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PARTIAL PURIFICATION AND CHARACTERIZATION OF L-HISTIDINE DECARBOXYLASE FROM FETAL RATS

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Abstract—Histidine decarboxylase (L-histidine decarboxylase, EC4.1.1.22) was purified more than 2500-fold from fetal rats. This is the first report of purification of mammalian histidine decarboxylase by modern biochemical techniques. The purification procedure consisted of ammonium sulfate fractionation, phospho- and DEAE-cellulose column chromatographies, Bio-Gel A-0.5m gel filtration, affinity chromatography using carnosine-coated agarose and Sephadex G-100 gel filtration. The purified enzyme was extremely unstable. DOPA-decarboxylase (L-3, 4-dihydroxyphenylalanine decarboxylase, EC 4.1.1.26) activity was removed during each step of the purification procedure. Pyridoxal 5′-phosphate was required for activity. As reported earlier for crude enzyme preparations, the pH value of the incubation medium influenced the K_m and V_{max} values of the purified enzyme. It was found that ionic strength also affected these parameters.

Histamine has important and numerous roles in the body and has attracted the interest of many workers. Two voluminous books have been compiled containing information on the chemistry, physiology, pharmacology and pathology of histamine [1, 2]. Recently, indirect evidence has been accumulated that histamine may be a putative neurotransmitter in the mammalian central nervous system [3–5]. However, histidine decarboxylase, the enzyme which forms histamine in mammalian tissues, has not been studied at a molecular level as extensively as the enzymes that participate in the formation of other biogenic amines. Håkanson [6] and Aures and Håkanson [7] studied histidine decarboxylase from fetal rats and concluded that the enzyme was too labile to allow its further purification by column chromatography. Hammer *et al.* [8] purified histidine decarboxylase from mouse mastocytoma 10-fold by chromatography on charged and non-charged *n*-alkyl derivatives of agarose.

As a first step in studies on mammalian histidine decarboxylase, we partially purified histidine decarboxylase from fetal rats and determined some of its characteristics.

MATERIALS AND METHODS

Materials

Pregnant rats were obtained from commercial sources. L-Carnosine was purchased from the Protein Research Foundation, Osaka, Japan. α-Methyl-DL-histidine and α-methyl-DL-DOPA* were obtained from Tanabe Pharmaceutical Co., Osaka, and Japan Merck-Banyu, Tokyo, Japan, respectively. α-Methyl-DL-histidine was purified by extraction from its alkaline solution with *n*-butanol to remove some fluorescent materials. The sources of other materials were as follows: Amberlite CG-50 (Organo Co., Tokyo), Sephadex G-100 and 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), Bio-Gel A-0.5m and 5m (Bio-Rad Lab., Richmond, CA, U.S.A.), phospho- and DEAE-cellulose (Whatman Inc., Maidstone, Kent, England), Diaflo ultrafiltration UM-20 membranes (Amicon Far East Ltd., Tokyo), and Collodion bag UH 100/25 (Schleicher & Schuel, Dassel, West Germany). Ammonium sulfate was enzyme grade and other chemicals were analytical grade.

Assay of histidine decarboxylase

The incubation mixture consisted of 100 μmoles potassium phosphate buffer, pH 6.8, 0.2 μmole dithiothreitol, 0.01 μmole PLP, 0.25 μmole L-histidine, and enzyme in a total volume of 1.0 ml. The reaction was started by adding L-histidine. After incubation at 37° for 60 min, the reaction was terminated by adding 0.05 ml of 60% perchloric acid. The mixture was placed in an ice bath for 10 min and then centrifuged briefly. The supernatant fraction was transferred to another test tube containing 0.8 ml of 0.5 M sodium phosphate buffer, pH 6.5, and a drop of 0.05% bromothymol blue in methanol as a pH indicator. The mixture was adjusted to pH 6.5 with 5 N KOH, placed in an ice bath for 10 min, and then centrifuged briefly. The supernatant fraction was applied to a column of Amberlite CG-50, Type I (0.4 × 2.0 cm) equilibrated with 0.2 M sodium phosphate buffer, pH 6.5. The column was washed with 4.5 ml of H_2O and 3.0 ml of 0.1 HCl, and then histamine was eluted with 1.0 ml of 0.5 N HCl. Histamine was measured by the *o*-phthalaldehyde method [9] using a Technicon autoanalyzer according to the method of Martin and Harrison [10], as modified by Yamatodani [11]. In this way as little as 50 pmoles histamine could be determined reproducibly and conveniently. The overall recovery of histamine in the procedure was 85–90 per cent. Tubes without enzyme and with 1 nmole histamine were set up in all assays. The enzymatic reaction proceeded linearly with respect to enzyme concentration and incubation time. When assaying a dilute enzyme solution (less than 2 μg/ml), 0.2 mg bovine serum albumin was added to the assay mixture; it did not influence the recovery of histamine

* Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; and PLP, pyridoxal 5′-phosphate.

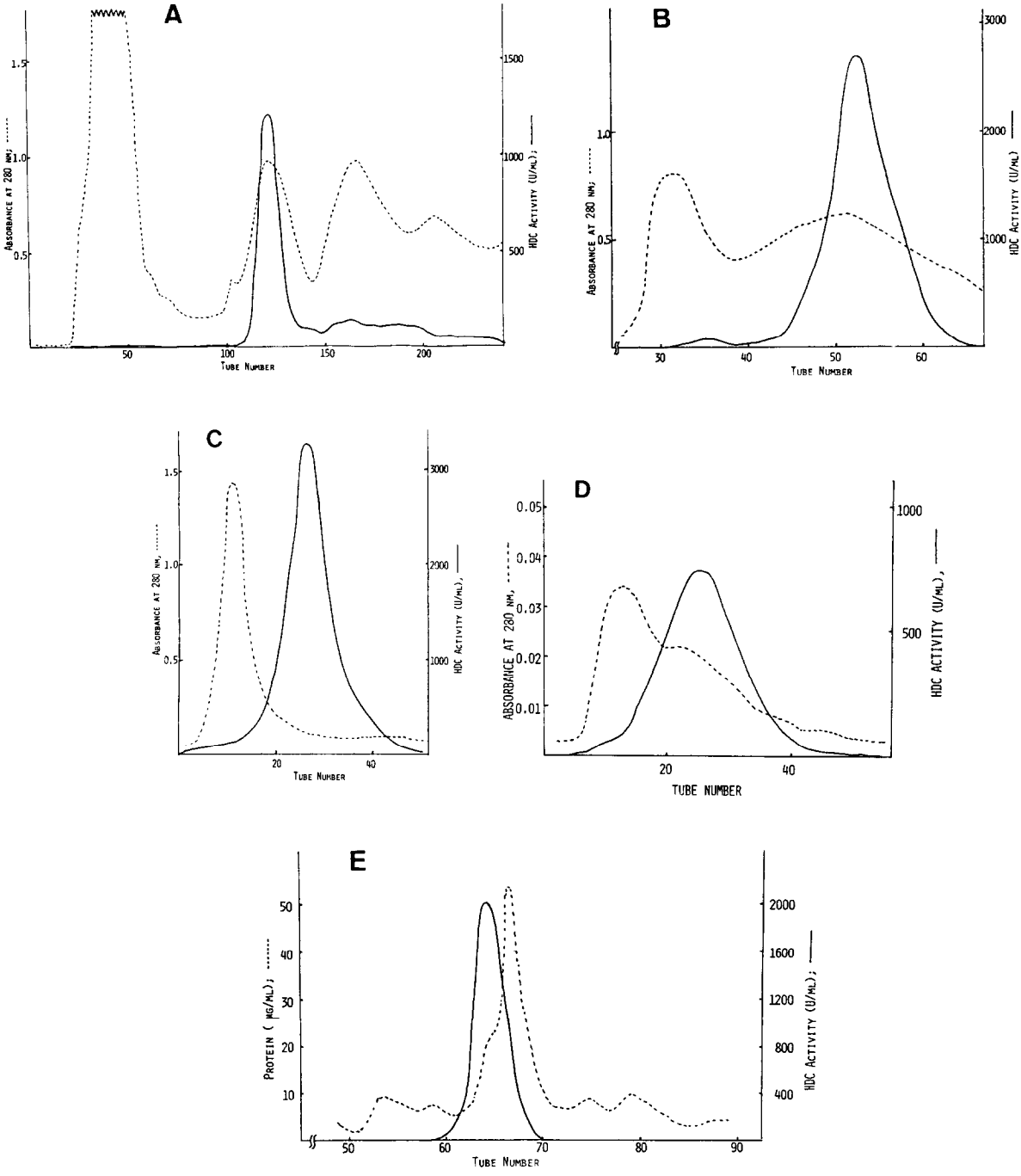

Fig. 1. Purification of histidine decarboxylase from fetal rats. (A) DEAE-cellulose column chromatography of histidine decarboxylase from fetal rats. Unadsorbed fractions from phospho-cellulose were concentrated. as described in the text. and applied to a DEAE-cellulose column. The column was developed with a linear gradient of potassium phosphate buffer from Fraction 30. as described in the text. The flow rate was 120 ml/hr and 17-ml fractions were collected. (B) Gel filtration through Bio-Gel A-0.5m. The active fraction from DEAE cellulose was concentrated and one-half (4.5 ml) was applied to a Bio Gel A-0.5m column, as described in the text. The flow rate was 17 ml/hr and 5.7 ml fractions were collected. (C) Affinity chromatography on carnosine-coated agarose. The active fractions from two runs on Bio-Gel A-0.5m of Fig. 1B were concentrated (4.5 ml) and applied to a carnosine-coated agarose column. as described in the text. The flow rate was 10 ml/hr and 4 ml fractions were collected. (D) Rechromatography on carnosine-coated agarose. One-half (30 ml) the active fractions of Fig. 1C was applied to a column of carnosine-coated agarose similar to the first and the column was developed similarly except that 4.4-ml fractions were collected. (E) Sephadex G-100 gel filtration. The active fractions from two runs of rechromatography on carnosine-coated agarose (Fig. 1D) was concentrated (1.2 ml) and applied to a Sephadex G-100 column. as described in the text. The flow rate was 3 ml/hr and 1.2-ml fractions were collected. Units: pmoles histamine formed/min. HDC: histidine decarboxylase.

