III. RESULTS

CHAPTER 1.1:

"Quantitative Radioisotopic Determination of Histidine Decarboxylase Using High-Performance Liquid Chromatography"

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Quantitative Radioisotopic Determination of Histidine Decarboxylase Using High-Performance Liquid Chromatography

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We have developed a procedure for the accurate measurement of histidine decarboxylase in tissues expressing low levels of enzymatic activity. Briefly, histamine is enzymatically synthesized from [^{3}H]-labeled histidine, followed by purification using high-performance liquid chromatography (HPLC) and quantitation by liquid scintillation counting. This method presents three advantages over previous techniques. First, prior to HPLC purification, excess precursor [^{3}H]histidine is removed on an anion-exchange resin. Second, purification by HPLC is considerably more selective than that of classical cation-exchange gravity columns or organic solvent extractions. Finally, the accuracy of this method is improved by including nonradiolabeled histamine as internal standard, which is quantified by ultraviolet detection. This simple procedure allows highly sensitive and accurate determinations of histamine synthesis. © 2000 Academic Press

Key Words: **histamine; histidine; high-pressure liquid chromatography; histidine decarboxylase.**

Histidine decarboxylase (EC 4.1.1.22) is a regulatory enzyme that catalyzes the synthesis of histamine from histidine. Expression of the enzyme is characteristic of specific cell types, including histaminergic neurons, mastocytes, and gastric enterochromaffin-like cells. Since histamine is involved in many relevant physiological actions, accurate determination of histamine synthesis could be important in research studies as well as in the process of new drug development. However, in some biological samples, histidine decarboxylase activity may be too low to be accurately determined using previously reported procedures. Previous methods for determination of histidine decarboxylase in tissues typically involve incubating samples under various conditions, followed by purification and quantification of the resulting histamine. Two major drawbacks of these techniques are the relatively poor selectivity of the purification of histamine and a lack of sensitivity of the quantification procedure. In general, these methods can be classified as fluorimetric, radioenzymatic, and radioisotopic.

Fluorimetric methods typically involve the derivatization of histamine with *o*-phthalaldehyde and quantification of the resulting fluorophore by spectrofluorometry (1–3). Unfortunately, neither the derivatization reaction nor the spectral characteristics of the fluorophore are highly selective, and thus the specificity of these techniques is largely based on the previous purification of histamine. Some authors have purified histamine on phosphocellulose columns (1, 2). However, the eluate volumes are relatively large in such columns, and the removal of histidine is incomplete, which reduces the sensitivity of this method. In more recent studies, histamine has been purified by HPLC. This method is very sensitive and selective, but it is also technically difficult and requires specialized HPLC equipment for postcolumn derivatization (4).

In a second approach, called the radioenzymatic method (5), once histamine is formed, it is then enzymatically converted to radiolabeled methylhistamine by adding the enzyme imidazole-*N*-methyltransferase and radiolabeled *S*-adenosylmethionine to the incubation medium. Radiolabeled methylhistamine is selectively extracted with organic solvents and then quantified by liquid scintillation counting. This method is both specific and sensitive, but it is also time-consuming, it requires expert handling, and, in our experience,

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the relative instability of radiolabeled *S*-adenosylmethionine may result in poor reproducibility.

Radioisotopic techniques are based on the utilization of radiolabeled histidine as the substrate in the enzymatic reaction and quantification of the resulting radiolabeled histamine (6–8) or CO_2 (9, 10). [^{14}C]CO_2 trapping has not been widely used since, in some tissues, the incubation of [^{14}C]histidine produces greater levels of CO_2 than histamine, suggesting that histidine might become transaminated to imidazole pyruvate and subsequently decarboxylated (11). On the other hand, methods based on the determination of [^{3}H]-labeled histamine suffer from the drawback that commercially available [^{3}H]histidine may contain impurities and have a relatively high radiolysis rate. Thus, this substrate frequently requires repurification and determination of specific activity. Also, given the low rate of transformation of substrate into histamine (0.1–0.5% in rat brain homogenates), the sensitivity of this approach requires a strict separation between histamine and the excess histidine. In previously published procedures, the purification of both commercial radiolabeled histidine and enzymatically produced histamine has been accomplished using cation-exchange gravity columns (Amberlite CG-50) (7, 8). Unfortunately, these columns do not provide effective purification of either substrate or product, giving rise to high background values and variable results.

