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α-FLUOROMETHYLHISTIDINE-INDUCED INHIBITION OF BRAIN HISTIDINE DECARBOXYLASE

IMPLICATIONS FOR THE CO_2-TRAPPING ENZYMATIC METHOD

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Abstract—The actions of *S*-α-fluoromethylhistidine (FMH), an irreversible inhibitor of the histamine biosynthetic enzyme histidine decarboxylase (HD), were studied on rat brain HD, as measured by a recently developed CO_2-trapping enzymatic method. As expected, FMH induced a virtually complete inhibition of HD in the hypothalamus both *in vivo* and *in vitro*. In the frontal cortex, however, maximal doses of FMH did not maximally inhibit HD, suggesting the existence of an FMH-resistant form of HD. Careful studies of the conditions under which the assays were performed (homogenate dilution, preincubation times, incubation times, temperatures), as well as experiments with inhibitors of other decarboxylases, were unable to provide an explanation for this. When comparable studies of the effects of FMH in these brain regions were performed by alternative methods for measuring HD activity, no evidence for the existence of an FMH-resistant form of HD could be found. Thus, even though the CO_2-trapping method appears to be accurate for measuring HD activity in rat hypothalamic homogenates, the present results show that this method may not be specific when studying brain regions other than the hypothalamus.

Key words: brain, histamine, histidine decarboxylase, α-fluoromethylhistidine

Histidine decarboxylase (HD†, EC 4.1.1.22) is the principal enzyme responsible for the synthesis of histamine in biological systems [1]. Although the central nervous system contains histamine in more than one type of cell [2], HD is a known marker for histaminergic neurons [3, 4]. Biochemical measurements of HD activity have been complicated by low levels of the enzyme in brain, as well as by difficulties in measuring the enzymatic product, histamine.

As part of a search for a convenient, sensitive and accurate method for measuring brain HD activity, our laboratory recently described modifications of a CO_2-trapping method which appeared to meet these goals [5]. As assessed by this method, HD activity in the rat hypothalamus was abolished by boiling, was suppressed completely by HD inhibitors such as *S*-α-fluoromethylhistidine (FMH), was resistant to the effects of other decarboxylase inhibitors, and yielded kinetic parameters known to be consistent with this enzyme [5]. Furthermore, the method yielded a regional distribution of HD in rat brain that was highly correlated with that found by a different HD method [5]. Presently, we report detailed studies of the effects of the irreversible HD inhibitor FMH on rat brain HD activity as assessed by this CO_2-trapping method. Since some of the findings were unexpected, critical experiments were repeated with two other methods for measuring HD activity.

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† Abbreviations: ANOVA, analysis of variance; FMH, *S*-α-fluoromethylhistidine; HD, histidine decarboxylase; HMT, histamine methyltransferase; and PLP, pyridoxal 5′-phosphate.

MATERIALS AND METHODS

Male Sprague–Dawley albino rats (160–400 g, Taconic Farms, Germantown, NY) were housed (2–3 animals per cage) and maintained in a 12-hr light–dark cycle with food and water freely available. For *in vivo* experiments, animals were injected with *S*-α-fluoromethylhistidine monohydrochloride hemihydrate (FMH, 100 mg/kg, i.p., or saline) and were decapitated 1–24 hr later. The *in vivo* experiments were designed so that animals were killed 2–7 hr into the dark portion of their light–dark cycle. For *in vitro* experiments, animals received no treatment and were killed at various times during the light portion of their cycle. No differences in FMH-sensitivity were found between light- and dark-cycle animals (data not shown). Brains were rapidly removed, placed on a chilled plate, and dissected. Regions were weighed and homogenized in 0.16 mL (hypothalamus) and 1.0 mL (frontal cortex) of ice-cold buffer containing 0.2 mM dithiothreitol, 0.1mM disodium EDTA and 1% polyethylene glycol (average mol. wt 300) in 0.1 M sodium phosphate, pH 6.8. This buffer ("CO_2-trapping buffer") was also used in other assays as described below. The homogenate either was used immediately, or was stored at −80° for up to 2 weeks. Upon thawing, homogenates were used only once before discarding; subsequent refreezing and thawing was avoided because this was reported to result in reduced HD activity [5]. Homogenates were assayed for HD activity by one of three methods, as described below.

