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INHIBITION OF HISTAMINE SYNTHESIS IN THE RAT BY α-HYDRAZINO ANALOG OF HISTIDINE AND 4-BROMO-3-HYDROXY BENZYLOXYAMINE*

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Abstract—Inhibitors of either specific or nonspecific histidine decarboxylase, or both, were studied *in vitro* and *in vivo*. Alpha-hydrazine analog of histidine (αHH) and 4-bromo-3-hydroxy benzyloxyamine (NSD-1055) were found to be potent inhibitors of the specific enzyme, while α-methyldopa and α-methyldopa-hydrazine had little activity. The latter two compounds as well as NSD-1055 were potent inhibitors of the nonspecific decarboxylase. Only αHH and NSD-1055 altered histamine levels *in vivo*. Administration of these compounds to female rats resulted in decreased levels of histamine in heart (30%), stomach (50%), and urine (45%) but did not alter histamine levels in peritoneal mast cells. Animals whose tissue histamine levels had been partially depleted by pretreatment with compound 48/80 showed little or no further lowering on administration of the histidine decarboxylase inhibitors. Administration of NSD-1055 to germ-free animals produced effects on tissue histamine similar to those in normal animals and decreased already low urinary histamine levels even further. The data suggest that histamine exists in rat tissues in at least three metabolic pools and that its synthesis *in vivo* is catalyzed primarily by the specific decarboxylase. Because of an apparent lack of toxicity, αHH should be a valuable research tool and warrants study as a potential therapeutic agent.

HISTAMINE in mammalian tissues is formed by intracellular decarboxylation of histidine.[1–3] Studies *in vitro* indicate that this reaction can be catalyzed by at least two distinct enzymes, a specific histidine decarboxylase§ and a non specific aromatic-L-amino acid decarboxylase.[4] The relative contribution of each of these enzymes to histamine synthesis *in vivo* has not been precisely determined. The recent development of potent enzyme inhibitors affords a means of determining this as well as evaluating the status of histamine pools in specific tissues.

The enzyme inhibitors used in the present studies were: two inhibitors of the non-specific decarboxylase, namely methyldopa and α-methyldopa-hydrazine;[7] 4-bromo-3-hydroxy benzyloxyamine (NSD-1055), previously shown to be a potent inhibitor

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§ Specific histidine decarboxylases have been prepared from several sources[4–6]. For purposes of presentation, specific histidine decarboxylase(s) in rat tissues will be considered to be a single enzyme.

of both the nonspecific and specific (hepatoma) histidine decarboxylase;[7] and α-hydrazino-analog-of-histidine (αHH), not previously reported upon. After preliminary studies *in vitro*, the effects of these compounds on tissue and urinary histamine levels in female rats were determined. For purposes of comparison, the effects of the histamine-releasing agent, compound 48/80 [8] were also examined, and some of the inhibitor studies were done in germ-free animals. Since the validity of existing chemical methods for assay of histamine in tissues, notably the brain, has been questioned recently,[9] it was also necessary to investigate this problem before undertaking the *in vivo* studies.

The conclusion is reached that the specific histidine decarboxylase is probably responsible for histamine synthesis *in vivo* and that histamine exists in rat tissues in at least three distinct metabolic pools.

MATERIALS AND METHODS

Drugs studied and their sources were as follows: D-2-Hydrazino-3-[4(5) = imidazole] propionic acid hydrochloride (α-hydrazino analog of histidine, αHH, MK-785); α-methyl-3,4-dihydroxy-L-phenylalanine (methyldopa); and racemic 2-methyl-2-hydrazino-3-(3,4-dihydroxyphenyl)-propionic acid (α-methyl-dopa-hydrazine, MK-485) were obtained from Merck, Sharp and Dohme Laboratories. Compound 48/80 was obtained from the Burroughs Wellcome Co., and 4-bromo-3-hydroxy benzyl oxyamine-dihydrogen-phosphate (NSD-1055) from Smith and Nephew, Ltd. All drugs were dissolved in aqueous solution immediately before use, dosage being calculated as mg of drug base; the volume of each injection was 1 ml/100 g rat.