or the blank value. In tests on the effects of PLP, inhibitors, pH, and ionic strength on the enzyme activity, these standard assay conditions were modified appropriately, as described in the legends to the figures.

Assay of DOPA decarboxylase

DOPA decarboxylase activity was assayed with a system similar to that used for histidine decarboxylase except that L-DOPA was used as the substrate. The reaction was stopped with perchloric acid and the supernatant fraction was adjusted to pH 6.5, as described previously. The supernatant fraction was applied to a column of Amberlite CG-50, Type I (0.4 × 3.0 cm) equilibrated with 0.2 M sodium phosphate buffer, pH 6.5. The column was washed with 5 ml of H_2O and dopamine was eluted with 2.5 ml of 4% boric acid. Dopamine was measured by the ethylene diamine method, as modified by Ogasahara *et al.* [12] without isobutanol extraction of the fluorophore.

Determination of protein

Protein was determined by the method of Lowry *et al.* [13] or Sedmak and Grossberg [14] with bovine serum albumin as a standard, or by measuring the absorbancy at 280 nm.

Preparation of carnosine-coated agarose

L-Carnosine-coated agarose was prepared by the method of Shaltiel *et al.* [15]. Sepharose 4B or Bio-Gel A-5m was used as agarose and gave similar results. Hydrolysis of the gel in 6 N HCl at 105° for 24 hr and analysis of histidine showed that the gel contained 10–15 μmoles L-carnosine/ml of packed gel.

Purification of histidine decarboxylase from fetal rats

Rats on days 15–20 of pregnancy were killed by placing them in a CO_2 atmosphere, and the fetuses were removed and stored at −80° until use. All procedures were carried out at 2–6°.

