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HISTIDINE DECARBOXYLASE MEASUREMENT IN BRAIN BY $^{14}CO_2$ TRAPPING

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Abstract—A method for measuring histidine decarboxylase (HDC) in crude rat brain homogenates was developed by modification of existing $^{14}CO_2$-trapping methods. The addition of EDTA to tissue homogenates and assay buffer reduced non-enzymatic decarboxylation, and improved assay sensitivity and reliability. Addition of polyethylene glycol (molecular weight 300, PEG300) to the homogenizing buffer increased enzyme stability, permitting storage of crude homogenates. Studies of time course, tissue dilution and blanks showed that up to 8 mg of tissue could be assayed successfully with a 3.5-hr incubation. *S*-α-Fluoromethylhistidine (FMH) and α-hydrazinohistidine, specific inhibitors of HDC, induced concentration-dependent reductions of enzyme activity by up to 90%, whereas inhibitors of other decarboxylases had little or no effect. Kinetic studies of the enzyme in crude homogenates yielded K_m and V_{max} values similar to those found previously with other HDC methods, although a poor fit was found to a single enzyme model. When determined by the new method, the distribution of HDC in seven regions of the rat brain agreed well with previous results. The method is rapid, simple to perform, and requires no specialized equipment other than a scintillation counter.

Histidine decarboxylase (HDC; EC 4.1.1.22) is thought to be the sole enzyme of histamine biosynthesis in organisms. In brain, where histamine functions as a neurotransmitter or neuromodulator [1–3], virtually all histamine-containing neurons also contain HDC, as assessed by independent immunochemical methods (see [1]). Thus, HDC is thought to be an excellent marker for histaminergic neurons.

Although a number of different methods have been used for measuring HDC, determination of this enzyme in brain homogenates is especially challenging because of the small amounts of enzyme present, the lability of the enzyme during storage and purification [4], and the distinct problems associated with measuring the enzyme's product, histamine. HDC assays performed by measuring endogenous histamine before and after incubation with the substrate histidine suffer from several drawbacks. These methods either lack sufficient sensitivity to measure the enzyme in small brain areas without pooling tissues (e.g. histamine determinations by spectrofluorometry [5, 6]), utilize an additional enzymatic methylation step [7–9], or require a complex, post-column derivatization HPLC system [10]. A radiochromatographic procedure employing [^{3}H]histidine incubations and subsequent isolation and quantification of the isotopic histamine formed [11] has been used extensively, although this method uses column purification of each sample assayed, and requires very low concentrations of labeled substrate for adequate analysis of small brain regions.

One of the simplest and most generally accessible methods for assaying any decarboxylase activity is the measurement of $^{14}CO_2$ release from the appropriate ^{14}COOH-labeled amino acid, a method first implemented by Kobayashi [12], and later improved by Beaven *et al.* [13]. However, this method was reported to be unsuitable for assay of brain HDC due to a lack of sensitivity (see Discussion in [6]). We have modified the $^{14}CO_2$-trapping method of Beaven *et al.* [13] and show that these modifications now permit the routine analysis of HDC in crude homogenates of brain tissue.

METHODS

Male Sprague–Dawley albino rats (200–500 g, Taconic Farms, Germantown, NY) were maintained in a 12-hr light–dark cycle and used for all experiments. Animals were decapitated and the brain regions were dissected, weighed and homogenized in 4 vol. of ice-cold buffer containing 0.2 mM dithiothreitol, 0.1 mM disodium EDTA and 1% polyethylene glycol (molecular weight 300, PEG300) in 0.1 M sodium phosphate, pH 6.8. The same buffer was also used for the HDC assay. The homogenate was either used immediately, or stored at −80° with a maximum of one thawing cycle.

HDC activity was measured by modifications of the $^{14}CO_2$-trapping method of Beaven *et al.* [13]. Brain homogenates equivalent to 8 mg of tissue (or less), along with any inhibitors, were pipetted into ice-cold 1.5-mL conical polypropylene Eppendorf tubes (total volume = 50 μL). To trap the $^{14}CO_2$ formed, the trapping agent Protosol® (20 μL, New England Nuclear, Boston, MA) was delivered to the bottom of 20-mL glass scintillation vials, and the

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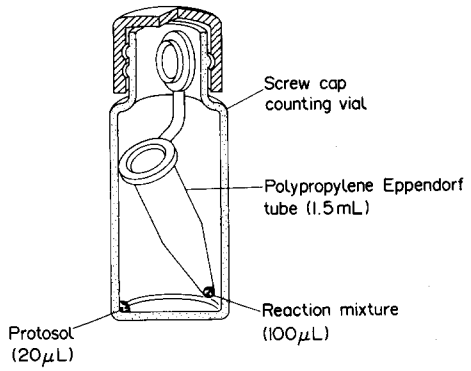

Fig. 1. $^{14}CO_2$-trapping technique used for measurement of brain HDC. Modified from Beaven *et al.* [13] and used with permission.

