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A SENSITIVE AND SPECIFIC ASSAY FOR HISTIDINE DECARBOXYLASE ACTIVITY

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Abstract—A method for assay of histidine decarboxylase activity is described; it is based upon using as substrate ^{14}C-histidine labeled in the carboxyl carbon and trapping in hydroxide of hyamine the $^{14}CO_2$ evolved during incubation. This procedure may be applied to assay of histidine decarboxylase activity in individual tissue specimens after gel-filtration chromatography of homogenates. For illustrative purposes assays of gastric tissue homogenates are presented. Among the advantages offered by this technique over those previously reported are simplicity, specificity, sensitivity, and the capability of expressing enzyme activity in terms of total histamine formed per unit of original tissue.

Histidine decarboxylases from fetal rat tissue and from gastric tissue were found to be stereospecific for L-histidine and not inhibited by high concentrations of histamine. Data are presented that suggest the presence of an unidentified soluble inhibitor of histidine decarboxylase in gastric tissue.

BIOSYNTHESIS of histamine in mammalian tissues may be catalyzed by a number of closely related or, perhaps, identical specific histidine decarboxylases and by a nonspecific aromatic L-amino acid decarboxylase.[1, 2] Over the years various techniques for assay of histidine decarboxylase activity have been suggested. Those methods that are based upon measurement of the rate of formation of nonradioactive histamine are too insensitive to be of value in assay of any but the most highly purified or most active preparations of the enzyme. Since both substrate and product have cationic imidazole rings, the degree of separation of radioactive histamine required for dependable enzyme assays has been difficult to achieve; a tedious procedure that requires crystallization of a dibenzenesulfonyl derivative has been recommended for this purpose.[3] More recently, the problem of separation has been obviated by using systems in which the $^{14}CO_2$ evolved by decarboxylation of uniformly labeled[4] or carboxyl-labeled ^{14}C-histidine[5] is trapped in suitable media such as Hyamine hydroxide[4] or phenethylamine[5] for subsequent scintillation counting.

The method for assay of histidine decarboxylase activity described in this paper is also based upon trapping of $^{14}CO_2$ evolved during incubation of enzyme with a carboxyl-labeled substrate. Incubation vessels are employed that are both simple in design and disposable. Homogenates of small specimens of tissue are prepared for assay by Sephadex gel filtration. This procedure offers several important advantages over previously described methods. First, since the resultant preparation is free from endogenous histidine, the initial specific gravity of the substrate is known. This

permits calculation of the total amount of histamine formed. Second, in contrast to previously recommended procedures for purification of this enzyme,[6] gel filtration provides quantitative recovery of tissue protein without loss of enzyme activity. This permits expression of results per unit of original tissue; e.g. units of enzyme activity per gram tissue of per milligram tissue protein. Finally, Sephadex chromatography resulted in a marked increase in sensitivity of the assay. The increase in apparent specific activity after gel filtration was much greater than could be accounted for solely on the basis of removal of endogenous substrate. This finding suggested the presence of soluble inhibitors of histidine decarboxylase activity in tissue.

The results of additional experiments designed to test the validity of this assay for histidine decarboxylase activity are presented. They include studies of pyridoxal-5'-phosphate dependency, stereospecificity, pH optimum, and effects of adding histamine, aminoguanidine, and known inhibitors of histidine decarboxylase to incubation mixtures.

MATERIALS

Reagents used in the incubations and their sources included the following: pyridoxal-5'-phosphate, D-histidine, and L-histidine were obtained from Sigma Chemical Co.; histamine dihydrochloride from Nutritional Biochemicals; aminoguanidine sulfate from Eastman Organic Chemicals; streptomycin sulfate from Charles Pfizer and Co.; D-2-hydrazino-3-[4(5)-imidazole]propionic acid (hydrazino analog of histidine, MK-785) from Merck Institute for Therapeutic Research; and 4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate (NSD-1055) from Smith and Nephew Research, Ltd. The radiochemicals used and their sources were ^{14}C-toluene standard, $4{\cdot}21 \times 10^5 \pm 2{\cdot}17$ per cent disintegration/min/ml from Packard Instrument Co.; and from California Corp. for Biochemical Research $Na_2{}^{14}CO_3$ (10 μc per μmole) and ^{14}C-DL-histidine (labeled in the carboxyl carbon). The specific activities of several batches of the radioactive histidine varied from 1·2 to 34 μc per μmole.

Animals from which enzymes were prepared were female Sprague-Dawley rats purchased from Blue Spruce Farms, Inc., Altamont, N.Y.