CO_2-trapping assay of HD activity. HD activity measured by this method was performed essentially as described in our previous report [5]. Aliquots of homogenates equivalent to 9–15 mg of tissue were preincubated with or without FMH at 37° for 30 min in a total volume of 0.05 mL. The CO_2-trapping agent Solvable (NEN Research Products, Boston, MA) was found to be equivalent to Protosol, used previously [5]. To start the reaction, aliquots (0.05 mL) of a reaction mixture containing [^{14}COOH]-L-histidine (0.04 μCi/tube, NEN Research Products), unlabeled L-histidine (diluted from a stock solution stored in 0.01 M HCl) and pyridoxal 5′-phosphate (PLP, weighed out for each experiment) were added to each microcentrifuge tube to achieve final concentrations of 13.6 μM L-histidine (total of labeled and unlabeled) and 10 μM PLP in a total of 0.1 mL of CO_2-trapping buffer. Following a 3 or 3.5 hr incubation at 37°, the reaction was terminated with perchloric acid (2 N, 0.05 mL), and the trapped labeled CO_2 was counted as described [5]. Enzyme activity was calculated as counts per minute from intact samples minus counts per minute from reagent blanks, expressed as picomoles of CO_2 produced per gram of tissue per hour.

Radioenzymatic assay of HD activity. This assay, based on the formation and detection of non-isotopic histamine from non-labeled histidine, was adapted from that described by Taylor and Snyder [6]. In this method, the histamine content (determined by the radioenzymatic histamine method described below) was measured in each sample before and after a 3-hr incubation with non-labeled histidine. Brain homogenates were preincubated with FMH and incubated with non-labeled histidine exactly as described above except that: (a) isotopic histidine was omitted and the total histidine concentrations were varied (see Table 1), and (b) the histamine methyltransferase (HMT) inhibitor metoprine (1 μM final concentration) was included in all samples. For each enzyme determination, activity was terminated by the addition of perchloric acid to different aliquots either before or after incubation with histidine to determine and correct for the histamine present before incubation. Following incubation, homogenates were diluted (1:10) with water, and aliquots (0.03 mL) were assayed in triplicate for histamine by a single isotope radioenzymatic method. This assay, based on the addition of a [^{3}H]-methyl group from labeled *S*-adenosyl-L-methionine (NEN Research Products) to histamine, was performed exactly as described [7]. HMT was purified from rat kidney as described by Bowsher *et al.* [8] up to the Sephadex G50 step, with a 148-fold purification. The histamine level in each set of triplicates was corrected for assay recovery, which was determined by the addition of histamine standards (0.05 ng) to a second set of triplicates for each unknown. Recovery (mean ± SEM) was 35.9 ±2.6%, calculated from a total of 32 separate determinations of recovery (each utilizing six tubes as above) from a total of three separate experiments.

Since a portion of the histamine formed during incubation could be methylated (and thus be undetected in this assay), it was necessary to inhibit the enzyme HMT. Pilot experiments showed that the concentration of metoprine used (1 μM) inhibited the metabolism of histamine formed during the HD incubation, and yet allowed the determination of histamine by methylation after the 1:10 dilution of the samples.

