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A DIRECT, SENSITIVE MICROASSAY FOR MAMMALIAN HISTIDINE DECARBOXYLASE

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Abstract—A microassay procedure for mammalian histidine decarboxylase based on the conversion of L-[^{3}H]histidine to [^{3}H]histamine, which were separated by an alkaline butanol extraction followed by thin-layer chromatography, is described. This assay is direct and simple to perform, in addition to being very sensitive and reproducible. It is useful for tissues containing high levels of endogenous histamine, because only newly formed radiolabeled histamine is measured. This report includes information on histidine decarboxylase activity at various pH levels, in different buffers, and in the presence of selected histamine active drugs. In addition, it describes histidine decarboxylase activity in several fetal rat tissues.

Histidine decarboxylase (HDC)† is an important enzyme because it catalyzes the single step in the formation of histamine (HA) from L-histidine (L-His). HA has prominent pathological and physiological roles in allergy, vascular permeability, neurotransmission, uterine contraction, and gastric acid secretion [1].

There are several methods available for measuring HDC activity. None of those HDC methods already described combines all the following features: high sensitivity, direct measurement, excellent reproducibility, simplicity, and low cost. Measuring $^{14}CO_2$ evolved from L-[*carboxyl*-^{14}C]His, an approach originally developed by Kobayashi [2] and modified by Ritchie and Levy [3], is the most rapid method, but it is not sensitive enough for tissues with low activity [4]. The method of Taylor and Snyder [5], a coupled radioenzymatic assay, has a high background because it measures endogenous HA as well as newly formed HA. One has to monitor the effect of exogenous agents on histamine-*N*-methyltransferase (HNMT) as well as on HDC. Keeling *et al.* [6] have developed a more novel coupled assay which combines the HDC and HNMT steps into one, using L-[^{3}H]His and unlabeled *S*-adenosylmethionine (SAM). Their assay requires purification of L-[^{3}H]-His, an extraction, a back-extraction, a column chromatographic step, and a liquid cation-exchange extraction. Baudry *et al.* [7] use a direct radiometric assay which requires large amounts of L-[^{3}H]His (5 μCi) per assay tube, purification of the substrate, and column chromatography. Weinreich and Yu [8] have developed a very sensitive direct radiometric assay for HDC which also requires several microcuries of isotope, and it involves an extraction and two chromatographic steps. Watanabe *et al.* [9] measure the HA formed via the fluorescent method using *o*-phthaladehyde, which is less expensive than radioisotopic assays but not as sensitive or as specific. Schayer's dilution assay for HDC [10] utilizes the ring-labeled L-[^{14}C]His, which is converted to [^{14}C]HA. The [^{14}C]HA is extracted in the presence of cold carrier and extracted to the benzenesulfonyl derivative. This method requires large amounts of tissue, and only 30–35 assays can be processed at one time.

We have developed a simple yet sensitive and reliable radioenzymatic assay for HDC. It is not as rapid as the trapping of radioactive CO_2, but one person can easily measure more than 100 tubes per day. This assay uses L-[^{3}H]His as a substrate and measures the formation of [^{3}H]HA, which has been separated by an alkaline butanol extraction followed by thin-layer chromatography (TLC). Our blank values are as low as 50–100 dpm above background after starting with 2.2×10^6 dpm. Taking into account recovery and "blank" values, our assay will yield counts that are double "blank" for the formation of 5 fmoles of HA.

MATERIALS AND METHODS

Chemicals. L-[2,5-^{3}H]His (40–60 Ci/mmole) and [2,5-^{3}H]HA (25–50 Ci/mmole) were obtained from Amersham (Arlington Heights, IL). L-[3-^{3}H]-His (10–25 Ci/mmole) was obtained from New Eng-

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† Abbreviations: HDC, histidine decarboxylase; HA, histamine; L-His, L-histidine; tMHA, telemethylhistamine; HNMT, histamine-*N*-methyltransferase; DAO, diamino oxidase; AADC, aromatic amino acid decarboxylase; α-methyldopa, α-methyldihydroxyphenylalanine; PMSF, phenylmethylsulfonylfluoride; MOPS, morpholinopropanesulfonic acid; HEPES, *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid; TES, *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; and PLP, pyridoxal 5′-phosphate.