In order to study histidine decarboxylase activity in small brain regions and subcellular fractions, we have developed an improved radioisotopic approach by devising more selective purifications of histamine and histidine. To do this, we took advantage of the high resolution of HPLC columns. In our experience, purification by HPLC is much more reproducible than that of Amberlite cation-exchange gravity columns, and it can be more easily monitored through the use of online UV detectors.

MATERIALS AND METHODS

Chemicals

During the course of this work, ring-labeled [2,5-^{3}H]L-histidine stocks were obtained from Amersham, ICN Pharmaceuticals, or New England Nuclear. We frequently found it necessary to repurify commercial radiolabeled stocks and to reevaluate their specific activity (see Purification of [^{3}H]Histidine Standards). Amberlite resin (Supelco) was purchased from Teknokroma (Spain). Other reagents were obtained from Sigma Chemical Co. or were of the highest purity available.

General Procedure

A simplified flow diagram of the procedure is shown in Fig. 1. Fresh brain regions were homogenized manually using a glass–Teflon homogenizer in 0.1–0.15 volumes (w/v) of ice-cold 10 mM potassium phosphate buffer (pH 7.4). Protein content of the homogenates was determined by the method of Lowry, using bovine serum albumin as a standard. Aliquots of approximately 0.2 mg of protein were incubated with 10 μM pyridoxal 5′-phosphate and 0.25 μM prepurified [^{3}H]histidine (1 μCi at a specific activity of 40–50 Ci/mmol). Homogenization buffer (40–60 μl) was added to bring the final reaction volumes to 100 μl. Reactions were initiated by the addition of [^{3}H]histidine and were incubated at 37°C for 1 h in a shaking water bath. After incubation, the samples were immediately placed on ice and deproteinized by adding concentrated trichloroacetic acid to a final concentration of 0.5% (w/v). Blank samples were prepared in exactly the same way, including the addition of trichloroacetic acid, but were not incubated. The trichloroacetic acid added to the reaction also contained 100 nmol of nonradiolabeled histamine per sample, which was used as an internal standard for the quantification. All samples were vortexed, chilled on ice, and centrifuged at 12,000g at 4°C for 10 min.

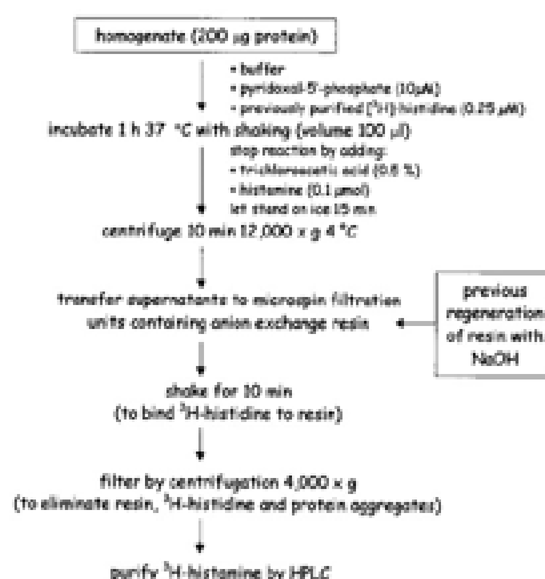

FIG. 1. Schematic of steps involved in quantitative determination of [^{3}H]histamine levels.

Twelve milligrams of Amberlite strong anion exchange resin (see Elimination of Excess Substrate with Amberlite Anion-Exchange Resin for details) was mixed with the deproteinized supernatant in the upper part of microspin filter tubes (LIDA, USA) containing 0.45-μm pore cellulose acetate membranes. The tubes were then vortexed for 10 min in an automatic shaker. During this step, the resin binds at least 95% of the [^{3}H]histidine through its carboxylic group, thereby clearing most excess precursor from the samples. The