The animals used in these studies were female Sprague-Dawley rats weighing 180 to 240 g. Female rats were chosen because they excrete large quantities of free histamine in the urine.[10] Unless otherwise noted, animals were allowed free access to drinking water and Purina rat chow. During periods of urine collection the animals were placed in glass metabolic cages and fed nothing but water. Urine was collected in glass vessels which contained 0·5 ml of 3 N HCl. Peritoneal mast cells were harvested, washed, and counted as described by Lagunoff and Benditt.[11] Other tissues were removed as quickly as possible after the animals were decapitated without anesthesia; the tissues were washed briefly in cold water and then placed on cracked ice for less than 30 min or frozen for less than 48 hr prior to assay.

Decarboxylase enzymes. Specific histidine decarboxylase was purified from homogenates of whole fetal rats (19 to 20 days' gestation) as described by Håkanson.[5] This preparation was chosen because it is relatively stable and is derived from normal rather than malignant tissue. Enzyme activity was assayed by the method of Weissbach *et al.* [4], modified in that the incubations were carried out at pH 6·5 and that pyridoxal-phosphate was added to a final concentration of $1{\cdot}3 \times 10^{-5}$ M. The finding of Håkanson that this preparation of histidine decarboxylase is absolutely dependent on the addition of pyridoxal-phosphate was confirmed.[5] The presence in the incubation mixture of pyridoxal-phosphate, $1{\cdot}3 \times 10^{-5}$ M, was found to be more than sufficient to produce a maximal rate of histamine formation.

Partial purification and assay of aromatic L-amino acid decarboxylase (nonspecific histidine decarboxylase) was carried out by the method of Lovenberg *et al.*;[6] purification was completed through the alumina Cγ gel adsorption and elution step. In contrast to the specific enzyme, the activity of this preparation is only slightly

enhanced by the addition of pyridoxal-phosphate; thus, no cofactor was added to these incubations.

Assay of histamine in tissues. Isotope dilution experiments were done to test the specificity of existing methods for the preparation of specimens for assay of histamine. Tissues were extracted into four to nine volumes of 0·4 N perchloric acid as described by Shore *et al.*[12] To each acid extract was added histamine dihydrochloride-2-(ring)-^{14}C (specific activity: 21·4 μc/mmole) (California Corp. for Biochemical Research) in sufficient amount to produce an estimated specific activity in the solution of the order of 1,000 to 5,000 cpm/μg histamine. These extracts were subjected sequentially to three procedures designed to separate histamine from other substances: (1) extraction from an alkaline medium into *n*-butanol and back into an acid medium, as described by Shore *et al.*;[12] (2) adsorption on a cation exchange resin (IRC-50) column and elution with acid as described by Oates *et al.*;[13] and (3) passage through an anion exchange resin (Dowex-1-carbonate) column, as described by Lovenberg) *et al.*[7] Aliquots of each extract were assayed for histamine by the method of Shore *et al.*[12], based on the fluorometric assay of the product of reaction of histamine with *o*-phthalaldehyde (OPT). Aliquots of each solution were also transferred to counting vials; a scintillation fluorophor solution[14] was added and the radioactivity determined in a scintillation spectrometer.

The results of these experiments are listed in Table 1. The specific activity of

TABLE 1. SPECIFIC ACTIVITY* OF HISTAMINE IN TISSUES AFTER SERIAL SEPARATION PROCEDURES

	Brain	Stomach	Heart
1. Butanol extraction	5,410	1,069	3,756
2. Elution from IRC-50	5,992	569	3,788
3. Effluent from Dowex-1-CO_3	6,908	584	3,674

* Specific activity = counts per minute per microgram apparent histamine. Internal standards were prepared by adding ^{14}C-histamine and authentic histamine dihydrochloride (Nutritional Biochemicals Corp.) to acid extracts of tissues. Also prepared were tissue and reagent blanks to which ^{14}C-histamine was also added. Each number represents the average result of two separate experiments and is corrected for recovery and quenching of ^{14}C-histamine. The concentration of histamine in each extract was changed less than 0·1% by the addition of radioactive material. See text for details of procedure.

apparent histamine from heart extracts remained constant through the three steps. The specific activity of apparent histamine from stomach decreased from step 1 to step 2, indicating that some substance(s) that quenches histamine fluorescence was removed in step 2 but not in step 1. The specific activity of apparent histamine from brain increased with each step, indicating that each procedure removed some substance(s) other than histamine that reacts with OPT to yield fluorescent product.

