LXXIII. HISTAMINASE.

IV. FURTHER STUDIES IN PURIFICATION.

V. SOURCE OF THE AMMONIA FORMED IN THE HISTAMINE-HISTAMINASE REACTION.

BY EARL WILLARD McHENRY AND GERTRUDE GAVIN.

From the School of Hygiene, University of Toronto.

(Received January 24th, 1935.)

IN a previous communication in which attempts to purify histaminase were discussed, the authors [1932] showed that when this enzyme causes the destruction of histamine, ammonia is liberated stoichiometrically. In this paper the results of further studies on methods of purification of histaminase and on the histamine-histaminase reaction are presented.

As in previous studies [Best and McHenry, 1930; McHenry and Gavin, 1932] kidney powder was used as a source of histaminase. The amount of enzymic activity was determined by measuring the amount of histamine remaining after incubation for 24 hours at 38° in a mixture which originally contained a known amount of histamine and enzyme preparation. All determinations of histamine have been made by the blood-pressure method in etherised cats [Best and McHenry, 1930].

IV. FURTHER STUDIES IN PURIFICATION.

As a basis for these investigations histaminase solutions were prepared by extracting 1 part of kidney powder with 25 parts of Sørensen phosphate buffer, p_H 7.2 for one hour at 38°. A single extraction yields 60 % of the enzyme contained in the powder, and the yield is increased to 80 % by a second extraction. Longer extraction periods (2–24 hours) gave no greater yields.

The authors [1932] reported that the presence of phosphate ions augmented the activity of histaminase. If this were due to the precipitation of traces of calcium, which have been shown by Best and McHenry to be inhibitory, one might expect that oxalate ions would have the same effect. Extraction of kidney powder with 1 % sodium oxalate was as satisfactory as extraction with phosphate solution. Varying the concentration of oxalate (0–0.53 g. per 100 ml.) during the incubation of histamine with histaminase gave the same type of curve as was found for the effect of the concentration of phosphate on the enzyme. It is likely that the similar effects shown by the oxalate and phosphate ions are due to the removal of calcium presumably contained in the crude histaminase.

The effects on histaminase of a number of substances known to activate or inhibit enzymes have been tested. Preservatives such as 0.3 % tricresol and 0.1 % sodium benzoate completely inhibited the enzyme. Methylene blue (0.2 %) gave 60 % inhibition at p_H 7.2 and the effect was not changed by the presence of 0.005 *M* potassium cyanide. Glutathione 0.1 %, cystine 0.01 %, cysteine 0.015 %, ergothioneine 0.005 % and phenolindophenol 0.008 % had no apparent effect on histaminase.

Several protein precipitants, in addition to those previously employed, have been used in an effort to fractionate histaminase solution. 40 % of the potency was recovered in the precipitate when just sufficient picric acid to cause a precipitate was added. Precipitation from a mixture containing 0.1 % flavianic acid destroyed all activity. Precipitation from a mixture containing 0.5 % tannic acid and extraction of the precipitate with cold aldehyde-free acetone to remove the tannic acid, gave complete recovery of the enzyme. This process increases the activity twenty-fold on a dry-weight basis.

Earlier work had shown that an ammonium sulphate precipitate of the enzyme could be dried to give a stable powder. As a basis for subsequent experiments in purification a large quantity of this precipitate was prepared as follows. 30 g. of kidney powder were extracted, first with 750 ml. and then with 600 ml. of phosphate solution. The combined filtrates were stirred for one-half hour with 4 g. carbex E (Pfanstiehl) per 100 ml. of solution and filtered. This removes about 10 % of the impurities and causes very little loss of potency. Ammonium sulphate to give 90 % saturation was added to the filtrate, the precipitate filtered off by suction and dried in a vacuum-oven at a temperature under 50°. A phosphate buffer solution of this dried precipitate was used in the following experiments. Ice-cold alcohol may be used to precipitate the enzyme. When the concentration of alcohol was varied between 30–90 % the amount of enzyme precipitated varied directly with the concentration of alcohol, a maximum recovery of 65 % being obtained with 90 % alcohol. On a basis of unit activity per g. this method gave little purification. From an ice-cold mixture of 10 parts enzyme solution, 10 parts alcohol, 6 parts ether, the precipitate contained 75 % of the original activity. Ice-cold aldehyde-free acetone also precipitated the enzyme. The percentage potency recovered increased in proportion to the acetone concentration with a maximum of 65 % activity with 90 % acetone. When the acetone, prior to admixture, was neutralised with sodium hydroxide, 95 % of the activity was recovered in the precipitate if the concentration of acetone was between 60–80 %. On a dry weight basis this procedure gave no apparent purification as phosphate was precipitated by concentrations of acetone above 50 %. By dissolving the ammonium sulphate powder in distilled water neutralised with sodium hydroxide and by treating this solution with acetone, a four-fold increase in activity on a dry-weight basis was obtained. A phosphate solution of this precipitate was stable for one month at refrigerator temperature.

