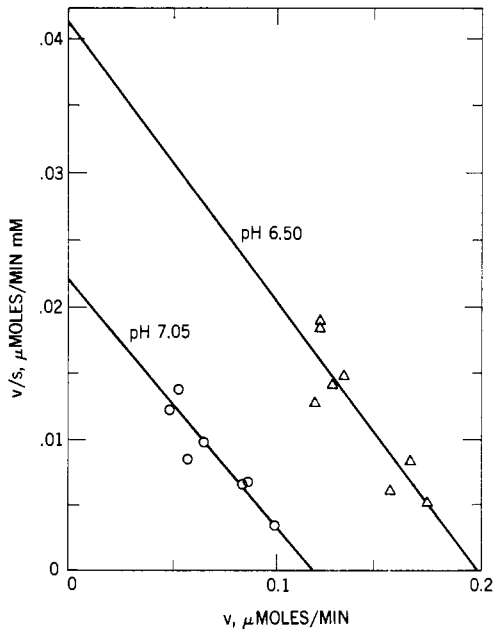


FIGURE 1: Relation of substrate concentration (*S*) to rate (*V*) of decarboxylation at pH 6.5 and 7.05. Enzyme (18.5 μg) was incubated with different amounts of histidine in 2.8 ml of 0.02 M potassium phosphate buffer in double-armed Warburg vessels at 37°. After 10 min, 0.2 ml of 6 N sulfuric acid was tipped in to stop the reaction and liberate CO_2.

Variation of the Kinetic Parameters of Histidine Decarboxylase with pH. Errors inherent in the manometric assay have so far prevented any detailed kinetic analysis of the action of histidine decarboxylase. Nevertheless, K_m values for histidine of about 5 mM were calculated for the reaction at pH 6.5 and 7.05 (Figure 1). This value is almost an order of magnitude larger than the value of 0.9 mM observed at the optimum pH of 4.8. The maximal velocities were calculated to be 65, 63, and 38 μmoles of CO_2/min per mg of protein at pH 4.8, 6.5, and 7.05, respectively. Although these values are only approximate, they indicate that a protonated group with pK_a between 5 and 6.5, *e.g.*, an imidazole group of the substrate or of the enzyme, is involved in substrate binding.

Substrate Specificity. When the substrate specificity of histidine decarboxylase was tested under conditions used for assay with histidine (*i.e.*, a few micrograms of enzyme and relatively low substrate concentrations) only L-histidine was decarboxylated (Rosenthaler *et al.*, 1965). We reexamined this result using very high amounts (milligram quantities) of enzyme and long observation periods (0.25–20 hr). Under these conditions several additional compounds were decarboxylated at a small fraction of the rate for histidine (Table IV). CO_2 evolution from each compound proceeded at a constant rate during the observation period and was followed until it exceeded the capacity of the manometers. At the termination of the incubations, reaction mixtures were examined chromatographically for the amine products (Table V). In each case, a new product not identical with histamine was formed, confirming the fact that decarboxylation of the substrate analog had occurred. The possible contamination of these substrates by histidine was examined by use of

TABLE IV: The Decarboxylation of Histidine Analogs by Histidine Decarboxylase.[a]

Compound	Experiment I[b]		Experiment II[e]	
	CO_2 Evolved (μmoles/min)	Act. (μmoles of CO_2/min per mg of enzyme)	Substrate Used (μmoles)	CO_2 Evolved (μmoles in 90 min)
L-Histidine[c]	0.67	67		
1-Methyl-L-histidine	0.61	0.78	2.7	2.4
(1,2,4-Triazole-3)-DL-alanine	0.55	0.71	3.2	0.9
β-(2-Pyridyl)-DL-alanine	0.41[d]	0.52[d]	5.4	2.3
(Thiazole-2)-DL-alanine	0.078	0.10	3.5	1.4
2-Thiol-L-histidine	0.021	0.026	2.4	0.4
3-Methyl-L-histidine	0.020	0.025	5.5	0.8
α-Methyl-L-histidine	0 ± 0.00015	0		
L-Histidyl-L-histidine	0 ± 0.00015	0		
Urocanic acid	0 ± 0.00015	0		