Materials used in the enzyme incubation included hydroxide of Hyamine (1 M in methanol) and 25-ml screw-cap polyethylene vials from Packard Instrument Co.; Whatman 3MM filter paper from Arthur H. Thomas Co. and 23-gauge, ⅝-in. disposable needles from Becton, Dickinson & Co.

Materials used for gel-filtration chromatography were Sephadex G-25 coarse form (particle size: 100–300 μ) from Pharmacia Chemicals and plain glass chromatographic columns (No. K-42000; internal diameter, 11·5 mm; length, 160 mm) from Kontes Glass Co.

METHODS

Preparation of enzyme. Specific histidine decarboxylase was prepared from whole fetal rats (19 to 20 days gestation) by a modification of the method of Håkanson.[6] The modification consisted of initial homogenization in 0·1 M sodium acetate buffer at pH 5·5 rather than 4·5. We found that when the lower pH was used there was occasional complete loss of enzyme activity. Use of the higher pH was suggested by Dr. Alan Burkhalter[7] and yielded uniformly satisfactory results.

Protein determinations were done by a modification of the phenol reagent method.[8]

FIG. 1. Incubation vessel used for assay of histidine decarboxylase activity.

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Carbon dioxide trapping system. A simple and efficient incubation vessel designed to trap $^{14}CO_2$ for subsequent scintillation counting was devised by Dr. Robert H. Roth;[9] a modification of this system was employed in the present experiments (Fig. 1). A straightened wire paper clip with a narrow loop on one end was forced through the center of the cap of a polyethylene vial so that the loop end projected from the lower part of the cap. A rectangular piece of Whatman 3MM filter paper measuring 1×3 cm was rolled into a cylinder and clamped firmly into the loop. Next, the paper was dipped into hydroxide of Hyamine and allowed to drip dry. It is essential that the loop be so positioned that when the cap is screwed onto the vial the filter paper is entirely in the upper third of the vial and does not touch the edges of the vial. The incubation is carried out at 37° in a Dubnoff metabolic shaker with the vial cap screwed on tightly. To terminate the reaction 2 ml of 6 N HCl is injected through the side of the vial through a 23-gauge disposable needle; the hole in the vial is sealed promptly with adhesive tape. Incubation is continued for 30 min after acidification to permit quantitative adsorption of CO_2 into the filter paper. Then the vial is opened; the filter paper is transferred to another vial containing 10 ml of a scintillation fluorophor solution[10] and the radioactivity determined in a scintillation spectrometer.

The vial cap assembly may be reused after acid washing; the lower part of the incubation vial and its contents are discarded.

Incubation procedure. Optimum conditions for the assay are achieved with incubation mixtures made up as listed in Table 1. The entire mixture with the exception of substrate was preincubated for 10 min. To start the reaction, substrate was added, and the cap of the vial was screwed on tightly. Further processing of the incubation mixture was described above.

TABLE 1. INCUBATION MIXTURE

Component	Final concentration
Enzyme preparation	4–10 mg protein/2 ml
Pyridoxal-5′-phosphate	$3{\cdot}7 \times 10^{-5}$ M
Streptomycin sulfate*	$1{\cdot}0 \times 10^{-4}$ M
Sodium phosphate buffer, pH 6·8	$12{\cdot}5 \times 10^{-2}$ M
L-Histidine	$2{\cdot}5 \times 10^{-4}$ M
^{14}C-DL-Histidine	0·25 μc/ml
Water	to final volume of 2·0 ml

* Streptomycin is added for the purpose of suppressing the growth of bacteria that may decarboxylate histidine; it does not affect histidine decarboxylase activity.

To facilitate precise timing of the start of the incubation, the substrate was prepared in advance as follows. In 10^{-4} N HCl, nonradioactive L-histidine and ^{14}C-DL-histidine were dissolved to final concentrations of 5×10^{-3} M (concentration is calculated to accommodate contribution of radioactive material to total L-histidine) and 5 μc per ml (2·5 μc per ml of ^{14}C-L-histidine) respectively. Thus, addition of 0·1 ml of this solution to the incubation mixture resulted in the desired concentration of substrate.

Unless otherwise specified, all data in this report were derived from experiments done on enzyme prepared from whole fetal rats. The incubation mixtures were as

listed in Table 1. Duplicate incubations were stopped by acidification at 30 and 60 min (to verify linear rates of reaction). Additional duplicates were prepared, differing only in that enzyme was replaced by water to calculate background. Data are reported as counts/min (corrected for background but not efficiency of counting) per hr per incubation.