land Nuclear (Boston, MA). L-His dihydrochloride, HA (free base), DL-α-methyldihydroxyphenylalanine (α-methyldopa), phenylmethylsulfonylfluoride (PMSF), imidazole, morpholinopropanesulfonic acid (MOPS), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), *N* - tris(hydroxymethyl) - methyl - 2 - aminoethane - sulfonic acid (TES), aminoguanidine, and pyridoxal 5′-phosphate (PLP) were purchased from the Sigma Chemical Co. (St. Louis, MO). Scintillation counting fluid was 3a20 (Research Products International, Elk Grove, IL). Dr. Kollonitsch of Merck Sharp & Dohme, Rahway, NJ, provided (*S*)-α-fluoro-methylhistidine (α-FMH). Impromidine was provided by John Paul of Smith Kline & French, Philadelphia, PA. Amodiaquin was a gift from Parke Davis, Detroit, MI.

Supplies. Sarstedt (Princeton, NJ) was the supplier of 400-μl polyethylene (PE) tubes and polypropylene vials used for scintillation counting. Microman M-50 was a Gilson product from the Rainin Instrument Co., Inc., Woburn, MA, and the Eppendorf Repeater was from the Brinkmann Instruments Co., Westbury, NY. The Hamilton Co. (Reno, NV) was the source for the repeating dispenser. Preadsorbent, channeled, silica thin-layer chromatography (TLC) plates were purchased from Whatman (LK5D) in Clifton, NJ, or from Baker (7009-4) in Phillipsburg, NJ. The TLC plate-scraping device has been described in detail [11].

Animals. Sprague–Dawley rats were obtained from the colony at the Waisman Center on Mental Retardation and Human Development. Fetal rat livers were from fetuses that were 18–20 days postconception.

Protein determinations. The method of Bradford [12] was employed to determine the amount of protein included in each assay, using bovine gammaglobulin as standard.

Preparation of HDC. Rats were killed by decapitation. Tissues were removed immediately and placed on ice. They were rinsed with ice-cold 0.9% NaCl, blotted dry, and weighed. Homogenizations were done with either a Brinkmann Polytron or a Sonifer Cell Disruptor from Heat Systems-Ultrasonics. The polytron was at setting 3 for 10–20 sec, and the sonifer was at setting 5 for 10 sec with a microtip. The homogenizing buffer (Buffer A) was 20 mM potassium phosphate, pH 7.2, containing 0.1 mg/ml PMSF. Following homogenization in 5 vol. of Buffer A, homogenates were centrifuged at 40,000 *g* for 30 min and the supernatant fraction was frozen and stored at −80°. In some instances, the whole homogenate was frozen and stored at −80°, thawed, and then centrifuged immediately before the assay. Brain homogenates were assayed without centrifugation. Dilution of supernatant fractions for tissues with high HDC activity was necessary before the HDC assay was performed.

HDC assay procedure. In this assay method, the production of [^{3}H]HA from L-[^{3}H]His was measured to determine HDC activity. The HDC reaction was carried out in 400-μl PE tubes in a volume of 40 μl containing: 0.2 μmoles potassium phosphate buffer (pH 7.2); 4 nmoles α-methyldopa, an amino acid decarboxylase inhibitor [5]; 4 nmoles impromidine, an HNMT and diamine oxidase (DAO) inhibitor [13]; 0.4 nmole PLP; 20 pmoles L-[^{3}H]His (0.2 to 1.2 μCi); enzyme; and test substances. Blanks routinely contained Buffer A. Alternate blanks containing either boiled enzyme or reaction mixtures containing enzyme but incubated in the presence of 0.4 N NaOH were comparable. The amount of radioactive L-His and the concentration of unlabeled L-His were sometimes varied. Twenty microliters of enzyme and 10 μl of test substance(s) or water were added to the 400-μl microfuge tubes with the Microman. The reaction was initiated with a mixture that contained the buffer, α-methyldopa, impromidine, PLP, and L-[^{3}H]His, using a Hamilton repeating dispenser with a 19-gauge needle that delivered 10-μl aliquots. The microfuge tubes were then briefly (<5 sec) centrifuged in a Beckman Microfuge B and incubated for up to 2 hr at 45°. The assay was terminated by adding a mixture of 5 μl of HA (2 mg/ml in absolute ethanol) and 5 μl of 4 N NaOH, using the 10-μl Hamilton repeating dispenser. The HA–ethanol solution was stored at −20° for up to a week. When stored for longer periods, the HA apparently broke down to a product(s) that was (were) no longer visible with ninhydrin spray. Either 100- or 200-μl aliquots of H_2O-saturated *n*-butanol were added to the terminated reaction with an Eppendorf Repeater, to extract the newly formed [^{3}H]HA. Assay tubes were capped, mixed with a Vortex–Genie mixer at top speed for at least 10 sec after initial mixing appeared complete, and then centrifuged for 5 min in the Microfuge B. This assay was most convenient to perform with the microfuge tubes racked in microfuge sliders for making additions to the tubes, for incubating the reaction, and for doing the extraction with alkaline butanol. Using this approach meant that tubes did not need to be racked and reracked to be centrifuged. In addition, eight tubes could be mixed at once with the vortex for the extraction. This mixing was facilitated by holding the tubes on their sides. Usually 50% of the butanol extract was spotted onto TLC plates. Before spotting, TLC plates were activated at 120° for at least 30 min and then allowed to cool to room temperature. Up to 50 μl was spotted at one time. If more than 50 μl of extract was spotted, the plates were heated in the oven at 120° for 5–10 min, allowed to cool to room temperature, and then respotted. Spotting was done with a Microman M-50. The positive displacement tips for this pipet were necessary to accurately deliver the butanol extract and fit into 400-μl tubes. A separate tip was used for blanks and samples. Between different samples, the tip was rinsed two to three times with H_2O. Using a separate tip for each sample did not improve the accuracy or reproducibility of the results. After the final spotting, plates were dried for 10 min in the oven, cooled to room temperature, and developed in chloroform–methanol–concentrated ammonium hydroxide, 12:7:1, at room temperature. Chromatographic development of TLC plates usually took about 45 min, but could take as much as 75 min on very humid days. The humidity also affected R_f values and the width of the visualized bands. However, the results remained unchanged. The R_f values also varied for different manufacturers' plates. Average