[a] All experiments were performed in 0.2 M ammonium acetate (pH 4.8). [b] Unless otherwise noted, 10 mg of each compound were incubated with 0.78 mg of histidine decarboxylase in Warburg vessels at 37°. [c] Enzyme (10 μg), bovine serum albumin (50 μg), and histidine hydrochloride·H_2O (5.0 mg) were used under standard assay conditions. [d] From an experiment in which 3.5 mg of substrate was used in a total volume of 1.0 ml. [e] The indicated quantity of each compound was incubated in a Warburg vessel with 1.56 mg of enzyme in 1.0 ml of buffer at 37° for 90 min.

the amino acid analyzer or by microbiological assay. Only 2-thiolhistidine, 1-methylhistidine, and 3-methylhistidine contained any free histidine, and the amounts present (0.14, 0.14, and 0.09%, respectively) were far too small to account for the CO_2 evolution from these substrates (Table IV). No sign of saturation of histidine decarboxylase by high concentrations of triazolealanine (42 mM), 1-methylhistidine (17 mM), or β-(2-pyridyl)-alanine (30 mM) was obtained; Lineweaver–Burk plots indicated that the K_m for each of these compounds was above 0.2 M. The low activity of these compounds as substrates may result largely from their failure to bind to the enzyme efficiently, a property which also explains their apparent inactivity as substrates or as inhibitors of histidine decarboxylase under the less stringent conditions tested previously by Rosenthaler *et al.* (1965).

Inhibition by Pyridine. To the many inhibitors of histidine decarboxylase previously found (Rosenthaler *et al.*, 1965) may be added pyridine, which inhibits competitively with a K_I value of 1.4 mM. This K_I value is comparable to that of 3.2 mM for imidazole and approaches the K_m value (0.9 mM) for histidine (Rosenthaler *et al.*, 1965). At pH 4.8, both pyridine (pK_a = 5.2) and the imidazole ring of histidine (pK_a = 6.1) are partially protonated.

Stereochemistry of the Enzymatic Decarboxylation of Histidine. Decarboxylation of tyrosine by tyrosine decarboxylase (a pyridoxal phosphate enzyme) proceeds stereospecifically in D_2O without inversion to yield only one of the two enantiomers of α-deuteriotyramine (Belleau and Burba, 1960). To determine whether the decarboxylation of histidine also was stereospecific, the reaction was carried out in D_2O and the histamine produced was examined to determine the number of deuterons introduced (by nuclear magnetic resonance) and the optical rotatory dispersion of the product.

1. NUCLEAR MAGNETIC RESONANCE STUDIES. L-Histidine·HCl (50 mg) in water at pH 4.8 was evaporated to dryness, then repeatedly dissolved in deuterium oxide and evaporated to dryness to remove exchangeable hydrogen. The residue was then dissolved in 1.5 ml of

TABLE V: Paper Chromatography of the Product of the Decarboxylation of Histidine Analogs.[a]

Substrate	R_F of Substrate	R_F of Product	Color of Product and Substrate Spots
Histidine	0.15	0.32	Purple
1-Methylhistidine	0.19	0.24	Green-purple
1,2,4-Triazole-3-alanine	0.19	0.36	Green-gray
β-(2-Pyridyl)alanine	0.36	0.54	Red-purple
2-Thiazolealanine	0.35	0.51	Brown
2-Thiolhistidine	0.20	0.29	Red-purple
3-Methylhistidine	0.18	0.30	Gray-purple

[a] At the end of incubation period used for each analog, 5 μl of each of the reaction mixtures of Table IV was applied to Whatman No. 3MM paper. Chromatograms were developed in 1-butanol–acetic acid–water (4:1:5, v/v) in the ascending direction. Spots were located by spraying the paper with a 0.1% solution of ninhydrin in 95% ethanol and then heating for 5 min at 110°.

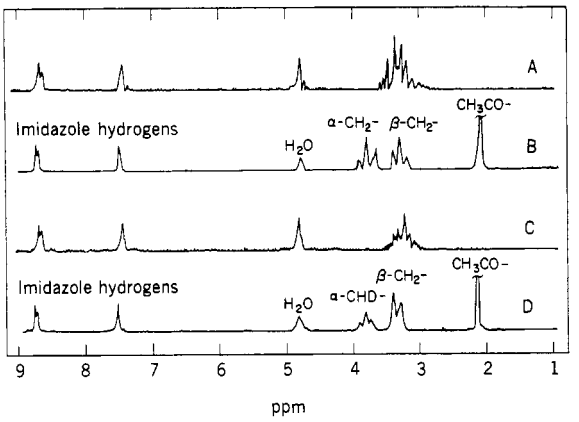
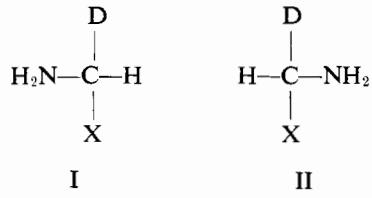