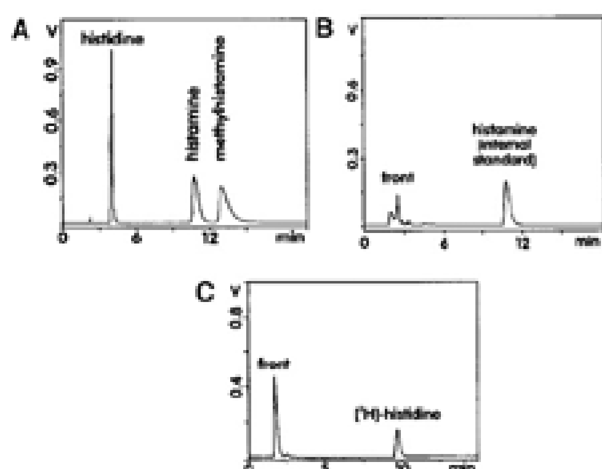

FIG. 2. Reverse-phase HPLC profiles, obtained by UV detection at 225 nm: (A) Standards of histidine, histamine, and *tele*-methylhistamine (100 nmol each). The mobile phase contained 10 mM octanesulfonic acid, 0.3 M sodium phosphate (pH 3), and 21% methanol. (B) Profile of a typical sample, showing the histamine used as internal standard (100 nmol) as the main peak. HPLC conditions were as in A. [^{3}H]Histamine was collected from the column during UV detection of the internal standard (nonlabeled) histamine. (C) Purification of a [^{3}H]histidine standard. Commercial [^{3}H]histidine (from Amersham, 0.2 ml stock) was injected into the HPLC, which was set up to make a linear gradient from 1 to 6 mM NaH_2PO_4. The histidine peak shown was estimated as 2.4 nmol against an external histidine standard (detector range was set up at higher sensitivity than in A). The peak was collected and its specific activity calculated for further use.

tubes were then centrifuged at low speed (4000*g*) for 5 min, and the filtrate recovered in the bottom half of the tube was injected into the HPLC system.

Histamine Purification by HPLC

[^{3}H]Histamine formed during the incubation reaction was purified by HPLC. The system consisted of a reverse-phase C_{18} column (Tracer Extrasil ODS2, 5-μm particle size, 25 × 0.46 cm; Teknokroma, Spain) and an ion-pair mobile phase, made up of 10 mM octanesulfonic acid and 0.3 M sodium phosphate buffer, adjusted to pH 3, plus 21% (v/v) methanol. The flow rate was 1 ml/min. This HPLC system completely separated standards of histidine, histamine, and *tele*-methylhistamine, the main histamine metabolite in brain (Fig. 2A). Because these standards contain imidazole rings, they could be detected by UV absorbance at 225 nm. However, the samples contained extremely low levels of radiolabeled histidine and histamine (several picomoles or less per injection) that were undetectable by UV absorbance. Similarly, endogenous histidine and histamine levels were also too low to be detected by UV. Thus, a typical UV profile of a sample consisted largely of one peak, the internal histamine standard (100 nmol per sample; Fig. 2B), completely devoid of interferences. The histamine fraction was recovered in a scintillation vial, mixed with Optiphase HiSafe III cocktail (Wallac), and [^{3}H]histamine was quantified in a liquid scintillation counter. The recovery of the internal standard in each sample (internal/external standard peak area) was quantified from histamine HPLC peak areas, obtained using Kontron data system 450 software. The amounts of [^{3}H]histamine formed during the histidine decarboxylase incubation were calculated from [^{3}H] dpm, the relative recovery of each sample, and the specific activity of the ring-labeled [^{3}H]histidine precursor used in the incubation. Average [^{3}H] dpm contained in blank samples were subtracted.

Elimination of Excess Substrate with Amberlite Anion-Exchange Resin

The relatively low percentage of [^{3}H]histidine converted to [^{3}H]histamine (see introduction) makes it advisable to remove most excess [^{3}H]histidine remaining in samples after incubation in order to prepare them for HPLC. To do this, deproteinized supernatants were simply vortexed in the presence of a strong anion exchange Amberlite resin (IRA 900, Supelco, mesh 16–50), a quaternary amine that efficiently binds carboxylic groups such as that of histidine.