There are published data that support the validity of specific techniques for assay of histamine in urine[13] and mast cells.[15] On the basis of these published data and the present findings it was decided to assay histamine in heart and mast cells by the

method of Shore *et al.*[12] and in stomach* and urine by the modified method of Oates *et al.*[13] It was further decided not to attempt assays of histamine in brain.

EXPERIMENTAL AND RESULTS

1. *Histidine decarboxylase inhibition* in vitro

Incubations of specific and nonspecific histidine decarboxylase were carried out in the presence of four inhibitors added to final concentrations of between 10^{-8} and 10^{-3} M. In studies of both enzymes, all ingredients of the incubation mixtures, including inhibitors, were preincubated at 37° for 10 min before the reaction was started by the addition of substrate. The degrees of inhibition produced by each concentration of each compound were plotted on graph paper; from these curves the relative potencies of inhibitors against both enzymes, expressed as concentrations required to produce 75% inhibition, were estimated (Table 2). NSD-1055 was the

TABLE 2. INHIBITION OF HISTIDINE DECARBOXYLASE *IN VITRO* BY FOUR COMPOUNDS

Compound	Concentration ($M \times 10^5$) producing 75% inhibition*	
	Specific enzyme	Nonspecific enzyme
αHH	7	80
NSD-1055	0·04	0·009
Methyldopa	>100	0·08
α-Methyldopa-hydrazine	20	0·09

* Numbers represent concentrations of each compound that produced 75% inhibition of activity, estimated as described in the text and multiplied by 10^5. Thus, low numbers indicate high potency as inhibitors. Each number represents the average of values obtained in at least two experiments. See text for procedures.

most potent inhibitor of both enzymes. The only compound found to be a more potent inhibitor of the specific enzyme than of the nonspecific was αHH. The degree of inhibition of the specific enzyme by each of these compounds was reduced in the presence of large excesses of pyridoxal-phosphate. Attempts to determine the nature of inhibition (competitive or noncompetitive) of the specific enzyme yielded uninterpretable data probably because, as observed by Håkanson,[5] the pH optimum for this enzyme changes with the concentration of the substrate.

2. *Effects of various compounds on histamine levels* in vivo

A. Single large doses. Alpha-hydrazino analog of histidine, NSD-1055, and methyldopa were administered i.p. in doses of 100 mg/kg. Urine collections were begun immediately after injection. The animals were sacrificed 24 hr later, and histamine levels in heart, stomach, and urine were assayed (Table 3). The two compounds that inhibited specific histidine decarboxylase, αHH and NSD-1055, produced significant decreases in histamine levels in heart, stomach, and urine. Methyldopa, which

* Subsequently we found that the problem of quenching fluorescence in extracts of gastric tissue was obviated if smaller specimens of tissue were prepared for assay by the butanol-extraction method. However, for purposes of continuity, the IRC-50 adsorption and elution step was used throughout these studies.

TABLE 3. HISTAMINE LEVELS BEFORE AND 24 HOURS AFTER INTRAPERITONEAL ADMINISTRATION OF SEVERAL COMPOUNDS

Compound	Dose (mg/kg)	Heart* (μ/g)	Stomach* (μg/g)	Urine* (μg/24 hr)
None (control)		4·3 ± 0·4 (35	19·7 ± 2·7 (20)	37·0 ± 4·8 (31)
αHH	100	3·2 ± 0·4 (12, P < 0·001)	12·0 ± 1·4 (8, P < 0·001)	22·4 ± 6·7 (8, P < 0·01)
NSD-1055	100	2·9 ± 0·3 (8, P < 0·001)	11·2 ± 1·4 (8, P < 0·001)	19·8 ± 2·2 (8, P < 0·001)
Methyldopa	100	4·1 ± 0·3 (5, P > 0·05)	25·2 ± 3·2 (5, P > 0·05)	41·1 ± 7·1 (5, P > 0·05)
48/80	2†	2·5 ± 0·4 (7, P < 0·001)	14·7 ± 1·3 (7, P < 0·01)	75·7 ± 13·4 (5, P < 0·01)

* Each value is expressed as the mean ± 1 standard deviation; in parentheses are the numbers animals studied and the probability of the chance occurrence of the differences from controls expressed as the P value.
† Administered in two doses (see text for details).

