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INHIBITION OF HISTIDINE DECARBOXYLASE AND DIAMINE OXIDASE BY 4-BROMO-3-HYDROXYBENZYLOXYAMINE

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Abstract—In freely fed rats, an intraperitoneal injection of 0·688 m-mole per kg of 4-bromo-3-hydroxybenzyloxyamine (NSD 1055) produces a transient drop in both the histidine decarboxylase activity of the glandular stomach and the diamine oxidase activity of the intestine. Both enzymes are inhibited by approximately 50 per cent within 30 min, but recovery to normal levels occurs within a few hours. The results obtained *in vivo* are in sharp contrast to the data *in vitro*, which show that the inhibitor is much more active against the decarboxylase. The transient effect of NSD 1055 against the two enzymes *in vivo* may be due primarily to the rapid clearance or metabolism of the drug, while the change in potency ratio from a situation *in vitro* to one *in vivo* may reflect the greater rate of turnover of histidine decarboxylase. The drug, however, is very effective in preventing the rise in histidine decarboxylase produced by insulin, pentagastrin or refeeding of starved animals. Presumably under these circumstances there is a greater opportunity for the drug to complex with the cofactor, pyridoxal phosphate.

THE VALUE of an agent which specifically inhibits histidine decarboxylase is twofold. First, it would facilitate the efforts of those investigators who are primarily concerned with unraveling the role of histamine in mammalian physiology. Second, in view of the possible involvement of histamine in various clinical conditions, such an agent is potentially of considerable therapeutic value.

Since the decarboxylation of histidine at any particular site will depend upon the presence of appropriate amounts of substrate, the coenzyme pyridoxal phosphate and the apoenzyme, interference with the supply or functioning of any of these components could lead to inhibition of histamine formation. Procedures involving the use of pyridoxine-deficient diets or nonspecific inhibitors such as semicarbazide have been shown to have pronounced effects on histamine metabolism,[1, 2] but these methods have not gained general approval because of the difficulty of interpreting the results. Inhibitors of protein synthesis, such as cycloheximide and puromycin, are very effective inhibitors of histidine decarboxylase in the gastric mucosa of the rat,[3–6] but the usefulness of these compounds is limited because of their high toxicity. A more specific inhibitor of histidine decarboxylase is obviously desirable, and Shepherd and Mackay[7] have recently reviewed the many attempts that have been made to find such a compound. The search has been rather disappointing, but one of the substances that has aroused considerable interest is 4-bromo-3-hydroxybenzyloxyamine, which is more

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often referred to as NSD 1055 or brocresine. This compound has been shown to be a very potent inhibitor of histidine decarboxylase *in vitro*[8–10] and it has been widely used, particularly in attempting to resolve the role of histamine in gastric functioning.[11–13] NSD 1055 has also been reported to inhibit histamine biosynthesis in man.[14] There is, however, some disagreement on the effectiveness *in vivo* of NSD 1055. Levine *et al.*,[15] for example, observed substantial reductions in tissue and urinary histamine in the rat after treatment with NSD 1055, whereas Johnston and Kahlson[16] did not find this drug to be an effective inhibitor of whole body histamine formation. Although Levine[17] has shown that rats from different sources may differ in their sensitivity to NSD 1055, more information is clearly needed on the ability of this compound to inhibit histidine decarboxylase *in vivo*. The present paper demonstrates that NSD 1055 may affect both the formation and catabolism of histamine. A preliminary report has been published.[18]

METHODS

Male Sprague–Dawley rats (Charles River) weighing 175–225 g were used throughout. Animals were killed by decapitation and the stomachs and intestines were removed and cleaned. For preparation of histidine decarboxylase, only the thick-walled glandular stomach was used. The first 12 in. of the small intestine was used for preparing monoamine oxidase and diamine oxidase. Tissues from four animals were pooled except where stated in the text.

Preparation of tissues. For the assay of intestinal diamine oxidase, 1 g tissue was homogenized in an Omnimixer in 24 ml of 0·1 M borate buffer, pH 7·8, and then centrifuged at 10,000 *g* for 30 min. The supernatant solution was decanted and diluted 1:1 with buffer to a final concentration of 1:50. Two ml of this solution was taken for analysis.