Table 1. Extraction of histamine from histidine into H_2O-saturated *n*-butanol*

Compound	Volume of extract (μl)	Extracted dpm × 10^{-3}	% Extracted
[^{3}H]HA	200	261 ± 7	72
[^{3}H]HA + 10 μg HA	200	290 ± 8	81
[^{3}H]HA + 10 μg HA	100	235 ± 6	65
[^{3}H]HA + 10 μg HA	300	289 ± 9	80
L-[^{3}H]His	200	122 ± 1	5
L-[^{3}H]His + 10 μg HA	200	123 ± 1	5

* Values are the mean ± S.E.M. of triplicates. Microfuge tubes contained the same reagents as assay blanks in 40 μl, except for either 359,000 dpm of [^{3}H]HA or 2,475,000 dpm of L-[^{3}H]His plus 5 μl of 4 N NaOH and 5 μl of absolute ethanol with or without 10 μg HA. Each tube was extracted with the indicated volume of H_2O-saturated *n*-butanol. After centrifugation of the tubes for 5 min in a Beckman Microfuge B, 50-μl aliquots of the butanol extracts were counted.

R_f values for L-His, HA, and tele-methylhistamine (tMHA) on Whatman LK5D plates were 0.15, 0.45, and 0.60 respectively. HA was visualized with a spray containing 0.2% ninhydrin in absolute ethanol. Heating the plates for 5 min at 120° was sufficient to develop the color. Using our TLC scraping device [11], a 19-channel plate took 4–5 min to scrape into counting vials. Next, 0.5 ml of a solution of 1 mg/ml of HA in absolute ethanol was added to the polypropylene vials, which were shaken on a New Brunswick Scientific Shaker at 100 rpm for 10 min. Finally, 5 ml of 3a20 scintillation fluid was added, and the radioactivity was determined in a scintillation counter.

Other investigators have reported that HA sticks to glass [14]; therefore, we exclusively used plastic tubes in preparing for and performing these HDC assays. In addition to HA sticking to glass, we observed a similar problem with L-His. This was a problem even when counting [^{3}H]HA and L-[^{3}H]-His. When aliquots of both isotopes were diluted with water in plastic tubes and then counted in both glass and polypropylene vials, the number of disintegrations per minute in glass vials was approximately 50% less than the number of disintegrations per minute in polypropylene vials for both [^{3}H]HA and L-[^{3}H]His.

HDC activities are reported at 0.5 μM exogenous L-His, which is subsaturating [15]. Subsaturating L-His was utilized to obtain maximal sample-to-blank ratios. Endogenous L-His was not removed from the homogenates or supernatant fractions. HDC activities were calculated based on the specific activity of the exogenous L-His added to the assay tubes and, therefore, the way in which the values are reported in this paper expresses relative differences. To obtain absolute values for HDC activities, one needs to either remove endogenous L-His or determine its concentration.