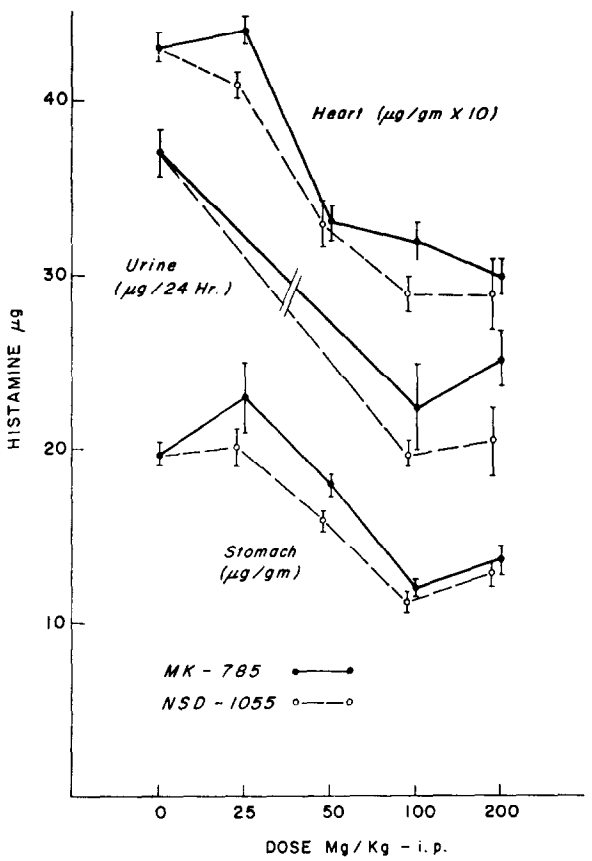

FIG. 1. Histamine levels in heart, urine, and stomach 24 hr after i.p. administration of various doses of MK-785 (αHH) and NSD-1055. Each point represents the average of at least four observations; brackets designate standard errors of means.

inhibited only the nonspecific decarboxylase, did not affect histamine levels significantly. For purposes of comparison compound 48/80, which acts by release of histamine, was administered twice i.p. in a dose of 1 mg /kg,24 hr and 16 hr before the animals were sacrificed. This treatment also resulted in decreased histamine levels in heart and stomach but increased urinary excretion of histamine (Table 3).

B. Dose–response relationships. Animals were sacrificed 24 hr after i.p. administration of various doses of αHH and NSD-1055. Histamine levels were determined in heart, stomach and urine. The minimal dose of either drug required to lower the histamine levels in heart or stomach significantly was greater than 25 mg/kg, and maximal effects were obtained with doses of 100 mg/kg (Fig. 1).

C. Onset and duration of effects. Histamine levels in heart, stomach, and urine were assayed at various times after i.p. injections of single doses of 100 mg of αHH and NSD-1055 per kg. Significant diminution of histamine levels was detected earlier in heart than in stomach, but maximal effects were observed at 3 to 6 hr in both tissues (Fig. 2). Histamine levels began to return toward normal within 24 hr; the return to normal was complete within 48 hr.

