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A New Histaminase Assay

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Diamine oxidase (diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6) catalyzes the following reaction:

$$RCH_2NH_2 + O_2 + H_2O \rightleftharpoons RC(H)=O + NH_3 + H_2O_2$$

When histamine is used as the substrate, the initial product of the reaction is imidazoleacetaldehyde (1) and the enzyme is referred to as histaminase. Estimations of histaminase activity have been based on determinations of oxygen consumption and aldehyde, ammonia, or hydrogen peroxide formation (2). A more sensitive and specific method for the determination of histaminase activity is based on the isotope-dilution techniques described by Schayer *et al.* (3,4). Although the isotope-dilution method is probably the most reliable, it is nevertheless, quite time-consuming and laborious. This paper describes a simple method for measuring the radioactive product(s) of the action of diamine oxidase on ^{14}C-labeled histamine. The present method for the measurement of histaminase activity is based on the ability of phosphocellulose columns to retain histamine while allowing the reaction products, imidazoleacetaldehyde and imidazoleacetic acid, to pass through.

MATERIALS AND METHODS

(*A*) *Chemicals*

Histamine (ring-2-^{14}C) dihydrochloride was purchased from Amersham/Searle. Aminoguanidine sulfate was obtained from Eastman Organic Chemicals and histamine dihydrochloride from Mann Research Laboratories. 4-Bromo-3-hydroxybenzyloxyamine dihydrogen phosphate (brocresine) was prepared by the Process and Analytical Research Section of Lederle Laboratories. Phosphocellulose (Cellex P) was obtained from Bio-Rad Laboratories and hog kidney diamine oxidase was purchased from Calbiochem. Isoniazid was obtained from Dr. L. Ellenbogen of Lederle Laboratories.

(B) Reagents

1. *Rat intestinal histaminase.* Adult female rats weighing approximately 250 gm each were used. The animals were killed by decapitation and the first 30 cm of the small intestine proximal to the stomach was removed, slit longitudinally, and rinsed in three changes of 0.9% saline at room temperature. After blotting, the intestines were homogenized for 1 min with 5 vol ice-cold 0.05 *M* sodium phosphate buffer, pH 7.5, in a Waring blender. The homogenate was centrifuged at 20,000*g* for 10 min at 4°C and the supernatant was poured through glass wool and frozen in 2 ml aliquots. The resulting preparation contained 10.6 mg protein/ml.

2. *Hog kidney diamine oxidase.* The commercially prepared enzyme was made to a concentration of 10 mg protein/ml in deionized water just prior to use.

3. *Phosphocellulose columns.* The resin was treated as described by Kremzner (5) and was stored at 4°C in 0.01 *M* sodium phosphate buffer, pH 6.0, containing 0.02% sodium azide. The slurry was poured into columns 0.4 cm(i.d.) × 9 cm with an attached reservoir which held approximately 15 ml. The resin was allowed to pack by gravity to a height of 4–5 cm.

4. *Substrate.* 50 μCi of histamine (ring-2-^{14}C) dihydrochloride (specific activity, 54.3 mCi/mmole) and 2.82 mg of nonlabeled histamine dihydrochloride were dissolved in 20.0 ml of 0.0001 *M* HCl to produce a final concentration of 8.1×10^{-4} *M* histamine containing 0.25 μCi/0.1 ml.

(C) Procedure

Into a 10 ml Erlenmeyer flask were pipetted 0.4 ml of 0.25 *M* sodium phosphate buffer, pH 7.5, 0.1–0.5 ml of the enzyme preparation, and sufficient deionized water to make a total volume of 2.5 ml. The mixture was incubated for 5 min at 37°C and the reaction was started by the addition of 0.1 ml of 8.1×10^{-4} *M* ^{14}C-histamine. The flasks were incubated at 37° in a gyratory shaker for varying periods of time. The reaction was stopped by the addition of 0.2 ml of 50% trichloroacetic acid. Blanks were run with each experiment. The blank flasks were identical to the experimental flasks except that the enzyme was added after the addition of the 50% trichloroacetic acid. The contents of the flasks were poured into 12 ml conical centrifuge tubes and the tubes centrifuged at 2,200*g* for 5 min. Then 2 ml aliquots of the supernatants were transferred into 12 ml graduated centrifuge tubes. One drop of 0.1% bromcresol green in ethanol was added to each tube and 1.0 *M* NaOH was added dropwise until the solution turned blue (pH 5.4). The contents of each tube were adjusted to a total volume of 7.5 ml with distilled water and were trans-

ferred to a phosphocellulose column. The solution was passed through the column into 10 ml volumetric flasks. The column was washed with 2 ml of 0.01 *M* sodium phosphate buffer, pH 6.0, and the volume of the combined eluate and wash was adjusted to 10 ml. Then 1 ml aliquots of the column eluates were transferred to scintillation vials for counting.