For the assay of histidine decarboxylase, glandular stomach tissue was finely minced with scissors, homogenized in 4 vol. of 0·25 M sucrose in an Omnimixer and centrifuged at 10,000 *g* for 30 min. One-ml aliquots of the supernatant fraction were tested for enzyme activity.

For the assay of monoamine oxidase, 1 g intestine was homogenized in 49 ml of 0·1 M borate buffer, pH 7·8, and then centrifuged at 10,000 *g* for 30 min. The particulate fraction was reconstituted to the original volume with buffer and 1-ml aliquots were used for assay.

Enzyme assays. Histidine decarboxylase was assayed as described previously.[19] Briefly, the reaction was carried out in a Warburg flask and the incubation mixture consisted of carboxyl-^{14}C-L-histidine (0·1 μc in 0·1 ml containing 5 μg L-histidine), 10 μg pyridoxal phosphate, 1 ml of enzyme extract and 0·1 M phosphate buffer, pH 7·0, to a final volume of 2 ml. Incubation was allowed to proceed for 2 hr at 37° in a shaking incubator, during which time the evolved $^{14}CO_2$ was absorbed on filter paper impregnated with hyamine hydroxide. The reaction was stopped by the addition of 0·2 ml of 1 M citric acid and shaking was continued for a further hour to allow complete absorption of $^{14}CO_2$. The filter strips were then removed, added to a toluene counting solution and counted in a liquid scintillation counter as previously described.[19]

Diamine oxidase was determined by using a modification of the method of Okuyama and Kobayashi.[20] Each assay tube contained 1 ml enzyme, ^{14}C-putrescine (0·1 μg in 0·1 ml containing 50 μg putrescine) and phosphate buffer (0·1 M, pH 6·9) to a final volume of 2·0 ml. Incubation time was 2 hr in air at 37°. The reaction was stopped by

adding a mixture of aminoguanidine (227 μg) and sodium bicarbonate (200 mg). In the original procedure, the reaction was stopped by saturating the reaction mixture with sodium bicarbonate, but it was subsequently found that if the extraction was delayed the reaction continued. The addition of aminoguanidine prevents further enzyme action and does not interfere with the extraction of the reaction product. After stopping the reaction, the end-product, Δ'-pyrroline, was extracted directly into a toluene–PPO (PPO = 2,5-diphenyloxazolyl) counting solution and counted in a scintillation counter.

Monoamine oxidase was assayed as described by Otsuka and Kobayashi.[21] Each assay tube contained 1 ml enzyme, ^{14}C-tyramine (0·1 μc in 0·1 ml containing 5 μg tyramine) and a final concentration of 10^{-5} M aminoguanidine and 10^{-3} M EDTA (disodium salt) and borate buffer (0·1 M, pH 7·8) to a final volume of 2·3 ml. Incubation time was 2 hr in air at 37°. Anisole–PPO was used to extract the radioactive end-product.

Assay for ^{14}C-histamine derived from ^{14}C-L-histidine. The assay for ^{14}C-histamine formed from ^{14}C-L-histidine is essentially the method of Schayer and Smiley.[22] In summary, it consisted of isolating the glandular portion of the rat stomach after the injection of labeled histidine, the addition of 66·4 mg of carrier histamine dihydrochloride, the homogenization of this tissue with carrier, the precipitation and removal of the protein with trichloroacetic acid, the extraction of the trichloroacetic acid from the solution with ether, and the extraction of the histamine into butanol from an aqueous alkaline solution. The butanol extract was then washed twice with 20-ml portions of an alkaline aqueous solution containing 20 mg of nonisotopic L-histidine to reduce the contamination due to radioactive histidine, and, finally, the butanol phase was extracted with HCl. The combined HCl extracts, which now contained the histamine, were then taken to dryness on the hot plate and the histamine was isolated as the picrate. The picrate was then recrystallized to constant specific activity.

Liquid scintillation counting of samples from the enzyme assays was carried out in a Packard 3002 or a Nuclear Chicago 725 scintillation spectrometer. Counting efficiency was 30 per cent for the histidine decarboxylase assay and 60 per cent for the diamine and monoamine oxidase assays. ^{14}C-histamine dipicrate was counted at infinite thickness in a low background gas flow counter (Nuclear Chicago 4318) with a background of 1·5 cpm. Under these conditions, 1 μg ^{14}C-histamine gives about 10,000 cpm.