Radiochromatographic assay of HD activity. This method, based on the enzymatic formation, separation and detection of [^{3}H]histamine after incubation of homogenates with [^{3}H]histidine, is similar to that described by Baudry *et al.* [9]. Brain homogenates were prepared as described above in 0.01M sodium phosphate buffer, pH 7.4, and assayed in 1.5-mL plastic Eppendorf tubes. The incubation mixture contained [^{3}H]L-histidine (0.09 μM, 50.7 Ci/mmol, NEN Research Products), PLP (8.9 μM), and unlabeled histamine (88.9 μM), with or without FMH (100 μM) in a total volume of 0.045 mL. Samples were incubated at 37° for 2 hr; separate experiments confirmed the linearity of the reaction up to this time. The reaction was terminated by the addition of 0.1 mL of 0.68 N perchloric acid containing 0.3 M non-labeled histidine. Samples then received 0.1 mL of 0.1 M KOH, and were diluted to a total volume of 1.0 mL with 0.05 M Tris–HCl buffer, pH 8.0. Following centrifugation, the supernatant fractions were added to a packed Bio-Rex 70 (50–100 mesh, Bio-Rad, Richmond, CA) cation exchange column (1 mL packed volume in polypropylene Econo-Columns, Bio-Rad) previously equilibrated with the Tris–HCl buffer. Each column was washed successively with 4 mL of 0.02 N HCl, three additional HCl washes (0.02 N, 10 mL each), and 2 mL of 1 N acetic acid. Labeled histamine (and possibly methylhistamine) was eluted from the columns by the further addition of 4 mL of 1 N acetic acid, which was collected directly into glass scintillation vials. Each vial then received 15 mL of scintillation fluid (Formula 963, NEN Research Products) and was then counted in a scintillation counter (Beckman LS-3801). Counts in each sample were corrected for reagent blank and histamine recovery. The reagent blank, measured by taking labeled histidine through the procedure without enzyme, was found to be 0.6% of the histidine added. The average recovery of labeled histamine added to the column was 58%.

In some cases, data were analyzed by analysis of variance (ANOVA) followed by comparisons with Student's *t*-test (CSS Statistica, Tulsa, OK). All procedures were reviewed and approved by the Albany Medical College Institutional Animal Care and Use Committee.

RESULTS

Systemic administration of FMH (100 mg/kg) resulted in a rapid, nearly complete inhibition of hypothalamic HD activity as assessed by the CO_2-trapping method (Fig. 1). The inhibition remained nearly 90% for 6 hr, after which partial recovery was observed at 12 and 24 hr. FMH also induced a rapid inhibition of HD in the frontal cortex, with a time course similar to that of the hypothalamus (Fig. 1). However, this inhibition (54% at 1 hr) was