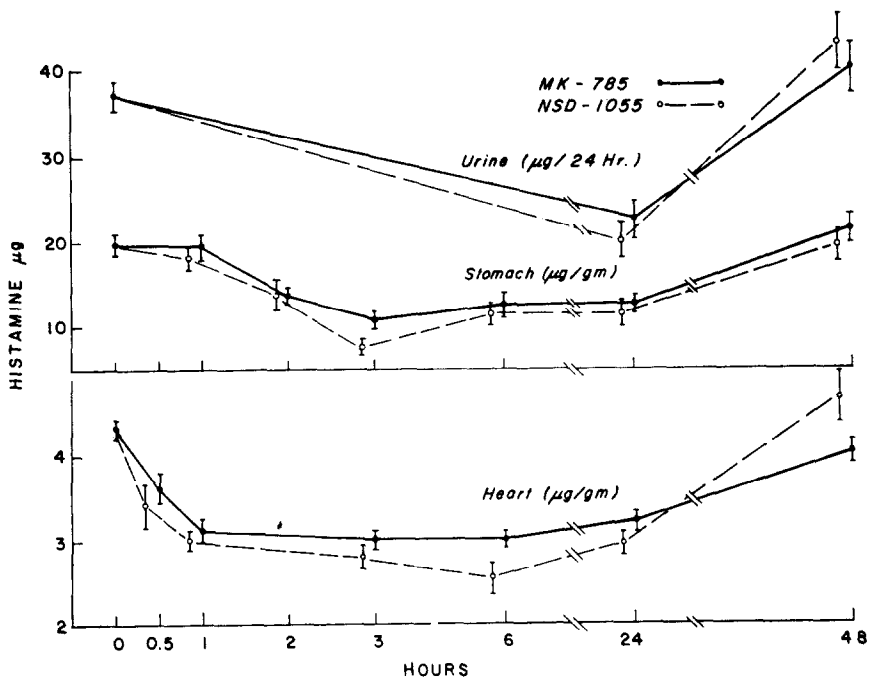

FIG. 2. Histamine levels in heart, stomach, and urine at various time intervals after i.p. administration of MK-785 (αHH) and NSD-1055), 100 mg/kg. Each point represents the average of at least four observations; brackets designate standard errors of means.

D. Repeated administration. NSD-1055 and αHH were each injected every 12 hr in a dosage of 100 mg/kg in four rats. A control animal was also studied. This dosage schedule of NSD-1055 was lethal to all four rats by the third day, whereas no toxicity was apparent with αHH during four days of study. Both drugs produced a marked decrease in urinary histamine excretion (Fig. 3). After 96 hr of αHH administration,

the average level of histamine in heart was 2·4 ± 0·4 μg/g, and in stomach 8·6 ± 1·4 μg/g. These values were slightly but significantly lower than the lowest values observed after single doses of this compound.

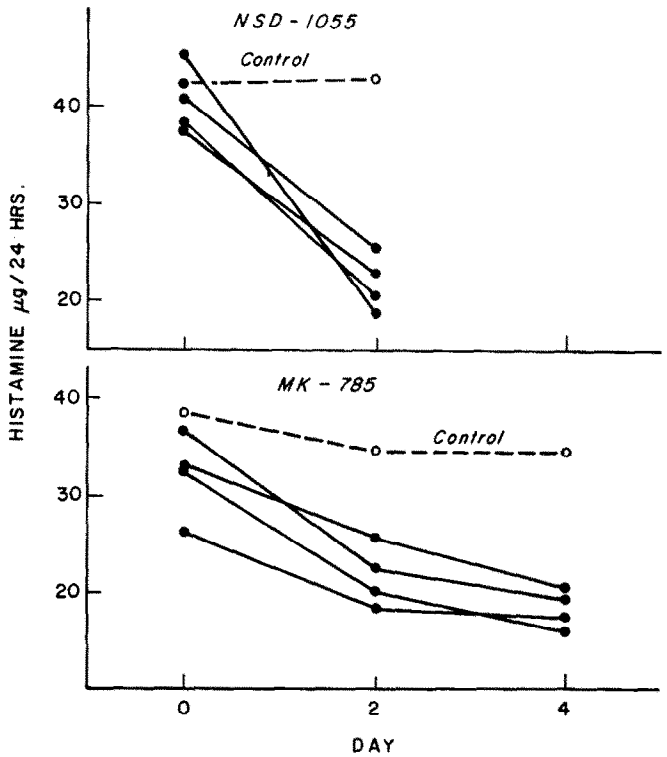

FIG. 3. Urinary excretion of histamine during administration of NSD-1055 and MK-785 (αHH), 100 mg/kg i.p. every 12 hr. Each line joins points representing values obtained in a single rat.