Step 1: Extraction of histidine decarboxylase from fetal rats. Fetal rats (1600 g) were homogenized in 3200 ml of 0.1 M sodium acetate buffer, pH 5.5 [16], containing 0.2 mM dithiothreitol and 0.01 mM PLP, in a Waring blender operating at maximum speed for 2 min. The homogenate was centrifuged in a Sakuma centrifuge at 9000 *g* for 30 min.

Step 2: Ammonium sulfate fractionation. To the supernatant fraction (3650 ml), 525 g ammonium sulfate was added slowly, and 10 min later the mixture was centrifuged at 9000 *g* for 20 min. The supernatant fraction (3850 ml) was mixed with 578 g ammonium sulfate, and the mixture was centrifuged, as before, 60 min later. The precipitate was dissolved in *ca.* 300 ml of 0.02 M potassium phosphate buffer, pH 6.8, containing 0.2 mM dithiothreitol and 0.01 mM PLP (buffer A) and dialyzed overnight against two 5 l. volumes of buffer A.

Step 3: Phospho-cellulose column chromatography. The dialyzate was applied to a phospho-cellulose column (5 × 40 cm) equilibrated with buffer A. Histidine decarboxylase was not adsorbed to the column under these conditions, and the active fractions were concentrated by the addition of 40 g ammonium sulfate/100 ml of solution. The precipitate obtained by centrifugation at 9000 *g* for 30 min was dissolved in about 300 ml of buffer A and dialyzed against buffer A.

Step 4: DEAE-cellulose column chromatography. The dialysate was applied to a DEAE-cellulose column (5 × 30 cm) equilibrated with buffer A. The column was developed with a linear gradient produced using 2200 ml of buffer A and 2200 ml of 0.2 M potassium phosphate buffer, pH 6.8, containing the same concentration of dithiothreitol and PLP as in buffer A. A typical elution pattern is shown in Fig. 1A. The major peak of activity (Fractions No. 113–131) was concentrated by precipitation with ammonium sulfate as before, and the precipitate was dissolved in 9.5 ml of 0.05 M potassium phosphate buffer, pH 6.8, containing dithiothreitol and PLP (buffer B).

Step 5: Bio-Gel A-0.5m gel filtrations. One half the solution (4.5 ml) was applied to a Bio-Gel A-0.5m column (2.5 × 90 cm) equilibrated and developed with buffer B. Figure 1B shows the elution profile on Bio-Gel A-0.5m gel filtration. The most active fractions (Fractions No. 49–59) from two runs were combined and concentrated with ammonium sulfate. The concentrated solution (4.5 ml) was dialyzed against 200 ml of 0.36 M sodium citrate solution containing 0.01 M potassium phosphate buffer, pH 6.8, 0.5 mM dithiothreitol and 0.01 mM PLP (buffer C). The sodium citrate solution was prepared by adjusting the pH of citric acid solution to 6.8 with NaOH to give a final concentration of 0.36 M citrate.

Step 6: Affinity chromatography on carnosine-coated agarose. The dialyzed solution was applied to an L-carnosine-coated agarose column (1.5 × 20 cm) equilibrated and developed with buffer C. The typical elution pattern in Fig. 1C shows that most proteins were not adsorbed under these conditions and that histidine decarboxylase activity was eluted in Fractions

Table 1. Summary of purification of histidine decarboxylase from fetal rats (1600 g)

Step	Total activity (units	Total protein (mg)	Specific activity (units/mg)*	Yield (%)
Crude extract	813,000	38,300	21.2	1100.0
Ammonium sulfate	812,000	15,200	53.5	99.7
Phospho-cellulose	656,000	6,950	94.5	80.7
DEAE-cellulose	269,000	195	1,380	33.1
Bio-Gel A-0.5m	188,000	64	2,930	23.2
First carnosine agarose	115,000	5.4	21,300	14.1
Second carnosine agarose	34,300	1.27	27,000	4.2
Sephadex G-100	12,100	0.25	48,400	1.5

* Units: pmoles histamine formed/min.