RESULTS

Several extraction conditions were tried to maximize the separation of [^{3}H]HA from L-[^{3}H]His (Table 1). Adding cold HA to the butanol extract helped the extraction of [^{3}H]HA and made it unnecessary to spot cold HA separately on the TLC plate for later visualization. Extracting with volumes of butanol beyond 200 μl did not improve the efficiency of the extraction. When the enzyme activity was high, extraction with 100 μl of butanol was sufficient. Isoamyl alcohol was 60% as effective as butanol in extracting the [^{3}H]HA, while chloroform was only 10% as effective (data not shown).

To calculate the activity of HDC, it was necessary to know the recovery of [^{3}H]HA from the TLC scrapings. Table 2 shows that adding HA to the ethanol improved the extraction of [^{3}H]HA. Extraction from Whatman LK5D plates was consistently about 10% better than from Baker 7009-4 plates. The Whatman LK5D plates are somewhat softer and, therefore, easier to scrape. Harder plates and plates with fluorescent indicators decreased extraction efficiency (data not shown).

Linearity with time for the conversion of L-[^{3}H]-His to [^{3}H]HA was examined using fetal rat liver supernatant fractions at both 37 and 45°. The HDC activity was 15% greater at 45° than at 37° and remained linear for at least 2 hr at both temperatures (Fig. 1).

The amount of HDC activity in the presence of various amounts of enzyme was examined (Fig. 2). There was a change in the slope of the line representing HDC activity. The break occurred at

Table 2. Recovery of histamine applied to thin-layer chromatography plates

Plate	HA (mg)	Extracted dpm × 10^{-3}*	% Extracted
Baker 7009-4	0	20.4 ± 0.9	28
Whatman LK5D	0	22.7 ± 0.3	31
Whatman LK5D	0.25	23.4 ± 0.6	32
Whatman LK5D	0.50	30.8 ± 0.4	42
Whatman LK5D	1.0	31.8 ± 0.1	44

* Values are the mean ± S.E.M. of triplicates. [^{3}H]HA (72,500 dpm) was applied to TLC plates and developed in chloroform–methanol–concentrated ammonium hydroxide (12:7:1). The HA was visualized with ninhydrin spray. The spots were scraped into polypropylene vials and extracted with 0.5 ml absolute ethanol with or without HA.

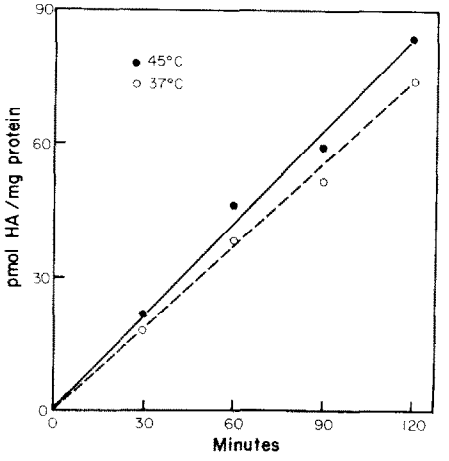

Fig. 1. Time course for HDC activity. Fetal liver 40,000 *g* supernatant fraction containing 60 μg protein/tube was assayed for up to 120 min as described in Materials and Methods. The points are the average of triplicates that varied less than 5%.

115 μg of protein, which corresponds to 20 μl of a supernatant fraction obtained from the homogenization of 100 mg of tissue in 2 ml. With 0.6 μM L-His and 230 μg protein at 45° for a half-hour incubation, about 7% of the substrate was converted to HA. HDC activity in brain homogenates was found to be linear up to at least 400 μg protein [16].

HDC has been shown to have a variable pH optimum dependent on substrate concentration [17]. Therefore, we determined the pH optimum under the assay conditions we described in Materials and Methods. Figure 3 illustrates a rather broad optimum from pH 7.0 to 7.8.

Enzyme activity measured *in vitro* is frequently dependent on the type and pH of buffer. For HDC, there was significantly more measurable enzyme activity with a phosphate buffer than with a sulfonic acid buffer at the same pH (Table 3). A potassium phosphate rather than a sodium phosphate buffer was used because potassium phosphate is soluble at higher concentrations, allowing storage of a more concentrated solution.