E. Effects on histamine levels in mast cells. NSD-1055 and αHH were injected s.c. to avoid artifacts due to local inflammatory reactions. The doses were 100 mg/kg given twice, 24 hr and 12 hr before sacrifice. Although urinary histamine excretion decreased significantly, indicating that administration of compounds by this route inhibited histamine formation, there was no effect on mast cell levels of histamine (Fig. 4). In other studies, mast cell histamine levels were found to be unaffected 3 hr after single doses of 200 mg of either drug per kg.

3. *Studies of the histamine remaining in tissues and urine after administration of histidine decarboxylase inhibitors*

A. Effects of histidine decarboxylase inhibitors after pretreatment with compound 48/80. The results presented above suggested the possibility that there could be two distinct pools of histamine in heart and stomach: one that was released by compound 48/80 and another that was depleted by inhibition of histidine decarboxylase. As shown in the following experiment, this hypothesis could not be substantiated.

Compound 48/80, 1 mg/kg, was injected i.p. at zero time and at 8 hr. At 24 hr some

animals received i.p. injections of αHH or of NSD-1055, 100 mg/kg. At 27 hr the animals were sacrificed, and tissue histamine levels were assayed (Fig. 5). In the heart, histamine levels after combined treatment were slightly lower than after administration of either compound alone. However, the levels did not approach zero. In the stomach, no additive effect was observed.

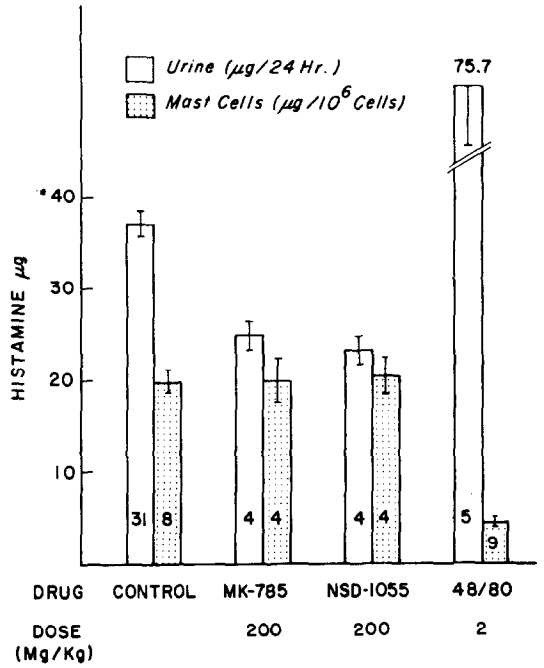

FIG. 4. The effects of MK-785 (αHH) and NSD-1055 injected s.c. in two doses of 100 mg/kg, 24 and 12 hr before sacrifice of animals, on histamine levels in urine and in peritoneal mast cells. For purposes of comparison, the effects of i.p. injections of compound 48/80 are also illustrated. The numbers in the bars indicate the numbers of observations; brackets designate standard errors of means.

B. Effects in germ-free animals. The following experiments indicate that during the administration of histidine decarboxylase inhibitors, a significant proportion of the histamine remaining in the urine was probably formed by intestinal bacteria. Fifteen germ-free female rats* of the Sprague-Dawley strain, weighing 160 to 180 g, were studied in groups of five. Control observations were made on the first group. Of the animals in the other groups, drugs were administered to four and one served as control. The timing of events is outlined below.

1:00 P.M.: Animals arrived in laboratory and were placed in glass metabolic cages which were not germ-free; they were permitted to ingest nothing but water.

5:00 P.M.: NSD-1055 or αHH, 100 mg/kg, was injected i.p.

9:00 A.M., day 2: Injections were repeated; 24-hr urine collection was begun.

*Born and raised under germ-free conditions in the Germ Free Animal Production Unit (Chief, Dr. Carl Miller), Division of Research Services, National Institutes of Health; these rats were made available to us by Mr. Howard Bohner.