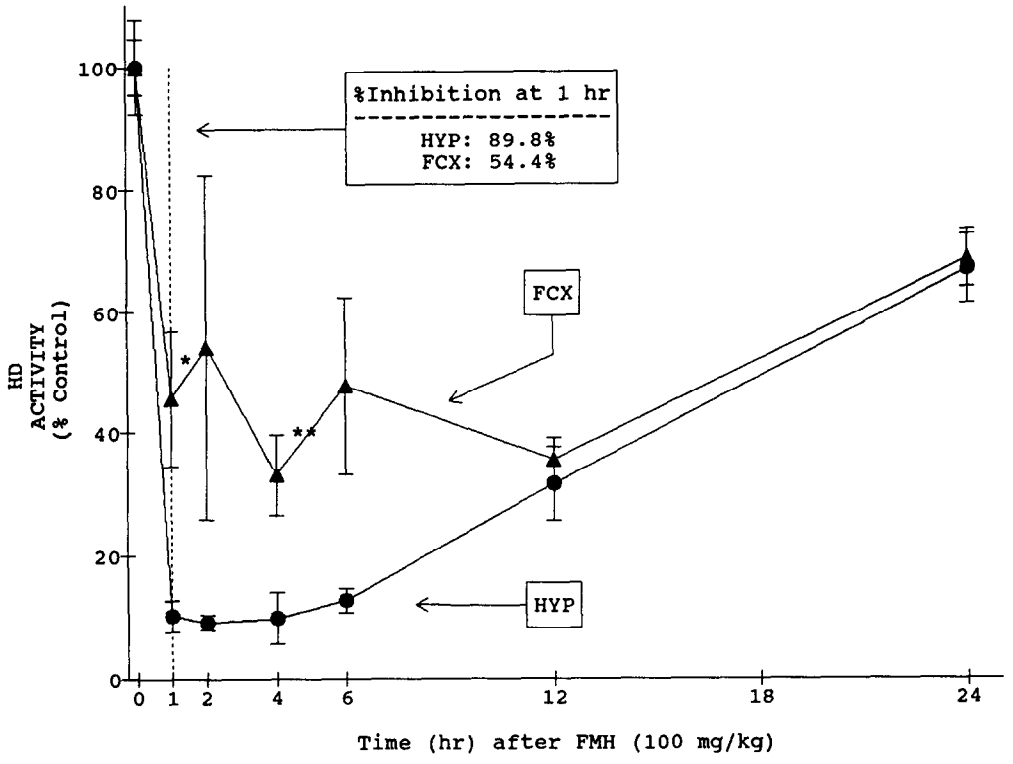

Fig. 1. Time course of FMH-induced inhibition of rat brain HD activity *in vivo*. Animals received FMH (100 mg/kg, i.p., or saline) and were killed after various time intervals (abcissa). Homogenates of hypothalamus (HYP) and frontal cortex (FCX) were assayed for HD activity by the CO_2-trapping method. For other reasons, these animals also received morphine (5.6 mg/kg, s.c) 1 hr prior to being killed, a treatment found in separate experiments to have no effect on HD activity in either region (not shown). For each region, HD activities from saline-treated control animals at different time points were not significantly different from each other. These values were pooled (139.4 ± 6.3 and 25.5 ± 1.9 pmol·g^{-1}·hr^{-1}, mean ± SEM, for hypothalamus and frontal cortex, respectively), and are shown as 100% at zero time. HD activities are thus expressed as percent of control values for each region (ordinate, mean ± SEM, N = 4–8). The percent of HD inhibition by FMH after 1 hr for each region is given in the box. A two-factor ANOVA (time, region) found highly significant differences ($P < 0.001$) for both factors. Key: (*, **) significant difference ($P < 0.05$ and 0.01, respectively) between the regions at the same time.

significantly less complete than that found in the hypothalamus (89%, Fig. 1). Inhibition of HD by FMH was similar in both regions at 12 and 24 hr (Fig. 1). In the same experiment, FMH inhibited HD activity by 71, 73, and 79% in the striatum, hippocampus, and midbrain, respectively, after 1 hr (data not shown).

In vitro experiments were performed with the CO_2-trapping method in order to characterize further this apparent difference in response to FMH (Fig. 2). FMH induced a concentration-dependent inhibition of hypothalamic HD; the maximum degree of inhibition (approximately 90%) was unchanged from 10 to 1000 μM (Fig. 2). Inhibition of HD in the frontal cortex was also observed over the same concentration range of FMH. In this case, however, the maximum inhibition was significantly less than that observed in the hypothalamus (53 vs 89%, respectively; Fig. 2).

Other *in vitro* experiments were performed to study this regional difference in the response to FMH (results not shown). Tissue dilution studies confirmed that both homogenates were on the linear part of the protein–activity curve, such that enzyme concentration did not explain the difference in FMH sensitivity. Similarly, the apparent FMH-resistant HD activity was observed over a range of preincubation and incubation times and at two incubation temperatures (37 and 45°, the latter having been used in some cases to assay HD, see Ref. 5). The difference also persisted when additional cofactor (PLP) was added before and/or after preincubation. Finally, experiments with inhibitors of DOPA decarboxylase (α-methylDOPA, 10^{-4} M; α-fluoromethyl-DOPA, 10^{-4} M), and ornithine decarboxylase (α-difluoromethylornithine, 10^{-4} M) were unable to identify the FMH-resistant activity in the frontal cortex.

Because the results from the CO_2-trapping HD assay suggested the existence of a novel, FMH-resistant form of HD activity in the frontal cortex, the effects of FMH on HD activity in the cortex and