The resin functional group is originally supplied equilibrated with chloride as a counterion. However, since carboxylic ions are not strong enough to displace chloride, we regenerated the resin to replace chloride with OH^- as the counterion. Resin regeneration was achieved by passing 20 volumes of 1 M NaOH through a large resin column (50 ml) and subsequently rinsing with 20 volumes of MilliQ water. The regenerated resin was then recovered and stored in MilliQ water.

The efficiency of histidine removal was calibrated using nonradiolabeled standards. Regeneration improved the binding of histidine to the resin by a factor of 20. The resulting resin binding capacity (about 0.5–1 mequiv/g) remained stable for several months of storage simply in water at room temperature. However, immediately prior to use, the resin was rinsed by changing its soaking water 10 times. Eighty microliters of resin bed (equivalent to 12 mg of dry-weight resin) was carefully pipetted onto the top part of microspin filter tubes with a standard yellow pipette tip cut 2 cm from the tip. Deproteinized supernatants were then mixed with the resin, which typically binds more than 95% of the excess [^{3}H]histidine. The anion-exchange resin also binds the trichloroacetate ion, raising the pH of the sample from 2 to 8. Efficiency of the removal of [^{3}H]histidine by the resin was controlled during sample processing by liquid scintillation counting of a 10-μl aliquot of each sample prior to injecting the remainder of the sample into the HPLC.

Purification of [^{3}H]Histidine Standards

Standards of ring-labeled [2,5-^{3}H]L-histidine (40–60 Ci/mmol) typically show a decomposition rate of at least 1–3% per month, as specified by the vendors, and this rate may even increase during storage. Such radiolysis generates unwanted by-products that must be separated from [^{3}H]histamine in samples (see Results and Discussion). To maintain a high degree of purity of the standards, we purified aliquots of the original stock by HPLC upon arrival and subsequently after every 3 months of storage. The system used for HPLC purification consisted of a reverse-phase C_{18} column (Tracer Extrasil ODS2, 5-μm particle size, 25 × 0.46 cm; Teknokroma, Spain) and a linear gradient mobile phase from 1 to 6 mM sodium phosphate buffer (pH 3) in 12.5 min. A low percentage of methanol (2%) and octanesulfonic acid (0.1 mM) were constant throughout the gradient. The flow rate was 1 ml/min. Under these conditions, histidine eluted at 9–10 min (Fig. 2C). Using a minor modification of this gradient, we observed that elution of histamine would require 20 mM phosphate, so our purified [^{3}H]histidine was not contaminated with [^{3}H]histamine. In a typical purification, 50 μCi of [^{3}H]histidine (2 nmol) was injected into the HPLC and the whole histidine fraction (0.5–1 ml) was collected. The amount of [^{3}H]histidine was quantified against an external standard calibration curve of nonradiolabeled histidine (Sigma) by UV absorbance at 225 nm. The limit of detection for the histidine standards by UV was about 50 pmol at maximum sensitivity. An aliquot of the purified fraction was subjected to liquid scintillation counting, so that the specific activity of the purified product could be obtained by dividing total dpm in the purified fraction by the amount of histidine detected. Since we recovered purified [^{3}H]histidine in a mobile phase containing a low concentration of octanesulfonic acid and methanol, we confirmed that such traces of these chemicals did not alter histidine decarboxylase activity assays (data not shown).

RESULTS AND DISCUSSION

The present method significantly improves previously published procedures to determine histidine decarboxylase activity by the radioisotopic method at three levels. First, we found that a much more selective purification of [^{3}H]histamine from samples is achieved using HPLC instead of cation-exchange gravity columns. This is critical, as the histidine substrate is in great excess relative to histamine formed during the incubation. The similarity between histidine and histamine molecules makes chromatographic separation of these compounds difficult, unless columns with much higher resolution are used, such as those for HPLC. Second, removal of most of the precursor [^{3}H]histidine from samples with an anion-exchange resin greatly increases the sensitivity. Finally, quantification of [^{3}H]histamine is carried out by including an internal standard, which further enhances the accuracy of the procedure and reduces the variability of sample processing.