To each scintillation vial was added 10 ml of Bray (6) solution and the samples were counted in a Nuclear-Chicago scintillation counter (Mark I) at 4°C. Counts/minute (cpm) were converted into disintegrations/minute (dpm) by using the per cent counting efficiency obtained from quench correction curves constructed from standard quenched ^{14}C standards.

Histamine was determined according to Shore *et al.* (7). Protein was measured by the biuret method of Gornall *et al.* (8).

RESULTS

Incubation Conditions. The effect of the pH of the incubation medium on histaminase activity was studied over the range of pH 6.0 to 8.0 using the rat intestinal preparation. The optimum activity of this preparation was found to plateau between pH 7.0 to 7.5. The pH of 7.5, which was arbitrarily chosen for the assay of the rat intestinal preparation, was also used for the assay of the partially purified hog kidney enzyme (9).

In order to study the effect of substrate concentration on enzymic activity, the concentration of ^{14}C-histamine was varied from 3.1×10^{-5} *M* to 2.5×10^{-4} *M* using both enzyme preparations. The use of substrate concentrations 3.1×10^{-5} *M* to 6.2×10^{-5} *M* resulted in maximal enzymic activity. When substrate concentrations above 6.2×10^{-5} *M* were employed, a loss of enzymic activity was found, similar to that described by Mondovi *et al.* (9). On the basis of these observations, 3.1×10^{-5} *M* ^{14}C-histamine was chosen as the standard substrate concentration.

Relationship between Histamine Disappearance and Product Formation. In order to demonstrate the quantitative recovery of the product by the present method, we compared the amount of histamine which disappeared with the amount of product formed. Histamine disappearance was measured fluorometrically and product formation was calculated by dividing the product radioactivity (dpm) by specific activity of the substrate in dpm/μg histamine at 0 time. As shown in Table 1, the product formed was in good agreement with the amount of histamine that disappeared.

Time Course of the Reaction. Table 1 also demonstrates that the formation of product is proportional to time of incubation. With the partially purified hog kidney enzyme, product formation was linear with

TABLE 1
Comparison of Histamine Disappearance with Product Formation

Enzyme source	Incubation time (min)	Histamine disappearance (μg/mg protein)	Product[a] formation (μg/mg protein)
Hog kidney[b]	30	0.88 ± 0.27	0.74 ± 0.03
	60	1.58 ± 0.12	1.49 ± 0.07
	90	2.52 ± 0.24	2.35 ± 0.07
	120	3.51 ± 0.11	3.03 ± 0.16
Rat intestine[c]	15	0.33 ± 0.02	0.54 ± 0.01
	30	0.78 ± 0.05	0.89 ± 0.03
	60	1.41 ± 0.17	1.46 ± 0.04
	90	2.08 ± 0.11	2.04 ± 0.06

Assays were carried out as described under "Methods." Incubation flasks contained either 1.0 mg hog kidney diamine oxidase or 1.06 mg rat intestinal preparation protein.

[a] Calculated as histamine.

[b] Average of 5 determinations ± S.E.M.

[c] Average of 3 determinations ± S.E.M.

time. On the other hand, when the rat intestinal preparation was used, product formation was curvilinear with time.

Product Formation as a Function of Amount of Enzyme. The relationship of product formation with amount of enzyme was also studied. With both the hog kidney diamine oxidase and the rat intestinal preparation, product formation was directly proportional to the amount of enzyme added (Fig. 1).