9:00 A.M., day 3: The experiment ended; animals were sacrificed and specimens were obtained in the usual fashion for histamine assays.

The results of these experiments are listed in Table 4. In untreated germ-free rats, histamine excretion in urine was 64% lower than in normal rats; histamine levels in

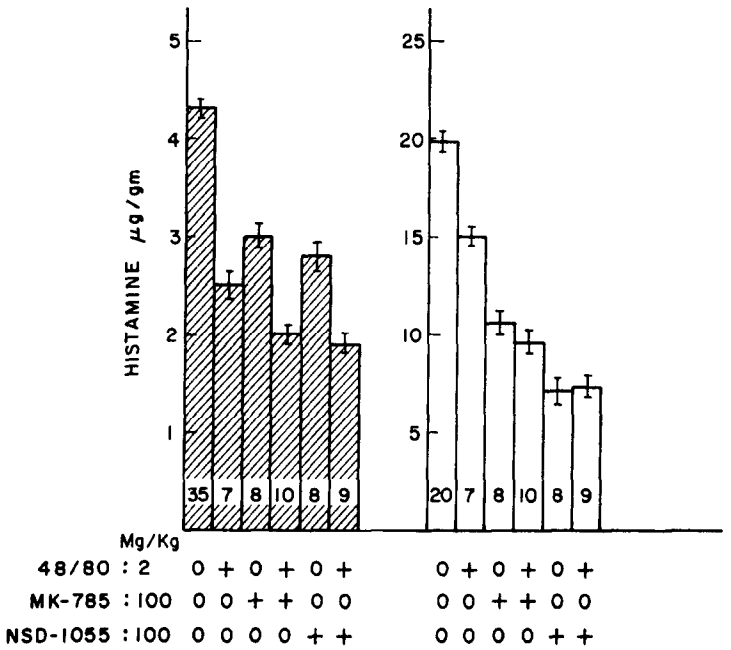

FIG. 5. Histamine levels in heart (hatched bars) and stomach (open bars) after i.p. injections of MK-785 (αHH) or NSD-1055 alone, or after pretreatment with compound 48/80, or after compound 48/80 alone. See text for details. The numbers in the bars indicate the number of observations; brackets designate standard errors of means.

TABLE 4. EFFECTS OF NSD-1055 AND α-HYDRAZINO ANALOG OF HISTIDINE ON HISTAMINE LEVELS IN GERM-FREE RATS

Compound	Dose* (mg/kg)	No. of animals	Heart† (μg/g)	Stomach (μg/g)	Urine (μg/24 hr)
None (control)		7	4·3 ± 0·4	22·2 ± 4·6	13·3 ± 4·6‡
αHH	200	4	2·8 ± 0·4 (P < 0·01)	15·9 ± 2·8 (P < 0·05)	lost
NSD-1055	200	4	3·1 ± 0·4 (P < 0·05)	15·5 ± 1·4 (P < 0·01)	4·7 ± 1·4 (P < 0·001)

* Administered in two doses (see text for details).

† Each value represents mean ± standard deviation. Numbers in parentheses represent the probability of the chance occurrence of the differences from controls expressed as P.

‡ Significantly lower than in animals that were not germ-free (P < 0·001).

heart and stomach were normal. Mast cell histamine levels were 19·8 ± 4·1 μg/10^6 cells, also not significantly different from normal. The effects of histidine decarboxylase inhibitors on levels of histamine in the heart were not significantly different from

those observed in normal rats. The decrease in stomach histamine levels after histidine decarboxylase inhibition in germ-free rats was slightly less than in normal rats. Urinary excretion of histamine in germ-free rats that received NSD-1055 was very low. Data are not available on animals that received αHH, owing to technical difficulties in the collection of their urine.