In general, radioisotopic methods are more specific and sensitive than fluorimetric or other methods of histidine decarboxylase determination. However, such specificity and sensitivity depend directly on the level of background (dpm) remaining in blanks. The blanks are critical in this assay, because the percentage of histidine converted to histamine is typically very low, and thus minor contaminants could greatly impair the reproducibility of results. Keeling *et al.* (8) described a radioisotopic method of histidine decarboxylase determination in a paper that critically addressed the high level of dpm in the blanks of the original method developed by Baudry *et al.* (7). However, Keeling's method was complex and also used ion-exchange gravity columns as a final step of purification. Because of the high resolution of HPLC, our method is much more selective than that of either Baudry or Keeling, with improved sensitivity and lower levels of dpm in the blanks. In fact, in our HPLC system, direct injection of 2.2×10^6 dpm of [^{3}H]histidine (the amount typically added to one sample) resulted in approximately 340 dpm (0.015%) in the histamine fraction. This was most likely due to "tailing" of [^{3}H]histidine impurities in the system. Such blanks would allow the injection of deproteinized samples into the HPLC without prepurification. However, to increase the sensitivity of the assay, we introduced a prepurification step in which the majority of excess [^{3}H]histidine was removed from the samples prior to HPLC. The Amberlite anion-exchange resin quickly and selectively binds histidine, but not histamine, through the carboxyl group. This step efficiently reduced blank dpm to the values shown in Table 1, and frequently even lower values were obtained. The use of other HPLC possibilities, such as gradient elution, could increase the sensitivity further still. As shown in Table 1, the dpm levels in the blanks were not significantly affected by varying the method by which the blanks were prepared, including the following: (A) incubating tissue in the presence of 0.5% trichloroacetic acid; (B) boiling tissue at 100°C for 30 min prior to incubation; (C) incubating samples in the presence of 0.5 mM α-(fluoromethyl)histidine, a specific histidine decarboxylase inhibitor (12, 13); (D) omitting sample incubation; (E) using no tissue at all in the incubation. These results indicate that there is no identifiable source of the low level of radioactivity remaining in blanks, which should be attributed mainly to impurities or radiolysis of [^{3}H]histidine standards, which "tail" long enough to reach the histamine retention time. Nevertheless, since the blank values shown in Table 1 would be the dpm equivalent of 0.7

TABLE 1

Dpm in Blanks (Run in Triplicate) Obtained by Different Methods: 0.5 mM α-(Fluoromethyl)histidine, 0.5% Trichloroacetic Acid through Incubation, Previously Denatured Tissue by Boiling, Buffer Instead of Tissue, and No Incubation[a]

Blank	Dpm 1	Dpm 2	Dpm 3	Mean dpm
No tissue	57	43	48	49
Preboiled tissue	27	39	43	36
α-(Fluoromethyl)histidine	53	49	63	55
No incubation	81	78	83	81
Trichloroacetic acid	84	65	60	69
Positive control	532	561	484	526

[a] All samples contained initially 2.2×10^6 dpm (25 pmol of purified [^{3}H]histidine). Positive controls are histidine decarboxylase activities of the same whole-brain homogenates. Incubation time, 1 h.

fmol of histamine (the specific activity of the [^{3}H]histidine used was 81 dpm/fmol), the detection limit of this assay would be about 2 fmol of [^{3}H]histamine (three times blank values).

As noted by the manufacturers, commercially available [2,5-^{3}H]L-histidine has a relatively high rate of radiolysis. The majority of radiolysis is likely due to the exchange of tritium from the partially aromatic imidazole ring to water or protons in the media, although it is also possible that a small percentage of decomposition could result from self-irradiation (14). In the studies of Garbarg *et al.* (15), radiolabeled substrate was purified immediately prior to use by passing it repeatedly through Amberlite cation-exchange columns similar to those used for histamine purification. This procedure would be predicted to eliminate any contaminants that could bind to the column at the same time as histamine. However, this method adds an additional experimental step to the determination procedure. It is also important to point out that Garbarg *et al.* (15) did not report the specific activity and concentration of the purified substrate. Perhaps their purification procedure did not significantly alter the specific activity of the substrate, but clearly this would be predicted to change its concentration, which is critical for reproducibility of the results. Accordingly, these authors did not express their results in absolute units, but only as dpm. With our method, [^{3}H]histidine could be efficiently purified by HPLC gradient elution, which provided simultaneous quantification of the histidine concentration. One batch of our purified product was used reliably for a period of 3 months. This purification procedure allowed us to successfully use batches from stocks that were several years old. On the other hand, since [2,5-^{3}H]L-histidine standard is ring labeled, decarboxylation to histamine would not change its specific activity. Thus, the results obtained are fully quantitative (expressing the absolute levels of histamine formed). Such quantitative radioisotopic determination of histidine decarboxylase has not been achieved by previous methods, which merely expressed the data as dpm obtained.