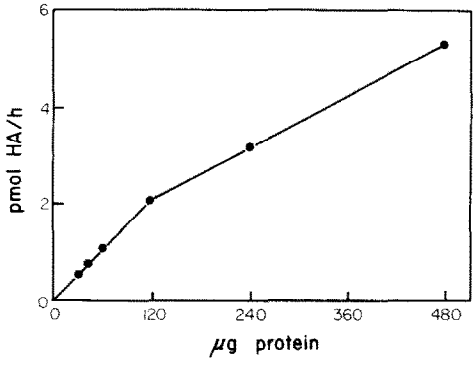

Fig. 2. Effect of protein concentration on the reaction rate. Various amounts of fetal liver 40,000 *g* supernatant fraction were incubated for 30 min at 45°. The points are the average of triplicates that varied less than 10%.

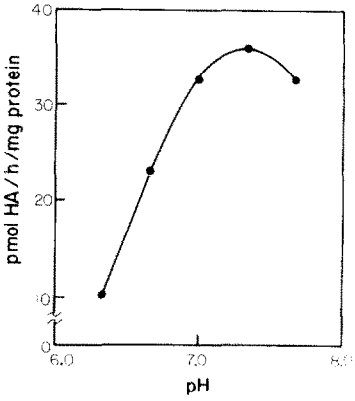

Fig. 3. Effect of various pH levels in potassium phosphate buffer on HDC activity. Fetal liver 40,000 *g* supernatant fraction was incubated for 120 min at 45°. The points are the average of triplicates that varied less than 10%.

Table 3. Effect of various buffers on fetal liver histidine decarboxylase activity

Buffer (50 mM at pH 7.0)	% of Maximum HDC activity*
Sodium phosphate	100
Potassium phosphate	96
Tris phosphate	80
MOPS	63
HEPES	56
TES	56

* HDC activity in sodium phosphate buffer is 54 pmoles HA/hr/mg protein. Each value is the mean of triplicates, which varied less than 10%.

Table 4 shows the effect of drugs known to influence the activity of HA metabolism. Although HA is predominantly formed from L-His by HDC, the nonspecific aromatic amino acid decarboxylase (AADC) can also decarboxylase L-His. α-Methyldopa is a potent inhibitor of the latter enzyme [3, 5]. Under our assay conditions, which include a low substrate concentration, there was no apparent conversion of L-His to HA via AADC. In peripheral

Table 4. Effect of various drugs on fetal liver histidine decarboxylase activity

Drug (100 μM)	HDC activity (pmoles HA/hr/mg protein)
Control*	56.3 ± 0.4
α-Methyldopa	55.5 ± 0.3
Aminoguanidine	32.3 ± 1.0§
Amodiaquin	59.6 ± 2.7
Impromidine	62.7 ± 0.7‡
α-Fluoromethylhistidine	1.5 ± 0.028

* Control was as described in Materials and Methods, except that no α-methyldopa or impromidine was included. Values are the mean ± S.E.M. of triplicates.

† $P < 0.005$, one-tailed *t*-test.

‡ $P < 0.05$, one-tailed *t*-test.

§ $P < 0.001$, one-tailed *t*-test.

Table 5. Histidine decarboxylase activity in rat fetal tissues at 19 days gestation

Tissue	HDC activity* (fmoles HA/hr/mg protein)
Brain	10 ± 0.1
Lung	218 ± 12
Heart	238 ± 15
Kidney	468 ± 15
Stomach	1,704 ± 238
Liver	13,255 ± 67

* Values are the mean ± S.E.M. from three different animals from two different litters.

tissues, HA is catabolized by HNMT and DAO [13]. Aminoguanidine is a DAO inhibitor [18], and our data indicated it also had a marked inhibitory effect on HDC activity. Amodiaquin is an HNMT inhibitor that did not inhibit HDC and did little to prevent HA from being catabolized. The apparent HDC activity was increased in the presence of impromidine, which has been reported to be an effective HNMT and DAO inhibitor [13]. In addition to obtaining more disintegrations per minute in the HA spot when impromidine was included in the assay, we found fewer disintegrations per minute in the spot corresponding to tMHA (data not shown). α-FMH is a specific irreversible inhibitor of HDC [19] and, as can be seen from Table 4, it almost totally inhibited our fetal liver HDC.

This assay can be used to measure HDC activity in fetal rat brain, which has very low activity (10 fmoles/hr/mg protein), as well as in fetal rat liver which has high activity (13,255 fmoles/hr/mg protein, Table 5). The lowest activity in 19-day rat fetuses was observed in brain and the highest in liver. Fetal rat lung, heart, kidney, and stomach contained much lower HDC activity than liver. However, fetal stomach had HDC activity about six times the activity of the lungs, heart, and kidneys. These data were consistent with those of Kahlson *et al.* [20], except that the latter authors reported relatively more activity in the lung.