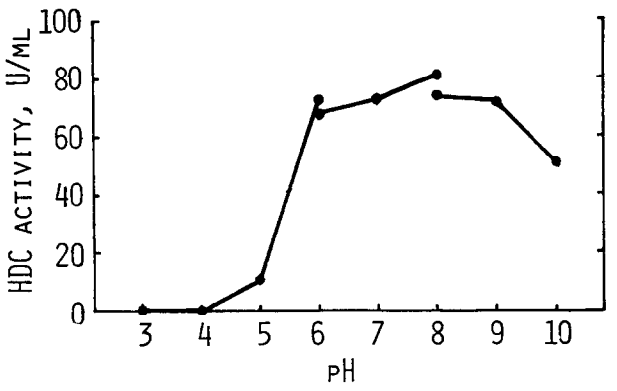

Fig. 2. Stability of histidine decarboxylase from fetal rats at various pH values. The ammonium sulfate fraction of histidine decarboxylase (6.0 mg) was incubated in 1.0 ml of 0.1 M buffers of various pH values containing 0.2mM dithiothreitol and 0.01 mM PLP, for 15 hr. The mixtures were dialyzed against two 2 l. volumes of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.2 mM dithiothreitol and 0.01 mM PLP, and aliquots from the dialysates were assayed for activity under the standard conditions. The buffers used were potassium citrate between pH 3 and 6, potassium phosphate between pH 6 and 8, and potassium borate between 8 and 10. Units: pmoles histamine formed/min. HDC: histidine decarboxylase.

No. 21–35. However, in view of the small amount of histidine decarboxylase present, the trailing edge of contaminating proteins could not be neglected and, therefore, the preparation was rechromatographed. For this purpose, the active fractions (60 ml) were divided in half and applied to two columns under the same conditions as described previously. The elution profile is shown in Fig. 1D. The eluate with activity (Fractions No. 19–39) from the two runs (180 ml) was dialyzed against buffer B and concentrated to about 20 ml by ultrafiltration, using an Amicon UM 20 membrane at 3.5 kg/cm^2 pressure, and then concentrated further to 1.5 ml by vacuum filtration in a collodion bag, UH 100/25.

Step 7: Sephadex G-100 gel filtration. The concentrate was applied to a Sephadex G-100 column (1.5 × 90 cm) equilibrated and developed with buffer B containing 0.5 mM dithiothreitol. Figure 1E shows the elution profile.

RESULTS

Purification of histidine decarboxylase from fetal rats

Histidine decarboxylase was purified from fetal rats, as described in Materials and Methods, and the summary is shown in Table 1. In this way, 2500-fold purification was achieved with 1.5 per cent recovery. This is the highest purification ever reported. The specific activity of about 0.05 μmole/min/mg of protein would be a minimum estimate, because the purified preparation was extremely unstable.

Stability of histidine decarboxylase

Håkanson [6] reported that histidine decarboxylase was extremely labile and that the preparation lost activity completely within a few days. In this work with dithiothreitol and PLP present throughout the purification, the crude preparation was rather stable between pH 6 and 9, as shown in Fig. 2. Håkanson [6] dialyzed the enzyme against H_2O after ammonium sulfate fractionation. We examined the effect of dialysis against H_2O on the stability, and the results are summarized in Table 2. As reported by Håkanson, dialysis of the enzyme solution against H_2O inactivated the enzyme, whereas dialysis against 0.1 M potassium phosphate buffer, pH 6.8, containing dithiothreitol and PLP preserved the activity for at least 6 days at 4° and two months at −20°. Therefore, the enzyme appears to be rather stable provided it is not purified extensively. However, as enzyme purification proceeded, the activity was lost progressively, particularly after the carnosine-coated agarose step, as shown in Table 1. One of the main reasons for this seems to be the dilution of the enzyme solution, because it was necessary to add

Table 2. Effects of dialysis solution on the stability of histidine decarboxylase*

Dialysis solution	Activity (%) remaining after 0 days	1 day	2 days	3 days	6 days
H_2O	100		72.6	71.1	50.0
Buffer	100	95.7	92.2	95.5	90.3

* Histidine decarboxylase obtained by ammonium sulfate fractionation (6.0 mg protein/ml) was dialyzed against H_2O, as described by Håkanson [6], or against 0.1 M potassium phosphate buffer, pH 6.8, containing 0.2 mM dithiothreitol and 0.01 mM PLP at 4°. Aliquots were taken on the days indicated for assay of activity remaining in the standard incubation mixture.