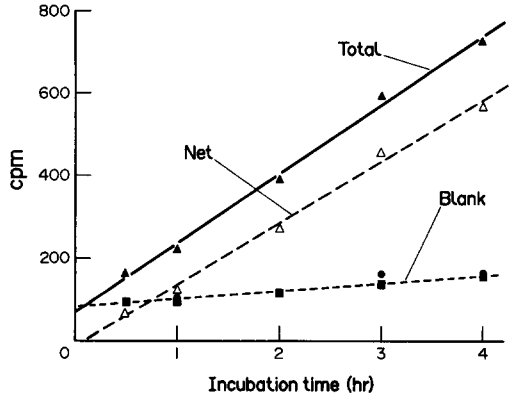

Fig. 2. Time course of $^{14}CO_2$ formation from [^{14}COOH]L-histidine. The HDC assay was performed as described with intact hypothalamic homogenate [8 mg tissue, (▲), labeled "Total"], boiled homogenate [10 min in boiling water, (●)], or buffer alone (■) for the incubation shown (abscissa). Data points represent mean cpm (ordinate) of duplicate determinations from one experiment. The experiment was repeated several times with similar results. Net cpm values (△) were obtained by subtracting total cpm for each time minus the buffer blank value at that time. The lines drawn were fitted by linear regression.

uncapped Eppendorf tubes containing homogenates were then placed into these vials (see Fig. 1). The scintillation vials were capped and preincubated in a shaker bath at 37° for 30 min. To start the reaction, aliquots (50 μL) of a reaction mixture containing [^{14}COOH]L-histidine (0.1 μCi/tube, New England Nuclear), unlabeled L-histidine (diluted from a stock solution stored in 0.01 M HCl) and pyridoxal 5'-phosphate (weighed out for each experiment) were added to each Eppendorf tube to achieve a final concentration of 29.4 μM L-histidine (total of labeled and unlabeled) and 10 μM pyridoxal phosphate in a total of 0.1 mL of buffer. The glass vials were capped tightly and incubated with shaking at 37° for up to 210 min. The reaction was terminated by addition of 2 N perchloric acid (50 μL), after which the scintillation vials were recapped, and the tubes allowed to incubate another 30 min at 37° to trap any remaining $^{14}CO_2$. The Eppendorf tubes were then removed, and 15 mL of scintillation fluid (Formula-963, New England Nuclear) was added. The vials were mixed and counted (5 min each) in a liquid scintillation spectrometer (Beckman LS-3801) with a ^{14}C window (0–670) for two cycles. Data from the first cycle were discarded. Enzyme blanks consisted of both reagent blanks (incubation of vials without enzyme) and tissue blanks (homogenate in boiling water for 10 min).

Data from substrate–velocity studies were fitted to both single enzyme and dual enzyme models. Fits to the single enzyme model were performed by linear regression of the data plotted as an Eadie–Hofstee plot (V/S vs V). Fits to the dual enzyme model were performed with the RS/1 procedure $FITFUNCTION, an iterative algorithm utilizing non-linear regression to yield estimates of two K_m (K_1, K_2) and two V_{max} (V_1, V_2) values [14].

RESULTS

Initial experiments confirmed earlier studies that brain HDC could not be measured accurately by previously described $^{14}CO_2$-trapping methods (see Discussion in [6]). Under these conditions (i.e. without EDTA in the buffer), high, inconsistent blank values (in the range of 400 to 900 cpm) were obtained (not shown). Addition of EDTA (0.1 mM) to the enzyme buffer lowered the blank considerably and reduced variability in blank values (see below). The assay sensitivity was not changed by variations in EDTA concentrations (0.01 to 1 mM, also not shown).