DISCUSSION

The HDC assay described in this report resulted in marked improvements in the reliability and economy of the assay, while maintaining high sensitivity. As little as 5 fmoles of [^{3}H]HA was recovered with twice the disintegrations per minute found in the blank. The improvement in variability can probably be attributed to the combination of the alkaline butanol extraction followed by TLC. Using our recently designed TLC scraping system [11], the ninhydrin spots were rapidly and efficiently scraped from TLC plates, allowing more than 100 assay tubes per day to be processed. A System 200 Imaging Scanner from Bioscan, Inc. (Washington, DC) counts tritium on TLC plates directly, which would potentially increase the efficiency of the assay further.

Yamada *et al.* [21] reported that protease inhibitors, including PMSF, prevented inactivation of HDC activity in several tissues. HDC activity in fetal rat liver was the same in the presence or absence of PMSF, with our assay. Since PMSF did not inhibit HDC activity in fetal rat liver and since it might provide some protective effect for other tissues, we routinely included PMSF in our homogenizing buffer.

Aures and Hakanson [22] have reported HDC from several tissues to be stable at 55° for a short time. Since we wanted to maximize the sensitivity of our HDC assay, we examined HDC activity at 37, 45, and 50°. As shown in Fig. 1, fetal liver HDC was linear for at least 2 hr even at 45°. Linearity was observed for only 30 min at 50°. In a study of HDC in brain hypothalamus, we also observed more activity at 45° than at 37° [16]. However, in brain there was a lag in HDC activity measured at 37° until 30 min, which was not observed at 45°. No lag was observed in fetal liver HDC at either temperature. Two possible explanations are differences in enzyme structure or differences in endogenous L-His concentrations.

The pH optimum of 7.4 at 0.5 μM L-His for fetal rat liver HDC is similar to that obtained by Hakanson [17]. He reported that, from 0.1 to 10 μM L-His, the pH optimum was quite constant and broad for HDC activity. We also found a broad pH optimum at low substrate concentration. Most investigators assay HDC using sodium or potassium phosphate buffers, which were definitely preferable to sulfonic acid buffers.

Since the affinity of AADC for L-His is about thirty times lower than that for HDC [3] and since the concentration of L-His in our routine HDC assays is many times below the K_m of AADC for L-His, it was not surprising that α-methyldopa did not inhibit the conversion of L-His to HA. Including impromidine increased the sensitivity of the assay somewhat by preventing a small amount of the newly formed L-[^{3}H]His from being metabolized to [^{3}H]-tMHA. These compounds are separated by TLC. Because α-FMH markedly inhibited the formation of HA from L-His and α-methyldopa did not inhibit the formation of HA from L-His, it is clear that what we were measuring with this assay was specific HDC and not AADC.

The usefulness of this assay for studying tissues with very low and very high HDC activity was demonstrated by our ability to measure fetal rat brain and fetal liver HDC activity. There is a 1300-fold difference in activity for these tissues. Since the K_m of HDC for L-His is in the range of 200–400 μM [15] and since endogenous L-His from the tissue preparations would not result in the actual concentration of L-His being greater than K_m, the activities reported in Table 5 are reflective of relative differences in HDC in various fetal tissues. Although we did not do a kinetic study of HDC in various fetal tissues, we have reported the kinetics of HDC in adult rat brain hypothalamus using this assay [16].

This method for assaying HDC is highly advantageous compared to any other reported method in terms of simplicity, sensitivity, reproducibility, reliability, and economy. Although the $^{14}CO_2$-trapping method is less time-consuming than this assay, it has the serious problem of the formation of CO_2 by routes other than direct decarboxylation, and is

approximately 10,000 times less sensitive [3]. The sensitivity of this assay is at least as good as any other described in the literature [21], yet requires only 1 μCi to assay as little as 5 fmoles of HA formed. Generally, purification of L-[^{3}H]His is not necessary to obtain a sensitivity of 5 fmoles. Replicates were usually within 5% of each other. Chances for variability with TLC seem to be less than for column chromatographic assays. This may be because there are larger differences between batches of resins used in column chromatography and because there are more places for error. With the assay procedure described here, measuring HDC activity in tissues with very low amounts of HDC can be reliably and conveniently accomplished.

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