Table 3. Comparison of histidine and DOPA decarboxylase activities during the purification procedure*

Step	Histidine decarboxylase (%)	DOPA decarboxylase (%)
Crude extract	100.0	100.0
Ammonium sulfate	99.7	64.0
Phospho-cellulose	80.7	46.6
DEAE-cellulose	33.1	5.8
Bio-Gel A-0.5m	23.2	3.86
Carnosine agarose	14.1	0.067

* Histidine and DOPA decarboxylase activities were assayed, as described in Materials and Methods. The activities of histidine and DOPA decarboxylases in the crude extract were 813.000 and 10.500.000 pmoles/min, respectively; these values were taken as 100.0 per cent.

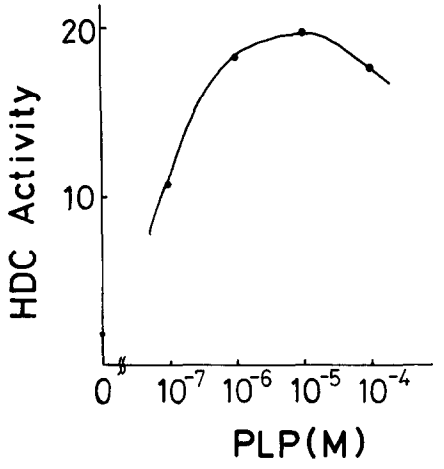

Fig. 3. Activation of "apo-histidine decarboxylase" by PLP. The "apo-enzyme" was obtained by purification under the standard conditions, except that PLP was omitted from the buffers. The "apo-enzyme" (5 μg) was preincubated with various concentrations of PLP in the assay mixture for 15 min; the reaction was initiated by adding L-histidine. HDC (histidine decarboxylase) activity is in arbitrary units.

bovine serum albumin to the incubation mixture for enzyme assay after the carnosine-coated agarose step.

Comparison with DOPA decarboxylase

Christenson *et al.* [17] reported that DOPA decarboxylase can catalyze the decarboxylation reaction of L-histidine at a very slow rate, and Aures and Håkanson [7] detected DOPA decarboxylase activity in a fetal rat extract. Therefore, DOPA decarboxylase activity was measured in parallel with histidine decarboxylase at each step of the purification procedure. For example, the main peak of DOPA decarboxylase was eluted earlier than histidine decarboxylase on DEAE-cellulose column chromatography (data not shown). Table 3 summarizes the results showing that, although DOPA decarboxylase activity was higher than histidine decarboxylase activity in the crude extract of fetal rats, as reported by Aures and Håkanson [7], it was efficiently removed from the fractions containing the latter activity during purification, and the final preparation contained essentially no activity under the assay conditions used. Thus, it is concluded that the final preparation purified by the above procedure is a specific histidine decarboxylase.

Effect of PLP on histidine decarboxylase activity

Histidine decarboxylase could be purified by our method equally well with or without PLP, but the enzyme purified in the absence of PLP showed little activity if PLP was omitted from the assay mixture. The effect of PLP on the activity of the apparent "apo-enzyme" is shown in Fig. 3. PLP was necessary for the activity, confirming the results obtained with crude enzyme. The PLP concentration required for 50 per cent activation was approximately 10^{-7} M.

Effects of inhibitors on histidine decarboxylase

α-Methyl-DL-histidine and α-methyl-DL-DOPA have been used to differentiate specific histidine decarboxylase from DOPA decarboxylase [18]. As shown in Fig. 4, α-methyl-DL-histidine was more inhibitory than α-methyl-DL-DOPA, confirming that the purified enzyme is specific for histidine.