When the assay was performed as presently described, $^{14}CO_2$-trapping activity in hypothalamic homogenates was linear with incubation time from 0.5 to 4.0 hr (Fig. 2), justifying the routine use of 3.5-hr incubations. Buffer blanks and boiled homogenates yielded nearly identical values (about 100 cpm, Fig. 2); incubation of intact enzyme at 4° also gave values similar to blank values (not shown). A slight, but detectable increase in blank values was found with increasing incubation times (Fig. 2), indicating the existence of a small amount of non-enzymatic decarboxylation. The fitted intercept for the total cpm curve (implying zero-time activity) was very similar to blank values, allowing for an intercept of approximately zero in the net cpm curve (Fig. 2). Activity was also linear with the amount of enzyme used (equivalent to 2–10 mg of tissue, Fig. 3), justifying the routine use of 8 mg of tissue. Under these conditions (8 mg tissue, 3.5 hr), hypothalamic homogenates typically yielded 630–860 cpm, with blank values ranging from 80 to 125 cpm. Based on these results, enzyme activity was calculated as counts per minute from intact samples minus counts per minute from reagent blanks, and expressed as picomoles of CO_2 produced per gram of tissue per hour.

The effects of several decarboxylase inhibitors were studied on the $^{14}CO_2$-trapping activity. *S*-α-Fluoromethylhistidine (FMH), the highly selective,

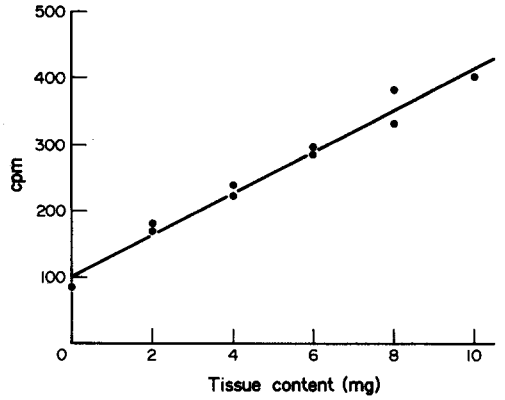

Fig. 3. Effect of tissue dilution on $^{14}CO_2$ formation from [^{14}COOH]L-histidine. Hypothalamic homogenates (equivalent to 0–10 mg tissue, abscissa) were assayed for HDC as described (2-hr incubation). Each point represents cpm obtained (ordinate) from an individual test tube. The experiment was performed a total of four times with similar results. The line drawn was fitted by linear regression.

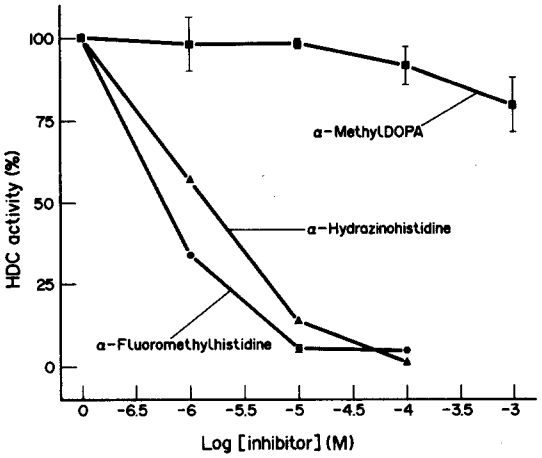

Fig. 4. Inhibition of rat hypothalamic HDC by decarboxylase inhibitors. Homogenates of rat hypothalamus were assayed for HDC (ordinate is percent of control activity) in the presence of increasing concentrations of inhibitors (abscissa). The HDC inhibitors α-fluoromethylhistidine and α-hydrazinohistidine, as well as the DOPA decarboxylase inhibitor α-methylDOPA, were preincubated for 30 min with homogenate and then assayed for HDC as described. Control activity was 249.9 ± 18.6 pmol/g/hr. Data points are either mean ± SEM (N = 3–5 for FMH and α-methylDOPA) or the mean of two duplicate experiments whose values ranged from 3 to 15% of the mean.

suicide inhibitor of HDC, induced a concentration-dependent inactivation of up to 95% of hypothalamic enzyme activity after a 30-min preincubation, with an estimated EC_{50} of less than 1 μM (Fig. 4). Another HDC inhibitor, α-hydrazinohistidine, showed similar inhibition with slightly less potency (Fig. 4). In contrast, α-methylDOPA, the inhibitor of DOPA decarboxylase, had no significant effect on enzyme activity up to 10^{-4} M (Fig. 4). In addition, α-difluoromethylornithine and chelidonic acid, inhibitors of ornithine [15] and glutamate [16] decarboxylases, respectively, reduced activity by only 11 and 6%, respectively, when tested as in Fig. 4 at 10^{-4} M (data not shown, results are means from two separate experiments).

