ACTA CHEMICA SCANDINAVICA 18 (1964) 2069–2076

Studies on the Enzymic Methylation of Histamine

ARNE GUSTAFSSON and GUSTAF PLYM FORSHELL*

Institute of Biochemistry, University of Lund, Lund, Sweden

The enzymic methylation of histamine has been studied by using an essentially homogeneous preparation of histamine-N-methyl-transferase. The enzyme did not appear to be a metalloenzyme, and no cofactors related to vitamin B_{12} could be detected in the enzyme preparation. The transferase has SH-groups essential for its enzymic activity, and the pH-optimum of the reaction is 8.8. The influence of some histamine analogues on the reaction as well as studies on substrate specificity are described. The methyl group transfer from S-adenosylmethionine to histamine probably occurs without a methylated enzyme as an intermediate.

Enzymes that catalyze the transfer of an intact methyl group from AMe** to histamine, thereby forming the physiologically inactive 1-methylhistamine, seem to occur in most vertebrates.[1] Brown *et al.*[2] have studied the reaction by using an enzyme preparation from guinea pig brain and Lindahl[3] has used pig liver and mouse liver as starting materials for the preparation. The purification procedure applied on pig liver has been further developed in this laboratory,[4] and the present paper describes some properties of the purified enzyme. Experiments have also been performed in order to study the mechanism of the N-methylation.

MATERIAL AND METHODS

AMe was prepared from bakers' yeast as described by Schlenk *et al.*[5] The product was analyzed on paper chromatograms developed in ethanol-water-acetic acid (65:34:1). The spots were located with UV-photography and with ninhydrine reaction. The R_F-values obtained were identical with those found in the literature.[6] The AMe solution was kept at $-20°C$ after partial lyophilization.

AMe-$^{14}CH_3$ was of commercial origin from Tracerlab S.A. S-Adenosylhomocysteine, unlabelled and tritium labelled in the adenine moiety, was kindly supplied by Dr. Duerre, Argonne Natl. Lab., USA. Of the histamine analogues α-methylhistidine and 1-methylhistamine were gifts from Dr. White, Lund; 2-methylimidazole from Houdry Proc. and Chem. Comp., Philadelphia, USA; 4-hydroxymethylimidazole, 4-methylaminomethyl-

* Present address: AB Leo, Hälsingborg

** AMe = S-adenosylmethionine

imidazole, 4-dimethylaminomethylimidazole, 4-piperidinomethylimidazole, 4-(β-4-morpholinyl)ethyl imidazole, 4-ethylaminoethylimidazole, 4-dimethylaminoethylimidazole and 4-piperidinoethylimidazole from Dr. Huebner, Ciba, Summit, N. J., USA; 3-β-aminoethylpyrazole, 3-β-aminoethyl-1,2,4-triazole, 4-(β-aminoethyl)-thiazole, 2-(β-aminoethyl)-pyrazine, 3-(β-aminoethyl)-pyridazine, 2-(β-aminoethyl)-pyridine, 2-(β-aminoethyl)-quinoline and 2-(β-aminoethyl)-quinoxaline from Dr. R. Jones, Lilly Research Lab., Indianapolis, Ind., USA.

Histamine-N-methyltransferase was prepared as previously described.[4] The enzyme solutions eluted from the DEAE-columns were stored at + 4°C without adjusting the pH (4.6). Storage at higher or lower temperatures seems to destroy the enzyme activity within 24 h. All preparations were tested for enzymic activity as described in a previous publication.[4] The protein content of the preparations used was about 0.3 mg per ml. The phosphor solution used for radioactive measurements consisted of 0.4 % 2,5-diphenyloxazole and 0.01 % 1,4-di[2-(5-phenyloxazolyl)] benzene in toluene.

RESULTS

Effect of pH. The activity of histamine-N-methyltransferase as a function of pH was determined. Maximal activity occurred at pH 8.8 but the curve seems not to be typically bell-shaped. Between pH 7 and 8 there is a plateau region which may be caused by ionization of the substrate or by dissociation of essential SH-groups. Similar results have been obtained by Lindahl when using a less purified enzyme preparation.[3]

Content of B_{12}-derivatives. Experiments have been performed to test if vitamin B_{12} may be bound as a prostethetic group of the histamine-N-methyltransferase. B_{12} was determined by using *Euglena gracilis* (z-strain) according to Killander.[7] Only very minute amounts (less than 0.4 mμg/ml) could

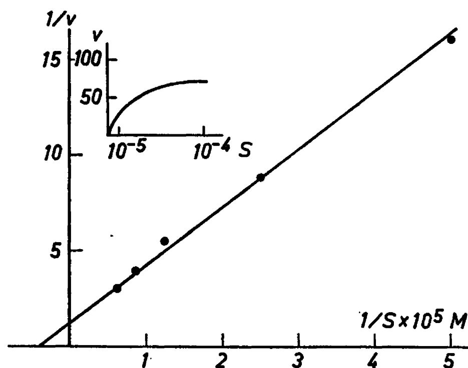


Fig. 1. Determination of K_M for histamine. 0.025 μmole AMe-$^{14}CH_3$ (21.5 μC/μmole), histamine as indicated, 100 μl enzyme solution and 100 μl 0.5 M Tris buffer, pH 8.1, were incubated for 6 min. Total volyme 250 μl. The reaction was stopped by adding 100 μl 10 N NaOH saturated with Na_2SO_4. Methylhistamine was determinated with the standard method.