Effect of Diamine Oxidase Inhibitors on Rat Intestinal Histaminase. In order to test further our assay for histaminase activity, we studied the effect of known diamine oxidase inhibitors (10–12) (Table 2). Both aminoguanidine sulfate and brocresine appeared to be effective inhibitors of intestinal histaminase activity (Experiment 1). However, the possibility existed that the product of histaminase activity may have been retained on the column in the presence of the inhibitors. To eliminate this possibility Experiment 2 was performed. In this case, a second control was added in which the products of histaminase activity were allowed to react with the inhibitors after stopping the enzymic activity. When compared to this type of control, aminoguanidine sulfate, brocresine, and isoniazid were all found to be inhibitors of histaminase activity by the present assay.

DISCUSSION

In preliminary experiments, we found that phosphocellulose columns were very efficient in removing labeled histamine. When mixtures con-

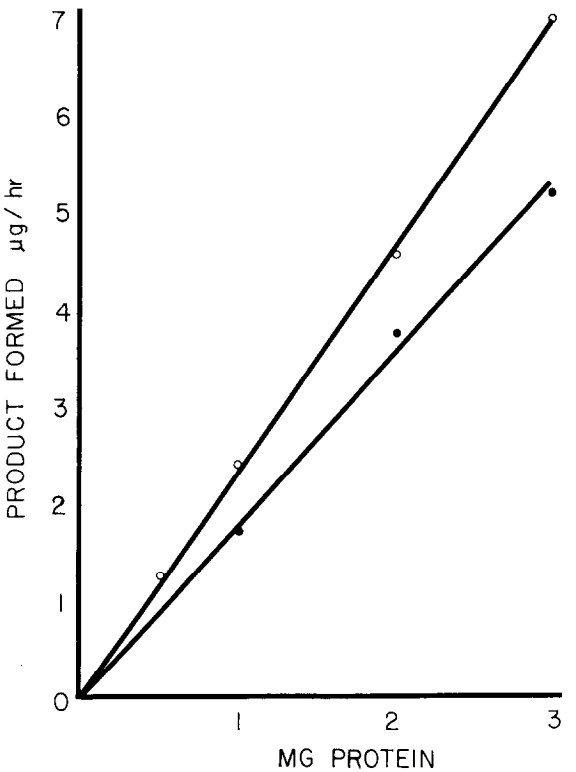

FIG. 1. Product formation versus amount of enzyme. Histaminase activity was measured as described under "Methods." Incubations were carried out in duplicate for 1 hr at 37°C. The ordinate represents amount of product formed calculated as µg histamine. Open circles represent intestinal preparation and closed circles represent hog kidney enzyme.

taining 0.0046 to 1.625 µmoles of ring-labeled ^{14}C-histamine were passed through the column as described under "Methods," 95.2 to 97.5% of the radioactivity we retained. The 2.5 to 4.8% of the ^{14}C which passed through the column did not behave as histamine since, upon rechromatography, 90% of this material was not retained on the resin. The amounts of histamine used in these experiments represented 0.08 to 28.01 times the maximal amount of histamine normally added to the column in the present assay.

As indicated above, a small amount of the radioactivity present in the labeled histamine passed through the column. This material, which was presumably a contaminant present in the ^{14}C-histamine, resulted in blank values which averaged about 1000 dpm/ml of column effluent. In studies of enzyme concentration versus activity, we have been able to

TABLE 2
Effect of Diamine Oxidase Inhibitors on Intestinal Histaminase Activity

Expt. No.	Inhibitors	Final concn. (M)	Product[a] formation (μg/mg/hr)	% inhibition
1	None	—	1.64	—
	Aminoguanidine sulfate	1×10^{-5}	0.02	98.8
	Brocresine	1.75×10^{-5}	0.28	82.9
2	None	—	1.77	—
	Aminoguanidine sulfate	1×10^{-5}	0.02	98.3[b]
	Aminoguanidine sulfate control	1×10^{-5}	1.21	—
	Brocresine	1.75×10^{-5}	0.50	71.4[b]
	Brocresine control	1.75×10^{-5}	1.75	—
	Isoniazid	1×10^{-4}	0.74	47.5[b]
	Isoniazid control	1×10^{-4}	1.41	—

[a] Calculated as histamine.

[b] Per cent inhibition as compared to the appropriate control.

Experiment 1: Inhibitors were preincubated with the enzyme for 5 min at 37°C. The reaction was started by the addition of ^{14}C-histamine and the incubation was allowed to proceed for 1 hr at 37°. Each flask contained 1.06 mg intestinal histaminase preparation. Control flasks were identical to experimental flasks that no inhibitor was added.