The majority of excess [^{3}H]histidine (approximately 95%) is readily removed from the sample by binding to the Amberlite resin after deproteinization. Just 12 mg of resin per sample is sufficient, although higher amounts of resin further increased histidine removal. However, very high amounts of resin could also decrease histamine recovery. Although histamine has no negatively charged groups and theoretically should not interact with the quaternary amine of the resin, we have observed that in the presence of high levels of Amberlite a low-affinity interaction can occur, perhaps via a hydrophobic affinity with the polystyrene matrix of the resin. Therefore, it is best to determine (using nonradiolabeled standards) the optimum amount of Amberlite to provide an efficient removal of excess histidine while maximizing the free levels of histamine in the samples. In our hands, the final recovery of histamine from homogenates subjected to the entire purification procedure was about 80%, and this percentage did not show significant variability between samples (see Table 2). Nevertheless, even if histamine recovery were reduced, the accuracy of the method is maintained by the inclusion of nonradiolabeled histamine as an internal standard within each sample, which would account for any changes in recovery.

In their studies, Baudry *et al.* (7) and Garbarg *et al.* (15) added nonradiolabeled histamine (0.1 mM) to the samples during incubation. However, we would not recommend this, especially since Savany and Cronenberg (16) and we ourselves (data not shown) have observed that 1 mM histamine can act as a competitive inhibitor of histidine decarboxylase. Instead, we added

TABLE 2

Intra-assay Variability of the Histidine Decarboxylase Determination[a]

Sample or blank	Dpm	Internal standard area (mV min)	Histidine decarboxylase activity (fmol mg protein^{-1} h^{-1})
Replicate 1	1155	79	196
Replicate 2	1350	93	196
Replicate 3	1285	94	184
Replicate 4	1399	98	192
Replicate 5	1263	88	192
Replicate 6	1382	94	200
Blank 1	165	106	—
Blank 2	131	103	—
Mean ± SD		94 ± 8.5	193 ± 5.5

[a] A rat hypothalamus homogenate containing 0.25 mg of protein was incubated for 1 h with 0.25 μM [^{3}H]histidine. Mean recovery of the internal standard was 81%.