Standardization. The efficiency of the CO_2-trapping system was determined as follows. To each of five vials all components of the incubation mixture other than substrate were mixed, and 6×10^{-3} μc of $Na_2{}^{14}CO_3$ was added. These vials were processed the same as a usual enzyme assay. In five other vials the same procedure was followed except that the $Na_2{}^{14}CO_3$ was added directly to the filter paper as it was placed in the fluorophor solution. With the latter five vials there were 8570 ± 85 (S.D.) count/min recorded. Following the usual incubation procedure yielded 8377 ± 265 counts/min. Thus the recovery of $^{14}CO_2$ was virtually quantitative ($97{\cdot}8 \pm 3\%$). The efficiency of counting ^{14}C-toluene in this system was determined as $59{\cdot}5 \pm 0{\cdot}8\%$.

If an enzyme preparation is used that is free from endogenous substrate, under the incubation conditions described above, these data permit the following calculation: the recovery of 655 counts/min represents the decarboxylation of 1·0 mμmole of histidine to histamine.

Replication. Five replicate 1-hr incubations yielded an average of 5460 counts/min; the standard deviation was 227 counts/min or 4·1%.

Validity of assay

The following experiments were designed to test some of the assumptions necessary to accept this procedure as a valid assay for histidine decarboxylase activity.

Linearity. Duplicate incubations were stopped at various time intervals up to 5 hr (Fig. 2). It was found that the reaction proceeded at a linear rate for up to 2·5 hr. This justified the use of 30- and 60-min incubations for routine assays. The reaction rate was also found to be a linear function of enzyme concentration (Fig. 2).

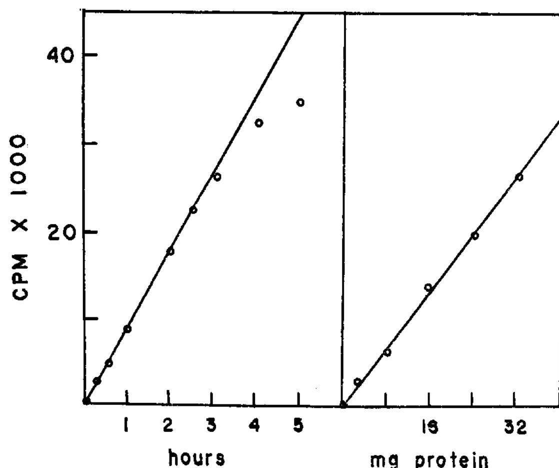

FIG. 2. Histidine decaboxylase activity plotted as a function of time (left) and protein concentration (right). The lines on each graph are straight lines of arbitrary slope drawn through the origin. See text for details of incubations.

Relation of $^{14}CO_2$ *recovery to histidine decarboxylase activity.* Omission of enzyme from the incubation mixture or boiling the enzyme before incubation resulted in complete cessation of the evolution of $^{14}CO_2$ (Table 2). In a nonradioactive incubation system, MK-785 and NSD-1055 have been found to be potent inhibitors of histidine decarboxylase activity;[11] addition of appropriate amounts of these substances to the incubation mixtures prevented the liberation of $^{14}CO_2$ (Table 2).

TABLE 2. RECOVERY OF $^{14}CO_2$ UNDER ALTERED CONDITIONS OF INCUBATION

Condition	Relative activity*
Control	100
Boiled enzyme	0
No enzyme	0
NSD-1055; 5×10^{-7} M	0
5×10^{-8} M	32
MK-785; 2×10^{-5} M	0
2×10^{-6} M	68

* Expressed as per cent of control.

Substrate and pH *relationships.* Håkanson[6] reported that the pH optimum of fetal histidine decarboxylase varies inversely with substrate concentration. Based upon his suggestion that the true substrate is the anionic form of histidine, the K_m was estimated at 6×10^{-7} M. His discussion seems to justify an arbitrary selection of substrate concentration if incubations are done at the pH optimum.

The substrate concentration of $2 \cdot 5 \times 10^{-4}$ M was chosen because it permits a convenient calculation and provides a suitable yield of $^{14}CO_2$. The pH optimum was found to be 6·8, which is in accord with the findings of Håkanson.[6]

Pyridoxal-5'-phosphate. Håkanson[6] reported that although this preparation of fetal histidine decarboxylase is highly dependent upon pyridoxal-5'-phosphate, very small amounts are required for saturation (approximately $7 \cdot 4 \times 10^{-7}$ M). The concentration of pyridoxal-5'-phosphate used in these assays ($3 \cdot 7 \times 10^{-5}$ M) is 50 times greater than that amount. Incubations of different preparations of enzyme to which no pyridoxal-5'-phosphate was added yielded between 3 and 16% of maximal activity. Increasing or decreasing the cofactor 10-fold from $3 \cdot 7 \times 10^{-5}$ M produced no significant change from maximal activity.