Kinetic studies of the enzyme in crude hypothalamic homogenates were also performed (Fig. 5). When the existence of a single enzyme was assumed, linear regression of the Eadie–Hofstee plot yielded K_m and V_{max} values of 540 μM and 5715 pmol/g/hr, respectively. However, the fit of these data to a single enzyme model was poor, and a better fit was obtained when a dual enzyme model was used (Fig. 5).

The distribution of the $^{14}CO_2$-trapping activity was determined in crude homogenates of nine regions of rat brain (Fig. 6). Regional activity varied over 10-fold from lowest values in the cerebellum (12 pmol/g/hr) to the highest activity in hypothalamus (219 pmol/g/hr). Activity was nearly the same in the caudate nucleus, hippocampus, cerebral cortex and in the remainder of the whole brain.

DISCUSSION

The results demonstrate that brain HDC can be measured accurately by the present $^{14}CO_2$-trapping method. The studies of time course (Fig. 2), tissue dilution (Fig. 3), and blanks show that the activity measured is enzymatic, whereas the remainder of the results demonstrate that the enzyme measured is HDC.

The sensitivity of the enzyme to FMH and α-hydrazinohistidine (Fig. 4) was very similar to results from previous studies of the enzyme measured by other methods. For example, EC_{50} values for FMH against fetal, brain and stomach HDC were reported to be in the low micromolar range [4], and concentrations of FMH greater than 10 μM caused nearly complete inhibition of HDC from brain [18], as well as from other tissues [19]. Similarly, α-hydrazinohistidine (10^{-4} M) was found previously to inhibit rat brain HDC by 89% [7] to 100% [6]. The slight inhibition of HDC by high concentrations of α-methylDOPA (Fig. 4), also observed in previous studies [5–7], is almost certainly due to inhibition of HDC by this substance, since α-methylDOPA effectively inhibits DOPA decarboxylase at much lower concentrations [20].

When our substrate–velocity results (Fig. 5) were fitted to a single enzyme model, the resulting K_m and V_{max} values resembled those reported by others for brain HDC. The K_m found for histidine (540 μM, Fig. 5) is in good agreement with the value of 400 μM, reported for crude homogenates of rat whole brain or hypothalamus [5, 6, 21]. Similarly, our V_{max} value for hypothalamic HDC (5.7 nmol/g/hr) is in agreement with previous results. Taylor and Snyder [7] found rat hypothalamic HDC activity at 0.1 M histidine (a concentration 250 times above the K_m, and thus very close to V_{max}) to be 28.8 nmol/hr/g protein, which is approximately 1.3 nmol/hr/g tissue. Schwartz *et al.* [6] reported a hypothalamic HDC value slightly below 6 nmol/g/hr at a substrate