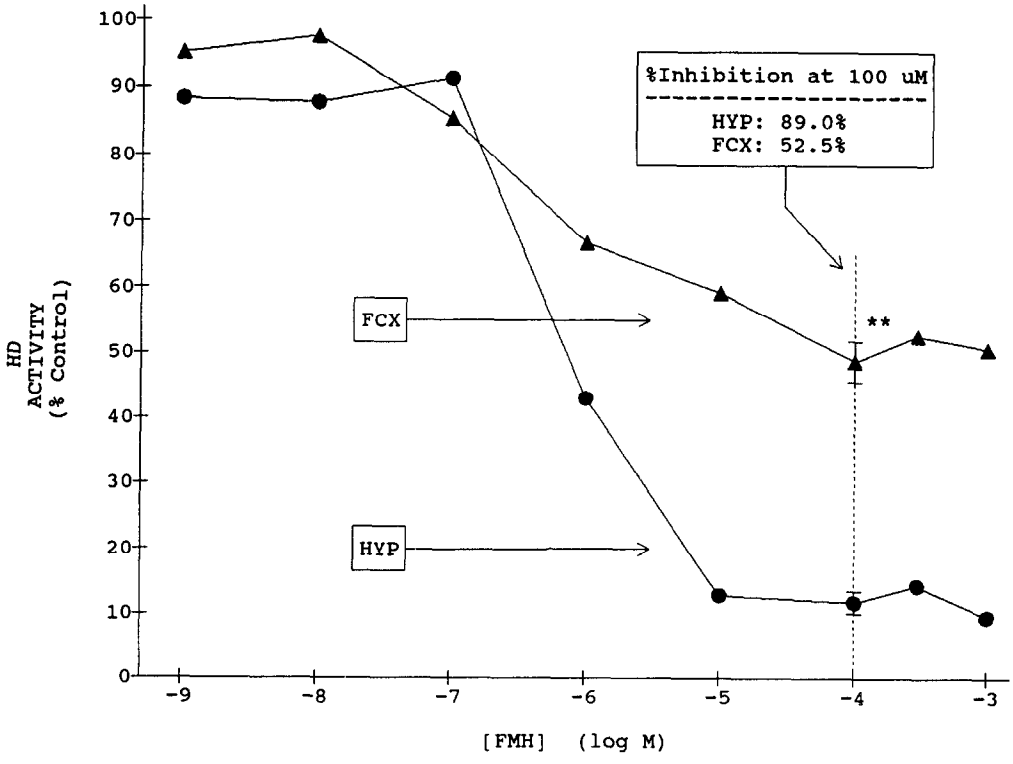

Fig. 2. Effect of FMH on rat brain HD activity *in vitro*. The hypothalamus (HYP) and frontal cortex (FCX) were harvested from naive rats. Regions were pooled and homogenates of each, in triplicate, were preincubated in the presence of various concentrations of FMH (abscissa) for 30 min. HD activity was then measured by the CO_2-trapping method as described. Results are expressed as percent of activity in the absence of FMH (ordinate). Except for the 10^{-4} M data, each point represents the mean of two or three identical experiments that yielded very similar results. At 10^{-4} M, the symbols represent means ± SEM for six separate homogenates. See Table 1 for the control activities ($pmol \cdot g^{-1} \cdot hr^{-1}$). The percent inhibition at 10^{-4} M FMH is shown in the box for each region. Key: (**) significant difference ($P < 0.01$) between the regions at this concentration.

hypothalamus were determined by two other enzymatic methods (Table 1). In contrast to the clear regional difference found in the effects of FMH (100 μM) as determined by the CO_2-trapping method, no such regional differences were detected by either the radioenzymatic or radiochromatographic method. Thus, as assessed by these other two methods, HD activity in both the hypothalamus and frontal cortex was virtually abolished by FMH (Table 1). The inhibition was demonstrated over a very wide range of substrate concentrations.

DISCUSSION

In a previous study, the CO_2-trapping method for HD was suitably modified for assays of brain homogenates [5]. Studies of homogenates of the *hypothalamus* showed that the method met virtually all of the criteria for an accurate and reproducible measurement of the activity of this enzyme [5]. The method also yielded a brain regional distribution of HD activity that was in good agreement with that found by another method for measuring HD activity, adding further credibility to the new assay [5].