FIGURE 2: Nuclear magnetic resonance spectra of histamine and its N^{α}-acetyl derivative produced by the enzymatic decarboxylation of histidine. (A) Histamine produced by decarboxylation in water. (B) *N*-Acetyl derivative of histamine produced in water. (C) Histamine produced by decarboxylation in deuterium oxide. (D) *N*-Acetyl derivative of histamine produced in deuterium oxide. For acetylation of histamine, the isolated samples of histamine hydrochloride were evaporated to dryness and dissolved in 1 ml of a solution of 0.1 N NaOH in saturated NaCl. Free histamine was extracted into three 2-ml portions of a 1-butanol–chloroform mixture (3:2). The extracts were evaporated to dryness and the residue was acetylated by allowing it to stand in 0.5 ml of acetic anhydride for 3 hr at room temperature. Excess acetic anhydride was destroyed by shaking with water. After evaporating the solution to dryness, 1 ml of 6 N hydrochloric acid was added and the mixture was evaporated to dryness at 40°. The residues of α-*N*-acetylhistamine hydrochloride were stripped of exchangeable protons by repeatedly evaporating to dryness from D_2O solution, and then dissolved in 0.9 ml of D_2O for nuclear magnetic resonance spectrometry.

D_2O, 1 mg of crystalline histidine decarboxylase in 20 μl of 50% saturated $(NH_4)_2SO_4$ was added, and the mixture was incubated at 37°. Deuterium acetate (2 N in D_2O) was added occasionally during the first 6 hr to maintain the solution at a glass electrode reading of pH 4.8. After 12 hr, 1 ml of 6 N HCl was added, the precipitated protein was removed by centrifugation, and the supernatant solution was evaporated to dryness. The residue was stripped of exchangeable protons by repeated solution in D_2O and evaporation to dryness, then dissolved in 0.9 ml of D_2O for nuclear magnetic resonance spectroscopy. A similar preparation of histamine prepared with water and acetic acid replacing D_2O and deuterium acetate served as control. In the nuclear magnetic resonance spectra obtained (Figure 2A,C) the signals of the hydrogens on the two methylene carbons atoms of histamine overlapped, making interpretation difficult. Acetylation of histamine shifted the proton signal of the α-methylene carbon downfield to −3.8 ppm, where it was completely separated from that of the β-methylene carbon at −3.3 ppm (Figure 2B,D). The signals at −7.5 and −8.7 ppm are assigned to the stably bound protons on the imidazole ring (Bhacca *et al.*, 1963). The −2.1-ppm peak in the spectra of *N*-acetylhistamine arises from the acetyl residue. The signal at −4.8 ppm results from traces of H_2O. Comparison of the integrated areas under the peaks of the nuclear magnetic resonance spectrum of the sample of histamine produced enzymatically in D_2O (Figure 2C) with that produced in water (Figure 2A) shows that the former has only three protons on its two methylene carbons; none of the protons of the imidazole ring are lost. When this sample of histamine was acetylated, the methylene signal was resolved into a doublet arising from two protons on the β-carbon atom and a triplet arising from one proton at the α position (Figure 2D). The doublet indicates that the signal of the β-methylene protons is split by a single neighboring proton (on the α-methylene group), whereas the triplet in the signal of the α-methylene proton indicates that it is being split by two neighboring protons on the β-methylene group. Both the area and the fine structure of the peaks thus indicate that only one deuteron has been introduced, and that this deuteron must be present on the α-methylene group. In the control preparation, as expected, both of the methylene proton signals are triplets, each arising from two protons (Figure 2B). The error in the determination of the protons responsible for the nuclear magnetic resonance signals is about ±10%.