Experiment 2: Identical to experiment 1 except that an additional set of control flasks were run. After 1 hr incubation at 37°C, the flasks were heated at 100° for 15 min to stop enzymic activity. After cooling to 37°, the inhibitor was added to one set of control flasks and all flasks were incubated for an additional hour at 37°. Trichloroacetic acid was added and the reaction products were recovered as described under "Methods." Each flask contained 2.12 mg intestinal histaminase preparation.

Experiments 1 and 2 were carried out in triplicate and duplicate, respectively.

measure conveniently the appearance of 1000 dpm/ml of column effluent after subtracting the blank value, which corresponds to about 2 nmoles of histamine disappearance. With prior purification of the ^{14}C-histamine substrate (5), amounts of product(s) corresponding to less than 2 nmoles of histamine disappearance could be detected.

In order to establish the recovery of the reaction product, the amount of product formed, calculated from the amount of radioactivity which passed through the column and the specific activity of the substrate, was compared with amount of histamine that disappeared. In these studies, two types of enzyme preparations were used, i.e., (*a*) purified hog kidney diamine oxidase and (*b*) the supernatant from rat intestinal homogenate. These preparations were chosen because they give rise to different reaction products. Thus the action of the purified hog kidney enzyme on histamine results in the formation of imidazoleacetaldehyde (1), whereas a mixture of imidazoleacetic acid and imidazoleacetaldehyde results from the action of the rat intestinal enzyme (13). In both cases, the calculated

amount of product formed was in good agreement with the amount of histamine that disappeared.

Both the relationship of the amount of product formed to the amount of enzyme and the time course of the reaction were studied with the two enzyme preparations. With either enzyme, the amount of product formed was linear with the amount of enzyme present. On the other hand, although the amount of product formed was linear with respect to time when the purified hog kidney enzyme was used, the use of the intestinal preparation resulted in a curvilinear relationship between the product formation and time of incubation. Although the reason for this effect is not known, it may be the result of some proteolytic activity present in the crude intestinal preparation.

By using the present method it was also possible to demonstrate the inhibitory effect of known diamine oxidase inhibitors on histaminase activity. However, it is important to run the proper controls when determining the effect of various compounds on histaminase activity by this method. Thus the incubation of aminoguanidine sulfate or isoniazid with the products of histaminase activity resulted in a decrease in the amount of radioactivity which was eluted from the column (Table 2). It is possible that these two inhibitors may have formed hydrazones with one of the products (viz., imidazoleacetaldehyde) which were positively charged and were retained on the column.

The method described above for the determination of histaminase activity was found to be relatively simple and sensitive. Presently, the most commonly used methods for the assay of histaminase activity are based on the disappearance of histamine or the conversion of ^{14}C-putrescine to ^{14}C-Δ'-pyrroline (14,15). More recently, Beaven and Jacobsen (16) described a specific and sensitive histaminase assay based on the measurement of tritiated water released from β-^{3}H-histamine by the action of histaminase.

When histamine disappearance is used as a measure of histaminase activity, it is difficult to determine the disappearance of small amounts of histamine. With the present method, the disappearance of 3% of the ^{14}C-histamine could be measured easily. Similarly, Okuyama and Kobayashi (14) and Beaven and Jacobsen (16) reported that 2% and 1% of the substrate disappearance, respectively, could be measured.

With the present assay, 2.25 nmoles of histamine disappearance could be easily detected with an error of 3%. By using a labeled histamine substrate of greater specific activity, it would be possible to increase the sensitivity of the assay.

In the present study, no attempt was made to examine the histaminase activity of various tissues. It has been shown that the pH optimum for

the measurement of histaminase may vary from one tissue to another (12) and that the substrate concentration required to produce the maximal activity must be determined since excess amounts of histamine may inhibit the enzyme (9). Thus, if optimum incubation conditions are determined for each tissue, the present method should provide a relatively simple and direct assay for the measurement of histaminase activity.

SUMMARY

A relatively simple and convenient method for measuring histaminase activity is described. After reaction of labeled histamine with histaminase, the unreacted histamine is removed by passage through phosphocellulose and the products are collected and counted directly.

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