Stereospecificity. In order to validate the assumption that, for purposes of calculation, the D-isomer of the ^{14}C-DL-histidine may be regarded as inert, it was necessary to demonstrate that this enzyme, like other mammalian decarboxylases[2] is stereospecific for L-histidine. Addition of L-histidine to the reaction mixture lowered the yield of $^{14}CO_2$ presumably because it lowered the specific activity of the substrate (Table 3). The addition of D-histidine had no effect, indicating that it is not a substrate for the enzyme.

Effects of histamine and aminoguanidine. It has been suggested that histamine may function as an inhibitor of histamine biosynthesis *in vivo*.[12] Many of the assays used for histidine decarboxylase depend upon measurement of the rate of formation of histamine. Aminoguanidine, an inhibitor of diamine oxidase, is often added to the

TABLE 3. STEREOSPECIFICITY OF HISTIDINE DECARBOXYLASE

	(counts/min/hr)
Standard incubation	5673
+ L-Histidine*	1780
+ D-Histidine*	5746

* The amount of L- or D-histidine added was 2·0 μmoles to incubations of final volumes of 2·0 ml.

incubation mixtures to prevent further metabolism of the formed histamine.[3] Therefore, it was important to determine whether increasing levels of the product formed during the incubation, particularly in the presence of aminoguanidine, resulted in inhibition of the enzyme.

Incubations in the presence of histamine (5×10^{-3} M) or aminoguanidine (5×10^{-4} M) or both together resulted in no inhibition of enzyme activity.

Assay of histidine decarboxylase in gastric tissue

Preparation of tissue. Female rats weighing 150 to 200 g were fed nothing but tap water for 16 hr before study. They were killed by decapitation; stomachs were removed and washed briefly in cold water. The squamous portion of the stomach was removed and discarded. The glandular portion (full thickness) was homogenized in 3 ml cold 0·1 M sodium acetate buffer. After centrifugation at 1000 *g* for 15 min the supernatant fluid was removed for further processing.

Sephadex chromatography. The procedure used to purify the fetal enzyme effectively removes virtually all endogenous substrate and any other substances of small molecular size that may influence the reaction. However, because it involves precipitation with heat and ammonium sulfate, its usefulness is limited to preparation of large amounts of enzyme for experiments in which estimation of the activity per unit of the original tissue is not required. Sephadex chromatography was found to be suitable for these purposes.

Columns were prepared containing Sephadex-G-25; the final dimensions of the gel bed were 11·5 mm internal diameter by 130 mm height. Aliquots of the supernatant of the tissue homogenate measuring 1·0 ml or less were applied to the column; as the meniscus settled into the gel bed, 10 ml of cold distilled water was added to the column. The colored protein fraction was collected to be assayed subsequently for histidine decarboxylase activity and for protein content.

To demonstrate that this procedure successfully removed substrate from the solution, tissue extract, 1 μmole L-histidine, and 1 μc ^{14}C-histidine were mixed in a final volume of 1 ml and applied to the Sephadex column. Less than 2% of the radioactivity was recovered in the protein fraction, indicating a highly efficient separation.

To demonstrate that this procedure had no adverse effect on enzyme activity, some preparations were subjected to this procedure twice, and aliquots were removed after each passage through the column, for enzyme and protein assay. While the enzyme activity per unit volume diminished with repeated chromatography, there was no significant change in the specific activity (counts/min/hr/mg protein).

Results of assay. The assay procedure was the same as that used for the fetal enzyme, with one exception. The amount of enzyme used was 1·0 ml of the final preparation which generally contained from 4 to 9 mg protein.

Experiments were done similar to those described for the fetal enzyme, to indicate that the reaction proceeded at a linear rate for at least 2 hr; required pyridoxal-5′-phosphate and that the amount used was in excess; was stereospecific for L-histidine; was inhibited completely by NSD-1055 (5×10^{-7} M) or MK-785 (2×10^{-5} M) but not at all by histamine (5×10^{-3} M); and had a pH optimum of 6·8.

The results of some typical assays of histidine decarboxylase activity in stomach tissue are listed in Table 4. The results are expressed as counts per minute per incubation or per milligram protein. Since the recovery of protein from the Sephadex columns is quantitative, by weighing the tissue prior to processing and assaying protein in the supernatant of the homogenate, it is possible to express results as cpm per g tissue. By the conversion factor 655 counts/min = 1·0 mμmole histamine (derived above), it is also possible to express results in terms of the amount of histamine formed.