2. OPTICAL ROTATORY DISPERSION OF MONODEUTERIOHISTAMINE. Monodeuteriohistamine, prepared from 500 mg of histidine hydrochloride by a scaled-up procedure essentially the same as that described in the preceding section, was extracted into 1-butanol from 0.1 N NaOH in saturated NaCl. After removing the butanol by evaporation, the resulting residue was dissolved in 2 ml of 2 N HCl and applied to a 2.5 × 8 cm column of cation-exchange resin (Dowex 50-X8, 200–400 mesh) which had been washed with 6 N HCl and equilibrated with 2 N HCl. The column was eluted first with 200 ml of 2 N HCl to remove any remaining histidine, and then with 150 ml of 4 N HCl to elute the histamine. The optical densities of the effluent fractions were read at 235 mμ and those containing histamine were pooled and evaporated to dryness at 50°. The residue was recrystallized from ethanol, washed with ether, and dried overnight; yield, 142 mg. The crystalline monodeuteriohistamine dihydrochloride softened at 215° on a hot-stage microscope and melted with some decomposition at 227°. A control reaction mixture in H_2O yielded 344 mg of histamine dihydrochloride which softened at 205° and melted with some decomposition at 224°. The optical rotatory dispersion curve of the monodeuteriohistamine in 4.0 ml of 1 N HCl was recorded with a Cary Model 60 recording spectropolarimeter at 25°. The specific rotation at 300 and 250 mμ was −12.2 and $-30.6°$ g^{-1} ml dm^{-1}, respectively; the control sample prepared by decarboxylation in water was optically inactive. We conclude that the decarboxylation of histidine by histidine decarboxylase proceeds stereospecifically.

If the deuteron occupies the position vacated by the carboxyl group, the monodeuteriohistamine should be *R* enantiomer (I), where X represents the 4(5)-imidazolylmethyl group. Although the absolute configuration of the enzymatically produced monodeuteriohistamine has not been determined, a tentative assignment can be made from the following considerations. Streitweiser and Schaeffer (1956) transformed optically active



I II

[1-²H]-1-butanol into an optically active [1-²H]aminobutane by use of a reaction which produces complete inversion of configuration. Both the butanol and the aminobutane displayed negative optical rotation at the sodium D line. Since the *S* enantiomers of both [1-²H]-ethanol (Lemeux and Houbard, 1963; Weber *et al.*, 1966) and [1-²H]-2-(*p*-hydroxyphenyl)ethylamine (Belleau and Burba, 1960) likewise exhibit negative optical rotations at the sodium D line, we may tentatively assign the *S* configuration to Streitweiser and Schaeffer's monodeuteriobutanol, so that their monodeuterioaminobutane must have the *R* configuration. Although the rotation of the enzymatically produced monodeuteriohistamine at the sodium D line is too small to measure accurately, the negative rotation at lower wavelengths indicates that it, too, should be negative. Since the imidazolium chromophore would not be expected to change the absolute value of the Cotton effect arising from the asymmetric carbon (Djerassi, 1960) and since a number of primary amines show shifts in the magnitude, but not in the sign, of their Cotton effects upon protonation (Lyle, 1960), we may tentatively assign the *R* configuration to the enzymatically produced monodeuteriohistamine. If this assignment is correct, the configuration about the α-carbon of histidine is conserved during decarboxylation, as it is in the monodeuteriotyramine produced from tyrosine by tyrosine decarboxylase (Belleau and Burba, 1960).

Attempts to Demonstrate Enzyme-Catalyzed Proton Labilization in Histamine. Glutamate decarboxylase from *Escherichia coli* and lysine decarboxylase from *Bacterium cadaverans* catalyze incorporation of deuterium from D_2O into γ-aminobutyrate or cadaverine, respectively (Mandeles *et al.*, 1954). To determine whether histidine decarboxylase acts in a similar way, two experiments were conducted. (a) Histamine (50 mg) was incubated in 1.5 ml of D_2O for 40 or 300 hr with 1 mg of crystalline histidine decarboxylase, or (b) monodeuteriohistamine prepared by enzymatic decarboxylation of 50 mg of histidine in D_2O was incubated at pH 4.8 in 1.5 ml of water with 1 mg of histidine decarboxylase for 44 hr at 37°. After removal of the protein, the residues were dried, dissolved in D_2O, and examined by nuclear magnetic resonance spectroscopy. The spectra were indistinguishable from those of the starting materials in each case. Histamine is known to bind at the substrate combining site of histidine decarboxylase, since it inhibits competitively the decarboxylation of histidine (Rosenthaler *et al.*, 1965). The absence of a demonstrable exchange of protons between the α-methylene groups of histamine and the solvent suggests that if this enzyme–histamine complex is analogous to the enzyme–product complexes proposed for pyridoxal phosphate requiring decarboxylases, it has a much higher energy of activation, relative to the product, than do the latter complexes.