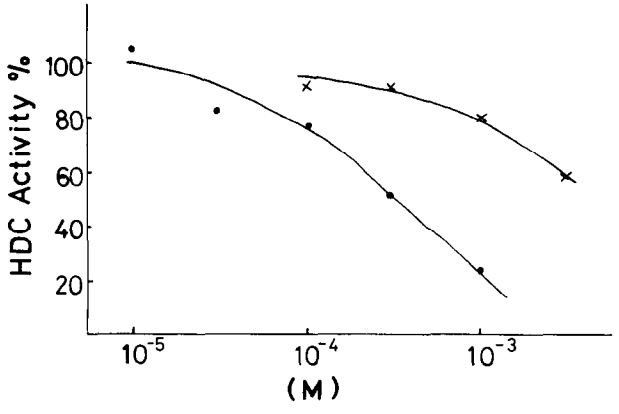

Fig. 4. Inhibition of histidine decarboxylase by α-methyl-DL-histidine (—●——●—) or α-methyl-DL-DOPA (—X——X—). The activity of the enzyme at Step 6 (3 μg) was assayed in the presence of various concentrations of the two compounds under the standard assay conditions. The activity in the absence of these compounds was taken as 100 per cent.

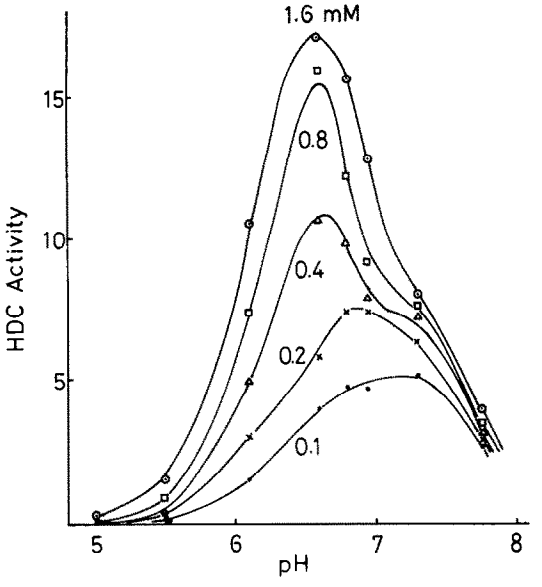

Fig. 5. Effect of L-histidine concentration on histidine decarboxylase activity at various pH values. The activity of the enzyme at Step 4 (50 μg) was measured with various concentrations of L-histidine in reaction systems containing 0.1 M buffers of various pH values and is shown in arbitrary units. Potassium acetate and potassium phosphate buffers were used for pH 5–6 and pH 6–8 respectively. HDC: histidine decarboxylase.

Effects of pH and ionic strength on the V_{max} *and* K_m *values of histidine decarboxylase for* L-*histidine*

The K_m and V_{max} values of crude histidine decarboxylases from a variety of sources are dependent on the pH value of the incubation mixture [19–22]. We examined the effects of pH value on the K_m and V_{max} values of the purified preparation. Figure 5 shows the pH curves of histidine decarboxylase at different substrate concentrations. As found by others with crude enzymes, the optimal pH was lower at higher substrate concentrations. The K_m values obtained from this figure are plotted against pH in Fig. 6. The higher the pH value, the smaller was the K_m value.

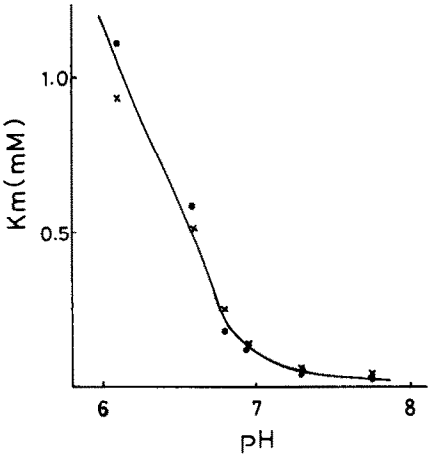

Fig. 6. Effects of pH on K_m values for L-histidine. The K_m values were calculated from the data in Fig. 5 when potassium phosphate buffers were used. The symbols (● and X) represent the results of different preparations of the enzyme.