Experiments were made on adsorption of the enzyme from a phosphate solution of the kidney powder. Histaminase was not adsorbed by Eastman's norite, darco, special carbon, Pfanstiehl's carbex E, or Lloyd's reagent. Kaolin in water, water and alcohol, phosphate buffer, phosphate buffer and alcohol showed no adsorption of the enzyme. The most useful method was the aluminium hydroxide procedure developed by Sherman *et al.* [1930], for pancreatic amylase. The gel was prepared as described by these authors. It was found advantageous to have both phosphate and alcohol present during the adsorption phase. The best proportions were found to be 6 parts gel, 5 parts alcohol, 4 parts phosphate buffer, 15 parts histaminase solution. This mixture was stirred for 15 minutes, centrifuged, and the supernatant fluid, which contained no potency, was discarded. Very little enzyme was eluted with 1 % ammonium hydroxide or with 1 % diammonium hydrogen phosphate. Elution with phosphate buffer p_H 7.2 and 8.8 gave 75 % recovery, while at p_H 5.9 there was a 50 % recovery. Increasing the concentration of phosphate three times or decreasing it to one-third normal gave lower yields. The most successful method of elution was to use a mixture

of phosphate buffers p_H 6.0 to 7.2 and glycerol. Yields of 85–90 % of the original activity were obtained when the glycerol concentration was between 40–60 %. If a phosphate solution of an ammonium sulphate powder was used in the above procedure only a 50 % yield was obtained. A still lower yield was secured if a solution of the acetone precipitate of the ammonium sulphate powder was used. Attempts to precipitate the enzyme after elution by tannic acid were unsuccessful.

V. SOURCE OF THE AMMONIA FORMED IN THE HISTAMINE-HISTAMINASE REACTION.

We have previously found that during the histamine-histaminase reaction the iminazole nucleus of histamine is ruptured and also that at p_H 7.2 one mol. of ammonia per mol. of destroyed histamine is liberated. If this ammonia were formed from the side chain, a change in the amino-nitrogen content during the reaction would be expected. In preliminary experiments to ascertain the source of the ammonia it was found that, owing to the presence of interfering substances in the solution, colorimetric methods of determining amino-nitrogen could not be used. Measurements with the Van Slyke gas analysis apparatus were more successful. With this method it was essential first to remove the proteins from the solution to be tested. The routine procedure of precipitation with sodium tungstate carried down a portion of the histamine present. Other protein precipitants, *e.g.* molybdic acid, tungstomolybdic acid, tannic acid, alcohol, zinc sulphate, magnesium sulphate, lead acetate, precipitated all or a part of the histamine. With Schenk's reagent and with copper sulphate no histamine was precipitated but the resulting solutions were unsuitable for use in the Van Slyke apparatus. The method finally adopted was precipitating with 1 volume of 10 % trichloroacetic acid to 1 volume of kidney solution and washing the precipitate with 5 % trichloroacetic acid solution. For each experiment 3 flasks were used: *A*, histamine + a phosphate solution of histaminase p_H 7.2 + toluene; *B* and *C*, a similar histaminase solution + toluene. These were incubated for 48 hours at 38° to destroy the histamine in flask *A*. After incubation histamine equivalent to that originally present in flask *A* was added to flask *C*. Flask *B* served as a control for the amino-nitrogen value of the enzyme solution. The proteins in each flask were then precipitated with trichloroacetic acid. Immediately before the gas analysis the trichloroacetic acid filtrates were neutralised and histamine

Changes in amino-nitrogen during enzymic destruction of histamine.

		Before incubation		After incubation		
		Mg. histamine initially present	Mg. amino-nitrogen theoretical	Mg. residual histamine (cat assay)	Mg. amino-nitrogen theoretical	Mg. amino-nitrogen as found
Histamine inactivated (flask A)	(a)	109	14	0	0	1.1
	(b)	109	14	1	0.1	0.8
	(c)	109	14	4	0.5	3.1
	(d)	87	12	11	1.4	2.0
	(e)	109	14	20	2.6	2.9
Control (flask C). <i>Note.</i> Histamine added after incubation	(a)	109	14	56	7.3	6.6
	(b)	109	14	98	12.8	12.4
	(c)	109	14	102	13.2	13.1
	(d)	87	12	71	9.2	8.1
	(e)	109	14	92	11.9	10.9

assays were made by the blood pressure method in etherised cats. It will be seen in the following tables that when large amounts of histamine were inactivated there frequently remained a small amount of amino-nitrogen in excess of that due to the kidney solution and any remaining histamine. Some inactivation of histamine took place in the control flasks probably owing to the high temperature in the laboratory during the experiments.

From these results it is evident that there is a very marked fall in amino-nitrogen value during the destruction of histamine. This would indicate that the ammonia formed during the reaction is derived from the amino-nitrogen of the side chain and that the rupture of the iminazole nucleus yields no amino-compounds.

SUMMARY.

A number of procedures for the concentration of histaminase have been described. During the histamine-histaminase reaction the amino-nitrogen value falls almost to zero indicating that the ammonia liberated is derived from the side chain of the histamine molecule.

Grateful appreciation is expressed to Miss Neta Thompson for valuable assistance and to Dr C. H. Best and Dr J. G. FitzGerald for their continued interest in the investigation.

REFERENCES.

- Best and McHenry (1930). *J. Physiol.* **70**, 349.
McHenry and Gavin (1932). *Biochem. J.* **26**, 1365.
Sherman, Caldwell and Adams (1930). *J. Biol. Chem.* **88**, 295.