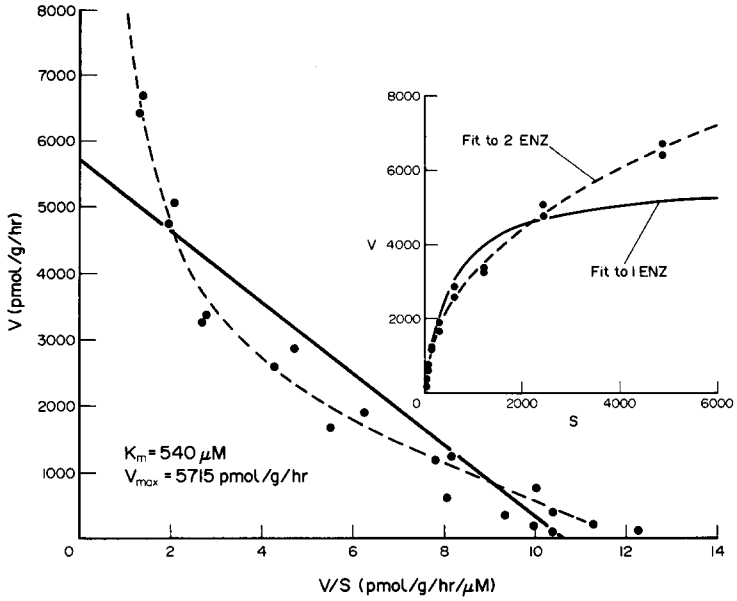

Fig. 5. Kinetic studies of rat hypothalamic HDC. Enzyme activity (V, pmol/g/hr) was determined as described in the presence of 0.1 μCi [^{14}C]histidine and various non-isotopic histidine concentrations, with total histidine concentrations used to define substrate concentration (S). Results are shown as an Eadie–Hofstee plot (V/S vs V). Shown are data from three different experiments, with each point representing the mean of duplicate determinations from a single experiment. The solid line shows the fit to a single enzyme model by linear regression (K_m = 540 μM, V_{max} = 5715 pmol/g/hr). The dashed line shows the same data fit to a two-enzyme model by non-linear regression (see Methods), which yielded two sets of kinetic constants (K_1, K_2 = 236, 9600 μM; V_1, V_2 = 2558, 12,319 pmol/g/hr). Inset: the same data and fitted functions are plotted as S vs V.

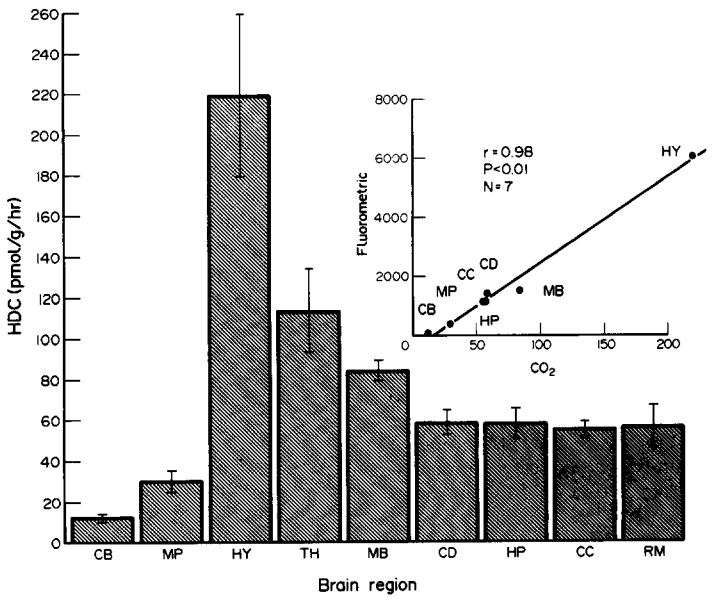

Fig. 6. Regional distribution of HDC activity in rat brain. HDC activity was determined by the CO_2-trapping method in nine regions (abscissa) of the rat brain. Bars shown are means ± SEM of HDC activity (ordinate) in each region (N = 4 animals). Abbreviations and average protein content (mg/g tissue [17]) for the regions are: CB, cerebellum (57); MP, medulla-pons (54); HY, hypothalamus (44); TH, thalamus (57); MB, midbrain (65); CD, caudate nucleus (44); HP, hippocampus (52); CC, cerebral cortex (61); and RM, remainder of brain (62). Inset: Correlation of the present regional brain HDC activity (abscissa) with regional HDC activity previously determined by a fluorometric method [6] (ordinate). The line drawn was determined by linear regression.

concentration of 10 mM. However, the present data are not persuasive of the existence of a single non-cooperative enzyme, since the HDC activity observed at higher substrate concentrations was greater than expected (Fig. 5). Previous kinetic studies of crude brain homogenates either did not include the actual data, or were not detailed enough to identify this pattern [5, 6, 21]. A K_m for histidine of 340 μM was reported from a purified rat brain HDC preparation, although no kinetic data were shown [4]. Multiple forms of HDC in gastric preparations have been observed by isoelectric focusing, although only a single form was observed in brain homogenates [4]. Clearly, more detailed kinetic studies of purified HDC are needed in order to clarify these results.

The regional distribution of HDC activity presently measured in rat brain (Fig. 6) is in very good agreement with previous results from a study utilizing a fluorometric method (Fig. 6 inset). A much higher substrate concentration was employed by the latter method than used presently, explaining why absolute regional values are higher (see discussion of substrate concentrations, below). Furthermore, in contrast to the present method, tissues were pooled to be able to measure HDC in some brain regions due to a lack of sensitivity of the fluorometric method [6].