RESULTS

In confirming the remarkable inhibitory potency of NSD 1055 against histidine decarboxylase *in vitro*, the drug was also subjected to a general screening against two other enzymes, diamine oxidase and monoamine oxidase. Figure 1 shows the effect of NSD 1055 on these enzyme systems *in vitro*. Histidine decarboxylase was prepared from the glandular stomach of the rat, while diamine oxidase and monoamine oxidase were prepared from the small intestine. In order to facilitate a rough comparison of the inhibitory potency of NSD 1055 on the different enzymes, the results are expressed as a percentage of the uninhibited control value. In addition to the expected inhibition of histidine decarboxylase, it is clear that NSD 1055 also produces a marked inhibition of diamine oxidase. There is, however, a pronounced difference in the effectiveness of the drug against histidine decarboxylase and diamine oxidase in that it is at least 10

times more active against the decarboxylase. Monoamine oxidase was not inhibited by NSD 1055 over the range of concentrations used.

Simply on the basis of these studies *in vitro*, it would be difficult to assess the relative effectiveness of NSD 1055 in inhibiting the histidine decarboxylase and diamine oxidase enzyme systems in the whole animal. The comparatively weak inhibition of diamine oxidase might indicate that this is a trivial action of the drug compared to its

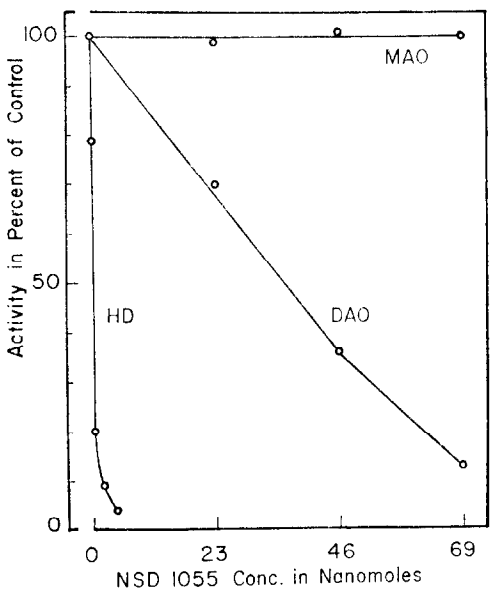

FIG. 1. Effect of NSD 1055 on histidine decarboxylase (HD), diamine oxidase (DAO) and monoamine oxidase (MAO) *in vitro*; 4·6 m-moles is equivalent to 1 μg NSD 1055.

effect on histidine decarboxylase. On the other hand, oxidative deamination by diamine oxidase is the major route of catabolism of histamine in the rat and, furthermore, the doses of NSD 1055 which have been used *in vivo* are quite substantial.

Freely fed rats were given the drug in a dose of 0·688 m-mole/kg intraperitoneally and the animals were killed at various times thereafter. A typical set of results is shown in Fig. 2. To facilitate comparisons, the results were again expressed as a percentage of the uninhibited control values. Concentrating on the first 3-hr injection, both histidine decarboxylase in the glandular stomach and diamine oxidase in the intestine were similarly depressed at 30 min, but in both cases there was some attempt at recovery. By 4–8 hr, the activity of both enzymes was back to normal. Monoamine oxidase activity in the intestine was not affected at this concentration of the drug, although it was noted in some experiments that higher doses of NSD 1055 (1·376 m-moles/kg) did produce a small transient decrease in the activity of this enzyme.

Although these results indicate that the effect of NSD 1055 on both enzymes is very similar, the essential point is that in freely fed animals the drug is as effective against diamine oxidase as it is against histidine decarboxylase. This is a very different situation from what was observed *in vitro*, where NSD 1055 was many times more effective against histidine decarboxylase.

In testing for potential inhibitors of histidine decarboxylase in the gastric mucosa, an alternative procedure to the use of freely fed animals is to use starved animals and to stimulate the decarboxylase with any one of a variety of agents. The inhibitor is then tested against an agent which is known to stimulate the enzyme. We routinely use three procedures to increase histidine decarboxylase activity in starved rats: (1) by giving insulin, (2) by giving pentagastrin, (3) by refeeding. The effect of insulin on histidine decarboxylase is probably mediated through vagal release of endogenous