Discussion

The spectrum of crystalline histidine decarboxylase is that of a simple protein, and no pyridoxal could be detected by chemical or microbiological assay of hydrolysates of the enzyme (Rosenthaler *et al.*, 1965). These indications that pyridoxal phosphate is absent are confirmed by the present findings of less than 1 g-atom of phosphate/mole of enzyme. The analytical results presented here also eliminate the possibility that other phosphorus-containing or metal ion cofactors are present. This is especially true since histidine decarboxylase appears to contain ten identical subunits/molecule of 190,000 daltons (Chang and Snell, 1968), so that each molecule should contain several active sites. The enzyme is not inhibited by incubation with avidin so that biotin also appears to be absent.

Each of the compounds decarboxylated by histidine decarboxylase has a heterocyclic nitrogen atom at the same position relative to its alanyl side chain. Only one such nitrogen atom is required for binding and for decarboxylation, as shown by the results with pyridine and pyridylalanine, respectively. Perhaps the imidazole binding site of the enzyme contains a negatively charged group which binds the 3(1)-nitrogen atom of histidine in the enzyme–substrate complex. The sharp increase in the K_m value for histidine between pH 4.8 and 6.5 is consistent with the binding of the protonated imidazole ring to the enzyme. The poor binding of triazolealanine may reflect the small fraction of triazole rings (pK_a = 2.6) protonated at pH 4.8. The relatively strong binding of pyridine and the weak binding of β-(2-pyridyl)alanine may be a consequence of a lowered pK_a of the pyridine N as a consequence of the presence of the electron-withdrawing side chain in the amino acid, an effect analogous to that seen in imidazole *vs.* histidine. The poor binding of 1-methylhistidine and the very low activities observed with 3-methylhistidine and 2-thiolhistidine would then reflect the relative inability of the bulkier groups to enter the imidazole binding site.

The fact that 3-methylhistidine is far less active as a substrate than 1-methylhistidine and the inactivity of α-methylhistidine indicate that the steric requirements close to the 3(1)-nitrogen and the alanyl side chain of histidine in the enzyme–substrate complex are far more stringent than those near the 1(3) imidazole nitrogen.

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Histidine Decarboxylase of *Lactobacillus* 30a. III. Composition and Subunit Structure*

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ABSTRACT: Histidine decarboxylase of *Lactobacillus* 30a has a $s^0_{20,w}$ of 9.2 S, a molecular weight of 190,000, and contains ten half-cystine residues per mole of native enzyme. In the presence of urea, ten sulfhydryl groups react with *p*-mercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid), or iodoacetic acid, indicating that there are no disulfide bridges in the enzyme. Mercaptidation of half of these sulfhydryl groups inactivates the native enzyme. Upon complete carboxymethylation or treatment with sodium dodecyl sulfate, the decarboxylase dissociates into ten subunits of mol wt 19,000, which are not further dissociated in 5 M guanidine hydrochloride. Digestion with carboxypeptidase A removes five tyrosine residues from native histidine decarboxylase without inactivating the enzyme. Similar treatment of *S*-carboxymethylhistidine decarboxylase liberates ten tyrosine residues and on longer digestion alanine and leucine. The results are consistent with a C-terminal sequence -Leu-Ala-Tyr for each of the ten subunits. Hydrazinolysis also indicates that tyrosine is the sole C-terminal amino acid. After acid hydrolysis of dinitrophenyl enzyme, dinitrophenylserine was the only α-dinitrophenylamino acid recovered, indicating that serine is present as an N-terminal amino acid. The low recovery of this amino acid leaves open the possibility of other terminal groups. A simplified procedure for discontinuous polyacrylamide gel electrophoresis in the presence of urea was used to separate the peptides resulting from cyanogen bromide cleavage of the decarboxylase. Results of cyanogen bromide cleavage, carboxypeptidase digestion, and tryptic peptide mapping support the presence of ten subunits in this enzyme, and indicate that these are either identical or nearly so.

Preceding papers (Rosenthaler *et al.*, 1965; Chang and Snell, 1968) have described the preparation of crystalline histidine decarboxylase from *Lactobacillus* 30a, its specificity of action toward histidine and histidine analogs, and the optically specific nature of this pyridoxal phosphate independent decarboxylation reaction. This paper summarizes studies on the amino acid composition and subunit structure of this enzyme, together with a preliminary correlation of its enzymatic activity with certain of its structural features.

Materials and Methods

Materials. Unless otherwise stated, all chemicals were reagent grade and were used without further purification. Samples of guanidine hydrochloride free from

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