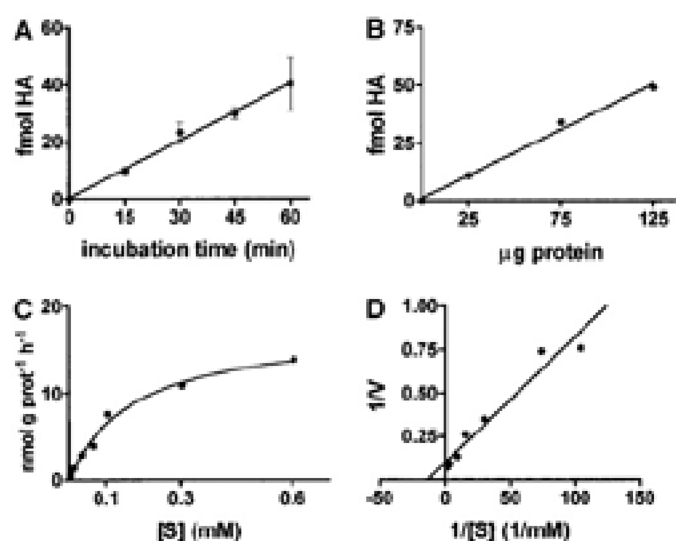

FIG. 3. Performance of the method. (A) Histamine formation increased linearly with incubation of rat hypothalamus homogenates up to 1 h. (B) Histamine formation increased linearly in relation to the amounts of protein in hypothalamus homogenates. (C) Michaelis–Menten representation of enzyme activity in whole-brain homogenates at pH 7.4 versus substrate concentration. (D) Lineweaver–Burk plot of C. Since homogenates contain endogenous histidine, we took into account the isotopic dilution of [^{3}H]histidine with endogenous substrate for these calculations. Endogenous histidine concentrations considered were 15 μg/g in whole brain or 25 μg/g in hypothalamus of rats (5). K_m obtained from the Lineweaver–Burk regression was 74 μM and V_{max} was 10.2 nmol g of protein^{-1} h^{-1}. The K_m calculated without considering endogenous histidine was about half that value.

histamine immediately after the incubation, in our case to be used as an internal standard. The use of an internal standard represents a clear improvement to the determination of [^{3}H]histamine. Our low variability (see Table 2) reduces the need of running large numbers of replicates of each sample, unless a great degree of accuracy is required. Quantification of the internal standard was carried out by taking advantage of imidazole ring UV absorbance of histamine, since it is present in large amounts (100 nmol/sample). In contrast, tissue histamine and [^{3}H]histamine are at concentrations at least 1000 times lower and thus did not interfere with UV absorbance of the internal standard. As expected, the recovery of added [^{3}H]histamine (from Amersham) and nonradiolabeled histamine (from Sigma) to samples (containing 0.3 mg of protein) displayed a linear correlation (Pearson's $r = 0.9999$, $n = 4$) and had similar recoveries (81 and 86%, respectively). Furthermore, since [^{3}H]histamine and the internal standard share the same HPLC properties, the internal standard is also useful to monitor column elution of [^{3}H]histamine.

Using homogenates derived from rat hypothalamus, we found the formation of histamine to be linear up to at least 1 h of incubation (Fig. 3A). In addition, incubation of increasing amounts of tissue produced increasing amounts of histamine (Fig. 3B). However, this relationship was not linear unless we corrected for the isotopic dilution of radiolabeled [^{3}H]histidine (0.25 μM) with the endogenous histidine from rat hypothalamus (25 μg/g of tissue, according to Ref. 5). Thus, increasing amounts of tissue contain increasing amounts of endogenous histidine, and therefore the relative amounts of [^{3}H]histidine substrate in the homogenates are proportionately decreased.

The catalytic activity we measured here is in close agreement with previously reported data for histidine decarboxylase. The optimum pH was 7.4 (data not shown) as previously reported by Garbarg *et al.* (15) at similar substrate concentrations. pH has been shown to influence the kinetics of histidine decarboxylase. The catalytic mechanism involves the formation of a Schiff base with the pyridoxal 5′-phosphate cofactor in such a way that the state of protonation of the substrate and the Schiff base would be critical for catalysis (17). This mechanism would also explain the fact that the optimal pH of the enzyme depends on substrate concentrations (2, 16, 17). Thus, K_m obtained at a different pH would not be comparable. Maintaining the pH at 7.4, we obtained a K_m value (74 μM, Fig. 3) that is in between that reported by Garbarg *et al.* (15) in human brain (30 μM) and that previously estimated in our laboratory using fluorimetry (approximately 200 μM; Ref. 2). Incubating the reaction at pH 6.5 would be expected to yield higher K_m values than at pH 7.4 (1, 2). The V_{max} value obtained here at pH 7.4 (10.2 nmol g of protein^{-1} h^{-1}) is similar to that which we previously reported in rat brain homogenates (6.7 nmol g of protein^{-1} h^{-1}, from Ref. 2). However, such V_{max} and K_m values are quite difficult to compare with literature values since these measures are greatly influenced by the factors mentioned above.

Determination of histamine synthesis may be necessary in many studies in both basic research and drug development. In particular, ligands of the recently cloned histamine H_3 autoreceptor (18) modulate histamine synthesis and release (19, 20). However, a very sensitive method is required to accurately quantify the low amounts [^{3}H]histamine formed in such studies. It is also useful that HPLC routines are easily automated by sample injectors and fraction collectors. The present method overcomes many limitations that impaired previous methods and promises to be useful for various types of studies of histaminergic function.

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