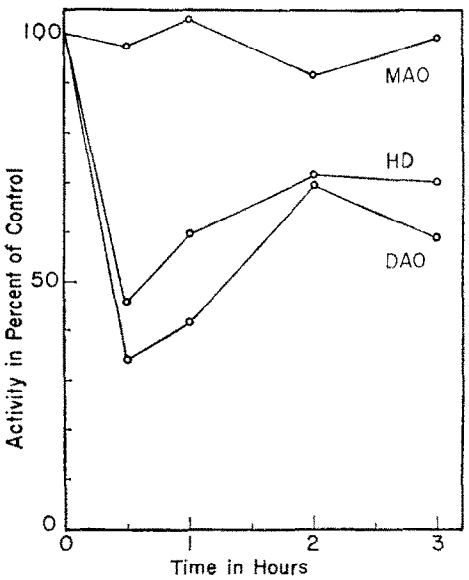

FIG. 2. Effect of NSD 1055 (0·668 m-mole/kg) administered *in vivo* on histidine decarboxylase (HD) diamine oxidase (DAO) and monoamine oxidase (MAO) activities. Each value is obtained from pooling the tissues from four animals.

gastrin,[4, 23] whereas pentagastrin, a synthetic analog of gastrin, stimulates the enzyme more directly. By refeeding we can study the response of the enzyme to a natural stimulus which includes, in addition to the release of endogenous gastrin, such factors as the physical distention of the stomach, which are known to affect the enzyme.[24]

Animals were starved for 24 hr and histidine decarboxylase was stimulated by insulin, pentagastrin or refeeding. Enzyme activity was determined 3 hr later. All three procedures caused a substantial rise in histidine decarboxylase activity relative to that of the starved control animals. As shown in Table 1, this rise is markedly inhibited when NSD 1055 is given at the same time as the stimulus.

There are two interesting features of these experiments relative to those shown earlier. First, the degree of inhibition obtained is much greater than when the drug is given to freely fed animals. Second, the inhibition is more prolonged. In freely fed animals where the food intake is not controlled, the sharp rise in histidine decarboxylase which is observed in starved animals given food is rarely seen. On this basis, NSD 1055 appears to be less effective against preformed enzyme than against histidine decarboxylase, which is being rapidly synthesized.

TABLE 1. EFFECT OF NSD 1055 (0·668 m-mole/kg) ON THE RESPONSE OF HISTIDINE DECARBOXYLASE TO INSULIN (5 units/kg), PENTAGASTRIN (200 μg/kg) OR REFEEDING*

Treatment	Histidine decarboxylase activity (cpm/2 ml ± S.D.)		
	Insulin	Pentagastrin	Refeeding
Control starved	477 ± 124 (6)	235 ± 86 (6)	825 ± 192 (4)
Stimulus alone	1564 ± 333 (6)	1224 ± 232 (6)	2262 ± 500 (4)
Stimulus + NSD 1055	426 ± 126 (5)	144 ± 64 (4)	985 ± 445 (4)

* The number of observations is indicated in parentheses.

Previous workers have attempted to correlate inhibition of histidine decarboxylase by NSD 1055 with changes in the histamine content of the mucosa[15] and also with changes in urinary histamine.[16] There does not appear to be any unanimity on these points and our own attempts to relate inhibition of histidine decarboxylase directly to the total histamine content of the mucosa were equivocal. In attempting to determine whether NSD 1055 did indeed inhibit the formation of histamine in the gastric mucosa *in vivo*, the following experiment was carried out.

Animals were starved for 24 hr and injected with insulin or insulin plus NSD 1055 as described previously. Two hr later, 20 μc of uniformly labeled ^{14}C-histidine (specific activity, 250 μc/μmole) was injected intraperitoneally and 60 min later the animals were killed and the ^{14}C-histamine in the glandular stomach was determined by isotope dilution. Using this procedure, the ^{14}C-histamine found in the stomach reflects the histidine decarboxylase activity. Thus, as shown in Table 2, the amount of ^{14}C-histamine found in the mucosa of an insulin-treated animal is greater than that in a control animal and, as shown in Table 1, the histidine decarboxylase is similarly increased under the same experimental conditions. The administration of NSD 1055 at the same time as the stimulus markedly reduces the amount of ^{14}C-histamine present in the mucosa.