TABLE 4. HISTIDINE DECARBOXYLASE ACTIVITY IN STOMACH TISSUE FROM RATS

(counts/min/incubation*)	(counts/min/mg protein)
3270	363
3561	419
1860	351
2237	520

* See text for description of incubations.

Assays were also done on supernatants of gastric homogenates that had not been subjected to gel filtration. Incubations containing up to 40 gm tissue protein resulted in small and erratic yields of radioactivity. The statistics of counting in this system did not permit meaningful estimates of the specific activity of histidine decarboxylase in crude tissue extracts prior to Sephadex chromatography.

DISCUSSION

In the past it appeared that even radioactive methods lacked the sensitivity required for dependable assays of histidine decarboxylase activity in individual tissue specimens. When these assays were used for study of crude tissue homogenates or extracts, very low amounts of radioactivity were recovered as product; commonly, final counting was done on samples containing less than 100 counts/min above background.[3,4] Confident interpretation of such results required unusual precision in all manipulations during the incubation and subsequent counting. The present findings indicate that this apparent lack of sensitivity was not, as previously believed, due to low enzyme activity in the tissue but rather to the presence of some interfering substance that can be removed by Sephadex chromatography.

As noted above, we were unable to estimate directly the apparent increase in specific activity of histidine decarboxylase accomplished by gel filtration, owing to our inability to assign meaningful values to the crude homogenates. Therefore,

for purposes of illustration we shall compare the results of the present method with the most closely related previously reported method. Kobayashi[4] used 8 to 10 times more extract of gastric tissue, incubations twice as long, and added substrate of approximately 3·5 times greater specific activity (after correction for that fraction of uniformly labeled histidine that is not labeled in the carboxyl position). Under these conditions he reported net counts/min above background ranging from 29 to 131 and averaging 72. These may be compared with typical net counts/min averaging about 2500 by the present assay. Presumably the difference may be accounted for, at least partially, by the fact that in the present experiments endogenous substrate was removed prior to incubation and thus was not present to decrease the specific activity of substrate. However, in order to produce so great a degree of decrease in specific activity the concentration of endogenous L-histidine would have to exceed 0·1 M, an obvious impossibility. Perhaps in crude homogenates, endogenous substrate is located strategically in relation to the enzyme so that it is metabolized preferentially; e.g. a loose binding to the enzyme that is broken by Sephadex chromatography. More likely is the possibility that Sphadex chromatography removes some as yet unidentified soluble inhbitor from the preparation.

Previously reported methods have proved quite satisfactory for the study of histidine decarboxylase activity in highly purified preparations of the enzyme.[6, 11] The only advantages offered by the present method for these purposes are simplicity and the convenience of a disposable incubation vessel. The enhanced sensitivity of the present procedure and the fact that it permits estimation of enzyme activity in terms of total histamine formed and expression of activity per unit of the original tissue make it particularly applicable to studies of histidine decarboxylase activity in individual tissue specimens. For example, this procedure should facilitate studies of such problems of current interest as estimation of the relative contributions of specific histidine decarboxylase and the nonspecific aromatic L-amino acid decarboxylase to histamine biosynthesis in different tissues and in different species; clarification of the purported role of increased histidine decarboxylase activity as a necessary correlate of various physiologic functions such as gastric acid secretion;[12] and studies of the effects of various drugs on histidine decarboxylase activity *in vivo*.

Preliminary investigations in this laboratory suggest the following precaution in using this procedure for study of histidine decarboxylase activity in homogenates of tissue obtained from animals to which inhibitors of this enzyme have been administered. We have found that some substances such as NSD-1055 are irreversible inhibitors; they are tightly bound to the enzyme and not removed by gel filtration. Other drugs such as MK-785 are reversible inhibitors and are removed by Sephadex chromatography. Therefore, use of this procedure would lead to the erroneous[11] conclusion that administration of MK-785 to animals results in no inhibition of histidine decarboxylase activity. This procedure obviously is not suitable for studies of effects of reversible inhibitors *in vivo*.

Finally, we should like to comment on the relation of histamine levels to histamine biosynthesis. It has been reported that release of histamine from skin[3] or from stomach[12] by any of a variety of pharmacologic or physiologic modalities is regularly accompanied by an increase of histidine decarboxylase activity. This finding prompted Kahlson *et al.*[12] to suggest a negative feedback relationship in the biosynthesis of histamine. The most simple of the possible explanations for such a relationship would

be direct inhibition of histidine decarboxylase activity by histamine. This simple explanation seems most unlikely in view of the present demonstration that histamine, in concentrations far greater than those present *in vivo*, has no effect on histidine decarboxylase activity. This negative feedback relationship is probably mediated by some as yet unidentified intermediate mechanism.

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