In vivo experiments with a large, single dose of FMH showed virtually complete inactivation of HD activity in the rat hypothalamus (Fig. 1), similar to previous results with mice [10, 11]. However, other results from the same experiment suggested the existence of an FMH-resistant enzyme in the frontal cortex of the *rat* (Fig. 1). Since a previous study reported complete FMH-induced inhibition of HD activity in the cerebral cortex of the *mouse* [10], the present results in the *rat* suggested the existence of a species difference, assuming the accuracy of the CO_2-trapping method. Alternatively, if no such species difference exists, these results would suggest that the method also detects non-HD activity in the rat cortex. Since, *at the time*, there was little reason to doubt the validity of the CO_2-trapping method, and, since all known forms of HD are inhibited completely by large concentrations of FMH (i.e. 10–100 μM [12, 13]), this discovery of FMH-resistant HD activity in the frontal cortex of the rat was studied in detail, as it may have been the result of a previously uncharacterized species-specific FMH-insensitive isoenzyme.

We also pursued the observations of Fig. 1 because

Table 1. Effect of FMH on HD activity in two rat brain regions by three methods

Method	[L-His] (μmol/L)	[FMH] (μmol/L)	L-Histidine decarboxylase activity (pmol·g tissue^{-1}·hr^{-1}) Hypothalamus (H)	Frontal cortex (F)	Ratio (F/H)
CO_2-trapping assay	13.6	0	173.7 ± 21.2 (6)	48.6 ± 4.9 (6)	0.28
		100	19.1 ± 1.9 (6) [89%]	23.1 ± 1.5 (6) [53%]	
Radioenzymatic assay	10,000	0	5,694 (2)	1,016 ± 141 (3)	0.18
		100	427. (2) [93%]	98 ± 50 (3) [91%]	
	30.0	0		189 (1)	
		100		0 (1) [100%]	
Radiochromatographic assay	0.089	0	6.4 (2)	1.0 (2)	0.16
		100	0.1 (2) [98%]	0.0 (2) [100%]	

Homogenates of hypothalamus and frontal cortex were prepared and assayed for HD activity by the three methods shown. For each experiment, the concentrations of substrate L-histidine (L-His) and FMH are given. HD activity values (means ± SEM) are given for the number of homogenates in parentheses. For each FMH experiment, the percent inhibition is given in brackets. For control experiments in the absence of FMH, the ratio of HD activity (frontal cortex/hypothalamus) is also given.

it seemed consistent with other unpublished studies in our laboratory. For example, FMH induced significant depletions of histamine in the hypothalamus, but had little or no effect on the levels of histamine in the frontal cortex. If these results do not imply the existence of FMH-resistant histamine synthesis, then the explanation for this finding would be that cortical HD is inhibited completely by FMH, but that the cortical histamine is in a metabolically inactive compartment. One such compartment is mast cells [11], although there is little histological evidence in the rat for the existence of these cells in the rat cortex [2].

The addition of FMH directly to brain homogenates (Fig. 2) showed that even extremely large doses of FMH did not inhibit HD completely in the frontal cortex. Thus, the *in vivo* results of Fig. 1 cannot be explained by the dose of FMH given, or by the inability of the drug to reach the tissue in adequate concentrations. As mentioned, we are also unable to explain the results of Fig. 2 based on inappropriate tissue dilution, preincubation time or incubation time. Since FMH requires the presence of PLP for its action [12], we also considered that homogenates of the frontal cortex may have a greater ability to inactivate PLP during preincubation. However, the same pattern of FMH-insensitivity in the frontal cortex was found when PLP was added before or after preincubation, or when it was added at both times (results not shown).