TABLE 2. INHIBITION BY NSD 1055 (0·668 m-mole/kg) OF THE STIMULATORY EFFECT OF INSULIN ON ^{14}C-HISTAMINE FORMATION IN THE GLANDULAR STOMACH*

Treatment	^{14}C-histamine (cpm/g tissue ± S.D.)
Starved control	33 ± 13·7 (6)
Starved + insulin	215 ± 46·5 (6)
Starved + insulin + NSD 1055	59 ± 39·6 (4)

* Each animal received 20 μc ^{14}C-histidine i.p. 60 min before removal of the tissue. The number of animals used is shown in parentheses.

DISCUSSION

The nonspecificity of NSD 1055 has been acknowledged by many of the workers who have used this drug. The ability of NSD 1055 to inhibit, for example, aromatic L-amino acid decarboxylase was reported several years ago,[8] but the lack of potential inhibitors of histidine decarboxylase is such that several attempts have been made to

use this drug, particularly in trying to unravel the role of histamine in gastric secretion.[12] The present report demonstrates that NSD 1055 inhibits diamine oxidase as well as histidine decarboxylase both *in vitro* and *in vivo*. In view of the predominant role of diamine oxidase in the catabolism of histamine in the rat, it is not surprising to find that some workers have had difficulty in trying to interpret the effects of this drug on parameters of whole body histamine metabolism such as urine estimations.[16]

The close similarity in the pattern of inhibition of both diamine oxidase and histidine decarboxylase in freely fed animals suggests that the transient effect of NSD 1055 is probably due to a rapid clearance or metabolism of the drug itself. A more interesting finding, however, was that the potency ratio of NSD 1055 against the two enzymes was different *in vitro* to that found in freely fed animals *in vivo*, and this probably reflects basic differences in the two enzyme systems. *In vitro*, NSD 1055 was much more active against the decarboxylase, but when the drug was administered to freely fed animals *in vivo* both histidine decarboxylase and diamine oxidase were inhibited to approximately the same extent. In this context, the most important difference between diamine oxidase and histidine decarboxylase, which would be evident *in vivo* but not *in vitro*, is the rate of enzyme turnover. Histidine decarboxylase in the gastric mucosa of the rat has been shown to be an enzyme with a high rate of turnover and the biological half-life has been calculated to be of the order of about 100 min.[5, 6] There is no evidence, either from our own studies or from the literature, to indicate that diamine oxidase in the intestine is turned over at a similar high rate. Most attempts to find an inhibitor of histidine decarboxylase have focused on the ability of a compound to inhibit the enzyme in a situation *in vitro*.[7] In the whole animal, however, the enzyme in the gastric mucosa is being continuously replenished and this high rate of turnover undoubtedly constitutes a major obstacle to effective and prolonged inhibition of histidine decarboxylase *in vivo*.

The most effective way to inhibit histidine decarboxylase would be to prevent the synthesis of the enzyme. The most potent inhibitors of this enzyme *in vivo* are, in fact, those compounds which inhibit the translational phase of protein synthesis, such as puromycin and cycloheximide,[5, 6] although the toxicity of these compounds renders them of little value except for short-term experiments. In view of the lack of information on the specific sequence of steps involved in the synthesis of histidine decarboxylase, the chances of finding a drug which specifically impairs synthesis is remote at the present time.

Alternative approaches to the inhibition of histidine decarboxylase center on impairment of the availability or functioning of the substrate or coenzyme. Substrate analogs such as α-methyl-histidine have been reported useful in reducing histamine formation,[2] although other investigators have not been so successful.[7] Furthermore, α-methyl-histidine is itself decarboxylated and, since it is a competitive antagonist,[2] a major problem is maintaining a high enough concentration in the blood to compete successfully with histidine. Attacking histidine decarboxylase through the coenzyme also appears to be of limited use because of the widespread requirement of many enzymes for pyridoxal phosphate. The ability of NSD 1055, for example, to inhibit diamine oxidase as well as histidine decarboxylase may be due, in part, to the fact that both enzymes require pyridoxal as the cofactor.[25] It is therefore probable that the majority of compounds which inhibit histidine decarboxylase through the cofactor will also affect diamine oxidase. Under certain conditions, however, histidine decarboxylase

may be much more susceptible than diamine oxidase to drug inhibition. Thus, in starved animals, NSD 1055 is very effective in blocking the rise in histidine decarboxylase produced by insulin, refeeding or pentagastrin. Both the degree and the duration of inhibition are considerably greater than those observed when the drug is administered to freely fed animals. Under conditions where the enzyme is being stimulated, therefore, it is very sensitive to drug treatment. One explanation for these results, suggested from studies *in vitro*,[9] is that NSD 1055 complexes with the pyridoxal. This would short-circuit the availability of pyridoxal for the apoenzyme, and furthermore, the complex itself might be inhibitory. Complexes of pyridoxal phosphate and NSD 1055 have been shown to be potent inhibitors of histidine decarboxylase *in vitro*.[9]