Table 4. Effects of pH and ionic strength on K_m and V_{max} values of histidine decarboxylase*

pH	K_m (mM)			V_{max}†		
	KCl (M)			KCl (M)		
	0	0.1	0.5	0	0.1	0.5
6.50	0.21	0.77		1420	1820	
6.95	0.05	0.15	0.95	860	1250	1780
7.40	0.04	0.07	0.25	500	1020	1320
7.80			0.14			1220

* Assay were conducted in 0.02M potassium phosphate buffer of the indicated pH values with 0, 0.1 and 0.5 M KCl.

† Arbitrary units, because the DEAE-cellulose fraction was used for kinetic studies.

In the course of these studies, we found that the ionic strength of the incubation mixture also influenced the K_m and V_{max} values. Table 4 summarizes the K_m and V_{max} values at three different ionic strengths of the incubation mixture. At a fixed concentrations of KCl, the K_m and V_{max} values decreased with an increase in pH value. At fixed pH values, with an increase in the KCl concentration the K_m and V_{max} values increased.

DISCUSSION

Recently, increased interest has been paid to histamine in the various fields of life sciences [1–5]. In view of this, it is rather surprising that attempts have not been made to purify histidine decarboxylase which forms histamine, since purification and characterization of the enzyme are essential for studies on phenomena in which the enzyme is involved, as demonstrated in a number of cases. Two reasons why this enzyme has not been purified are its low content in tissues and its great instability. In this work, we partially purified the enzyme from fetal rats, which are one of the richest sources of the enzyme and which can be obtained rather easily in large numbers as a starting material. DEAE-cellulose column chromatography (Fig. 1A) gave a minor peak(s) of activity in addition to the major peak. Currently, we do not know whether these peaks might represent multiple forms of histidine decarboxylase present in the whole rat fetus or artifacts produced during purification. This remains to be studied. The origin of our enzyme preparation should also be determined in the future (for example, stomach, liver, mast cell, etc.).

The specific activities calculated for the purified preparations may not be accurate and are probably underestimations, because the enzyme is extremely unstable when purified. The final preparation was estimated to have a specific activity of 0.05 μmole/min/mg of protein under the standard assay conditions, in which a concentration of substrate almost equal to the K_m value was used to minimize the influence of DOPA decarboxylase, if present in the crude preparations. This value may correspond to about 0.2 μmole/min/mg under the best conditions, judging from Fig. 5 and Table 4. The latter value is comparable to the specific

activities estimated for other amino acid decarboxylases from mammalian sources: those of mouse brain glutamate and hog kidney DOPA decarboxylases are 2.0 [23] and 8.9 [17] μmoles/min/mg of protein respectively. On polyacrylamide gel electrophoresis, our final preparation gave several distinct bands, when protein was stained by Coomassie brilliant blue, and one symmetrical peak of activity corresponding to one of the protein bands when the activity was assayed from 2-mm slices of the gel (data not shown). The purity of the present preparation was estimated to be approximately 10%, and this is consistent with its lower specific activity in comparison to the other amino acid decarboxylases. Although our final preparation did not have a yellow tint characteristic of PLP enzymes, the apoprotein purified in the absence of PLP showed little activity without PLP in the assay mixture, while it did show activity with PLP. This result indicates that PLP is a coenzyme of the histidine decarboxylase which we have purified.

The recovery was not satisfactory, and suitable conditions must be established for stabilizing purified samples. Under conditions giving greater stability and with a larger amount of starting material, purification should be more successful. Because the enzyme was so unstable, some of its properties had to be studied using the enzyme at the DEAE-cellulose step. These properties must be confirmed using a homogeneous preparation. So far, we confirmed general enzymatic properties determined on more crude preparations from various sources, such as rat fetus, rat stomach, etc. [6, 7, 16, 18–22]. Moreover, the crude enzyme from rat brain also has similar properties [24, 25], suggesting that rat brain histidine decarboxylase is very similar to that of fetal rats and that it might cross-react with antibodies elicited against fetal enzyme. In preliminary studies, it was found that the rat brain enzyme could be purified in the same way as the fetal enzyme.

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