Although the present method differs only slightly from that described by Beaven *et al.* [13], our results show that the changes made are necessary for the accurate assay of HDC in brain. Addition of EDTA to the assay buffer caused a lower and more consistent reagent blank, implying the existence of a metal-catalyzed non-enzymatic degradation of histidine, which results in CO_2 liberation. Although the apparent role of metals was not heretofore recognized, previous studies also found evidence for non-enzymatic CO_2 production from histidine [22, 23]. Curiously, histamine was not found as a co-product, and the reaction was reported to be accelerated by the addition of either pyridoxal phosphate, epinephrine or ascorbate [22, 23]. The reported stimulation of this reaction by reducing agents is particularly intriguing, since we found a dramatic increase in reagent blank values after addition of hydrogen peroxide, an oxidation reagent (unpublished). Whatever the reaction is, conditions for it are favored in boiled homogenates, since boiled samples prepared without EDTA often showed more CO_2 liberation than unboiled aliquots containing HDC (our unpublished studies, also found by others [22]). In the presence of EDTA, however, boiled samples behaved as reagent blanks (Fig. 2). Imidazoles like histidine are known to bind some metals with good affinity [24]. Thus, in the absence of EDTA, a histidine–metal complex may undergo a small amount of non-enzymatic chemical destruction. The reaction need not be quantitatively very important for it to reduce dramatically the assay sensitivity by increasing blank values. Further studies are needed to identify the metals involved. EDTA has been used previously during purification and assay of HDC without any apparent justification [21, 25], although CO_2-trapping was not employed in the assay of brain HDC in these studies.

Since the nature of the chemical reaction(s) that gives rise to the blank values in this assay remains poorly understood, caution must be used in extrapolating results from brain homogenates to other tissues. Even in the presence of EDTA, tissue components other than HDC may be able to contribute to blank values, and it should not be assumed without verification that the method is suitable for measuring HDC in tissues other than brain.

We have also included in the homogenizing buffer PEG300, a substance found to stabilize purified fractions of HDC [26]. Although extensive stability studies have not been performed, preliminary results found that brain homogenates prepared as described with PEG300 showed no loss of HDC activity when stored up to 10 weeks at −80°. Even under these conditions, enzyme loss was evident with repeated freezing and thawing. In the absence of PEG300, HDC activity was rapidly lost over a few days of storage at this temperature even with only a single thawing (unpublished results).

In the present method we have used the trapping agent Protosol® instead of hyamine hydroxide [13], the former having been found to be superior for CO_2-trapping studies [27]. Even though the Protosol® is added as a 20-μL drop as shown in Fig. 1, it often runs all the way around the bottom of the vial. Because of this, the Protosol® solution sometimes evaporates to dryness. In controlled experiments, we found that evaporation of the Protosol® solvent had no effect on the trapping efficiency when compared to non-evaporated replicates (unpublished results). It should also be noted that glass scintillation vials are available with either aluminum foil or polypropylene (gas tight) caps. We found no difference in CO_2 recovery between these types of caps (unpublished results), and thus recommend the less costly foil caps.

As with all radiometric enzymatic assays, the sensitivity of the CO_2-trapping method depends on the substrate concentration used. Although lowering the substrate concentration in the presence of a fixed amount of isotope will increase assay sensitivity [28], endogenous histidine levels in the homogenate must also be considered. If the amount of exogenous histidine is not large compared to the levels contributed by the homogenate, variations in endogenous histidine levels could change the specific activity of the substrate, with ensuing errors in estimating HDC activity. Assuming an average brain histidine level of 15 ng/mg tissue [7], it can be calculated that, under the present conditions, brain histidine levels contribute approximately 7 μM histidine (in addition to the 29 μM histidine added). Under these circumstances, a treatment that incidentally resulted in a 30% change in endogenous histidine levels (a very large change) would change the apparent HDC activity by less than 10%. For these reasons, the use of histidine concentrations lower than 29 μM is not recommended for assays of crude homogenates at these tissue dilutions.

The present CO_2-trapping method has several advantages over other methods for measuring HDC in brain. The method is simple, since it does not require solvent–solvent extraction or column chromatography, and the only instrument needed is a scintillation counter. Despite the 3.5-hr incubation,

the method is not time-consuming: a 40-tube assay can be completed in 5–6 hr.

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