Johnston and Kahlson[16] found that NSD 1055 reduced the amount of ^{14}C-histamine appearing in the urine after an injection of ^{14}C-histidine without producing any significant effect on the total urinary histamine. The argument has been repeatedly waged that the excretion of histamine in the urine faithfully reflects the rate of histamine formation.[26] It was on this premise that Johnston and Kahlson[16] raised the question as to the usefulness of the isotopic method in the determination of histamine formation *in vivo*. Since the resolution of this point is important as a guide to which method should be used in future work, the limitations of urinary estimations of histamine should be understood.

The urinary excretion of histamine has proved to be a useful measure of changes in histamine metabolism, but it represents the overall effect and does not distinguish either the source or the mechanisms involved in producing the change. An increase in urinary histamine, for example, may occur in at least three different ways: (1) the release of bound histamine from mast cells by injection of compound 48/80;[27] (2) increased histidine decarboxylase activity as found during pregnancy;[28] (3) inhibition of diamine oxidase by aminoguanidine.[29] An increase in urinary histamine is not necessarily a consequence of increased formation. Furthermore, urinary estimations of histamine, while useful as a guide to the predominant effect on whole body histamine metabolism, tend to conceal other changes in histamine metabolism which may be critical to a particular locus of action of histamine. For example, during pregnancy in the rat the urinary histamine is raised due primarily to the high histidine decarboxylase in the fetal liver,[30] but the diamine oxidase activity in the uterus, placenta and plasma is also dramatically increased.[31, 32] Similarly, the increase in urinary histamine produced by aminoguanidine through inhibition of diamine oxidase obscures the fact that prolonged treatment with this agent produces a very marked inhibition of histidine decarboxylase in the gastric mucosa.* It follows that the failure of an alleged inhibitor of histidine decarboxylase such as NSD 1055 to produce any apparent change in urinary histamine may be due, in part, to multiple actions on histamine metabolism. In relation to the physiological function of histamine in a specific tissue, parameters of whole body metabolism may be misleading and so also may be the multiple actions of a single drug. The ability of NSD 1055 to inhibit diamine oxidase in the intestine, for example, may be of little consequence in events occurring in the stomach, for this is a tissue which contains little or no diamine oxidase. Nevertheless, this point raises the more general question of the effect of diamine oxidase inhibition in one tissue on the histidine decarboxylase activity in another. Since the consequences of diamine oxidase

* Y. Kobayashi and D. V. Maudsley, to be published.

inhibition are, in essence, increased circulating levels of histamine, the question raised is what effect does histamine itself have on histidine decarboxylase activity? Evidence for a feedback coupling between histamine and its synthesizing enzyme has been provided by several laboratories.[26, 33, 34] If this is correct, then higher circulating levels of histamine would tend to enhance the inhibition of histidine decarboxylase. The problem, however, is not as clear cut as Kahlson and Rosengren[26] have indicated, because the procedures used to demonstrate the feedback coupling have utilized injections of large doses of histamine. There has been no satisfactory demonstration that variations of endogenous histamine exert a physiological control over histidine decarboxylase activity.

Let us now consider the usefulness of the isotopic method for the determination of histamine formation *in vivo*. The value of a single injection of ^{14}C-histidine rests primarily on the assumption that the injected histidine mixes uniformly with the endogenous amino acid. This assumption appears reasonable in view of the fact that stimulation of histidine decarboxylase by insulin corresponds with an increased amount of ^{14}C-histamine found in the gastric mucosa. Conversely, the increase in histidine decarboxylase produced by insulin can be blocked by NSD 1055 and the ^{14}C-histamine is also reduced under the same experimental conditions. The correlation between the changes in histidine decarboxylase and the ^{14}C-histamine content of the tissue indicates, therefore, that the latter is a satisfactory index of histamine formation *in vivo* in the rat. Work carried out by Reilly and Schayer[35, 36] permits a similar conclusion.

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