The persistence of the apparent FMH-resistant HD activity, as characterized by the CO_2-trapping method, required confirmation by alternative methods for measuring HD activity. In contrast to the CO_2-trapping method (which measures CO_2 formation), we utilized two additional methods for measuring HD activity, both of which measure *histamine* formation. Surprisingly, when HD activity was measured by the radioenzymatic method, there was no such FMH-resistant component. Thus, FMH (100 μM) inhibited HD in the hypothalamus and frontal cortex by 93% and 91%, respectively (Table 1). Because the radioenzymatic method utilizes a much higher substrate concentration than that of the CO_2-trapping method (10,000 vs 13.6 μM, respectively), it was still considered possible that two different enzymes in the frontal cortex could be involved: an FMH-sensitive HD detected with millimolar amounts of substrate, and an FMH-resistant form measured with micromolar amounts of substrate. This was also not the case, however, since cortical HD activity measured by the radioenzymatic method was inhibited completely by FMH even when the substrate concentration was lowered to 30 μM (Table 1). Furthermore, the same results were obtained with the radiochromatographic method for measuring HD activity, an assay utilizing very low substrate concentrations (Table 1). Since both the radioenzymatic and radiochromatographic methods for measuring HD are based on histamine formation and both methods yielded complete FMH-induced inhibition of HD in the frontal cortex (Table 1), it seems clear that the CO_2-trapping method detects an FMH-resistant CO_2-producing activity in the frontal cortex that is not a histamine-forming enzyme like HD. It should be noted that while the *absolute* HD activities resulting from the radiochromatographic method may not be accurate (owing to the potential contribution of endogenous histidine to the substrate level; see Ref. 5), the comparison between activities in the presence and absence of FMH remains valid as long as the inhibitor (added *in vitro*) does not affect endogenous histidine levels significantly.

The nature of the enzyme(s) responsible for the FMH-resistant formation of labeled CO_2 from carboxyl-labeled L-histidine is unclear. Another relatively obscure pathway of histidine metabolism is its transamination to imidazole pyruvate [14, 15]. The FMH-resistant CO_2 formation observed presently could result from the formation and subsequent decarboxylation of imidazole pyruvate, producing imidazole acetic acid and CO_2. However, the importance of this pathway as a source of CO_2 formation is unknown, since this pathway has not been demonstrated in the brain. The extreme

chemical reactivity of imidazole pyruvate and the lack of selective inhibitors of these pathways have impeded efforts to confirm this mechanism.

Although previous studies had shown that the CO_2-trapping assay for HD met our criteria for a successful method when studying the hypothalamus [5], the present results suggest that the method may not be specific for measurement of this enzyme in other brain regions. However, if cortical homogenates contained another enzyme in addition to HD that was being measured by this method, a regional distribution of the total of these activities would not have been expected to parallel the known distribution of HD. In fact, an excellent correlation across seven brain regions was found between the regional distribution of the CO_2-trapping activity and HD measured by another method ($r = 0.98$, $P < 0.01$ [5]). One possibility is that in the absence of FMH, the CO_2-trapping assay measures only HD (and not the other enzyme), but that the addition of FMH completely inhibits HD and "shunts" labeled histidine through an alternate, FMH-resistant CO_2-producing pathway. According to this explanation, the method might still be accurate for measuring HD activity in the frontal cortex in the *absence* of HD inhibitors (explaining the correct regional distribution), but it would fail to meet the FMH criteria for specificity, since in the presence of FMH another enzyme would contribute activity.

A closer examination of the control data of Table 1 may help to resolve this issue. If the CO_2-trapping activity in the frontal cortex (but not that of the hypothalamus) represents both an HD and non-HD component, then the ratio (cortex/hypothalamus) of control HD activities for this method should be higher than the ratio found with the other methods, which presumably measure only HD in both regions. Table 1 shows this to be a likely possibility, as the CO_2-trapping method yielded a ratio of 0.28 vs 0.18 and 0.16 for the radioenzymatic and radiochromatographic methods, respectively. Previous studies with these methods found comparable ratios of 0.25 (CO_2-trapping [5]) vs 0.22–0.23 (radioenzymatic method [6, 9]), 0.17 (radiochromatographic method [9]), and 0.19 (fluorescence method [16]). Moreover, the deviation of the cortical/hypothalamic ratio for the CO_2 method, compared with the other methods (0.28 vs 0.18), approximates the size of the FMH-resistant component in the cortex. Thus, it seems likely that the CO_2-trapping method detects enzymatic activity in the frontal cortex (and possibly elsewhere) in addition to HD. In our paper describing the development of the brain CO_2-trapping method [5], caution was advised against the indiscriminant use of the method in tissues other than the brain. It now appears that the same advice may apply to brain regions outside of the hypothalamus.

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