DISCUSSION

The present findings indicate that tissue levels of histamine may be lowered by administration of αHH and NSD-1055 in rats and that these changes result from inhibition of histamine synthesis by specific histidine decarboxylase. The fact that urinary histamine decreased concomitantly with the lowering of tissue levels favors an effect on synthesis, since comparable changes in tissue levels produced by the releasing agent, compound 48/80, were accompanied by a substantial increase in urinary histamine excretion.* That histidine decarboxylase inhibitors blocked histamine synthesis nearly completely was indicated by the very low levels of urinary histamine excretion produced in starved germ-free rats in which the contribution of histamine absorption from the gastrointestinal tract was minimized. Further, histamine-depleting activity of different compounds *in vivo* paralleled their activities on specific histidine decarboxylase *in vitro*. Alpha-hydrazino analog of histidine was found to be a potent inhibitor of the specific enzyme only. While NSD-1055 is also a potent inhibitor of the general aromatic-L-amino acid decarboxylase, a selective inhibitor (methyldopa) of the latter enzyme failed to lower tissue or urinary histamine levels. A corollary conclusion is that the synthesis of histamine *in vivo* is catalyzed primarily, if not exclusively, by specific histidine decarboxylase.

It is likely that inhibition of enzyme activity by αHH and NSD-1055 depends upon an interaction of inhibitor with cofactor. The degree of inhibition produced by each of these compounds *in vitro* was reduced in the presence of excess pyridoxal-phosphate. It has been shown that NSD-1055,[6] α-methylamino acids,[16] and hydrazine types of inhibitors in general[6] react with pyridoxal-phosphate, Furthermore, Kahlson *et al.*[3] have shown that the feeding of a pyridoxine-deficient diet to female rats resulted in lowering histamine levels in urine and in some tissues. These effects were enhanced by additional administration of semicarbazide, a toxic carbonyl reagent which reacts with pyridoxal. These workers also noted that administration of large doses of α-methylhistidine, a relatively weak inhibitor of specific histidine decarboxylase[3,4,6] resulted in decreased urinary excretion of histamine; effects of this agent on tissue levels of histamine were not reported.

The present findings also indicate that histamine must exist in various tissues of the rat in at least three distinct metabolic pools, the first of which has a very short half-life, as indicated by rapid depletion (3 to 6 hr) of histamine in heart and stomach by the inhibition of histamine synthesis. That this pool of histamine is also released by compound 48/80 was indicated by the finding that histidine decarboxylase inhibitors produced little or no further lowering of tissue histamine levels in animals pretreated with 48/80. Evidence that compound 48/80 releases histamine from a pool that is rapidly turning over has also been provided by Riley, who reported that the histamine

* R. W. Schayer (personal communication) found that αHH was much more effective than α-methylhistidine in blocking the formation of ^{14}C-histamine, measured 2 or 3 days after injections of ^{14}C-L-histidine, in skin of mice and lung of guinea pigs.

content of gastric tissue returned to levels that were even higher than normal within 6 days after the cessation of compound 48/80 administration.[8]

The other two metabolic pools of histamine turn over more slowly, and thus were not depleted by the acute inhibition of histamine synthesis. One of these pools is located in mast cells and according to Schayer[1] has a half-life of approximately 50 days. The present findings confirm previous reports[8] that this pool is also depleted almost completely by compound 48/80. The other pool with slow turnover is located in organs such as heart, stomach and, as suggested by the data of Kahlson *et al.*[3], in lung. Since it is not released by compound 48/80, it is presumed not to be in mast cells. Furthermore, mast cells are rare in the lung and stomach of the rat.[8] Also consistent with this concept of histamine pools are the findings of Kahlson *et al.*[3] in female rats whose histamine-forming capacity was diminished by pyridoxine deficiency. Significant lowering of gastric histamine levels was detected within 24 hr, but only after 140 days in tissues such as skin that are rich in mast cells, and not at all in lung.

The finding that tissue levels of histamine in germ-free rats were not different from those in normal rats confirms similar findings of Gustafsson *et al.*[10] However, the subnormal urinary histamine excretion in female germ-free rats does not agree with their observation in one female germ-free rat that the urinary excretion of histamine was higher than normal. They found normal urinary excretion of total histamine in male germ-free rats.

Finally, since αHH selectively inhibits specific histidine decarboxylase *in vitro* and, in doses that are without apparent toxicity, it effectively inhibits histamine synthesis *in vivo*, this compound is obviously a valuable research tool; further studies are planned to evaluate it as a potential therapeutic agent.

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