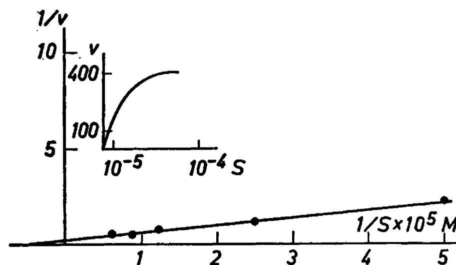


Fig. 2. Determination of K_M for AMe. The reaction mixture contained histamine-^{14}C at a concentration of 10^4 M, AMe as indicated, 100 μl enzyme solution and 100 μl 0.5 M. Tris buffer, pH 8.1, in a total volume of 250 μl. Incubation time and other experimental conditions were as described in Fig. 1.

Table 1. The influence of metals and some anions on the methylation reaction. Incubation was performed under standard conditions with the additions indicated. The reaction mixture was incubated for 60 min.

Salt added	% inhibition at a salt concentration of 10^{-4} M	10^{-3} M	10^{-2} M
$CuCl_2$	63	100	100
$NiCl_2$	3	48	100
$CoCl_2$	13	48	100
$MnSO_4$	−8	−9	−12*
$CaCl_2$	—	0	0
$MgCl_2$	—	0	2
$Zn(Ac)_2$	59	76	100
KI	—	5	5
KCN	—	0	—
NaN_3	—	0	—

* Indicates activation

be detected in the enzyme preparation (0.25 mg protein/ml.) In view of this result it appears unlikely that vitamin B_{12} or any of its derivatives is present in the methyltransferase.

Determination of K_M for histamine and AMe. The initial velocity of the histamine methylation as a funtion of histamine concentration was determined as described in Fig. 1. AMe-$^{14}CH_3$ was used in order to get the same amount of radioactive material in all the test tubes. The concentration of histamine varied between 2×10^{-6} and 1.6×10^{-5} M, and the values obtained were plotted according to Lineweaver and Burk.[8] The K_M-value for histamine was calculated to 2.2×10^{-5} M. The K_M for AMe was determined by varying the concentration of unlabelled AMe between 2×10^{-6} M and 1.6×10^{-5}M while the concentration of histamine-^{14}C was kept konstant (Fig. 2). K_M for AMe was calculated to 1.6×10^{-5} M.

Dependence on metal ions. In order to test the influence of metal ions and some other ions on the methylation reaction experiments described in Table 1 were performed. Neither intense dialysis of the enzyme against aqueous EDTA nor the addition of this compound to the reaction mixture had any effect on the enzyme activity.

Influence of sulfhydryl reagents. The effect of common SH-inhibitors on the histamine methylation have been reported elsewhere.[4] In all cases the inhibition was prevented by adding reduced glutathione to the reaction mixture before preincubation. Cystein at a concentration of 10^{-3} M activates the reaction to 33 % but thioglycollate has no influence on the methylation reaction.

Inhibition by chlorpromazine. Chlorpromazine has been reported as an inhibitor of histamine-N-methyltransferase.[2] The results in Table 2 indicate that part of the inhibition can be counteracted by addition of reduced glutathione to the reaction mixture.

Table 2. Inhibition studies with chlorpromazine. Incubation was performed under standard conditions for 60 min. The drug was preincubated with the reaction mixture except substrate for 10 min.

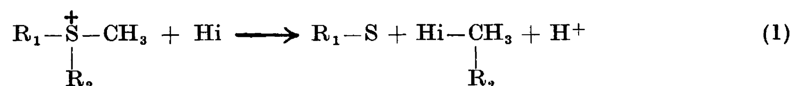
Chlorpromazine conc. M	GSH* added before preincubation, M	GSH added after preincubation, M	Inhibition %
	—	—	16
10^{-5}	10^{-5}	—	0
	—	10^{-5}	10
	—	—	50
10^{-4}	10^{-4}	—	15
	—	10^{-4}	21
	—	—	74
10^{-3}	10^{-3}	—	38

* Reduced glutathione

Inhibition by histamine analogues. The incubation mixture contained 0.025 μmole histamine-^{14}C, 0.164 μmole AMe, 100 μl enzyme solution, histamine analogues as indicated in Table 3 and 0.5 M Tris buffer, pH 8.1, to a total volyme of 250 μl. The histamine was added to the reaction mixture after 10 min preincubation of the other components. The methylhistamine formed was determined with the standard method. The results are summarized in Table 3. Of the compounds tested dimethylaminoethyl-imidazole was shown to inhibit the reaction in a competitive manner. The inhibition could be reversed by adding increasing amounts of histamine.

Studies on substrate specificity. Most of the histamine analogues above were also tested for their ability to function as substrates for the histamine-N-methyltransferase. In these cases the reaction mixtures contained 0.025 μmole AMe-$^{14}CH_3$, 100 μl 0.5 M Tris buffer, pH 8.1, 100 μl enzyme solution and histamine analogues to a final concentration of 10^{-4} M. Total volume 250 μl. After 60 min the reaction was stopped by adding 100 μl 10 N NaOH saturated with Na_2SO_4, and methylated products were extracted into 1000 μl butanol (previously saturated with water). A 200 μl aliquot of the butanol extract was transferred to a vial containing 800 μl of ethanol and 5 ml of phosphor solution and the radioactivity measured in a liquid scintillation counter. Control incubations, in which the substrates were omitted, were run concurrently to correct for the small amounts of AMe-$^{14}CH_3$ that are extracted into the solvent. The results are summarized in Table 3 (the right part).

Experiments on the mechanism of the transmethylation reaction. The overall reaction catalyzed by histamine-N-methyltransferase may be formulated as follows:



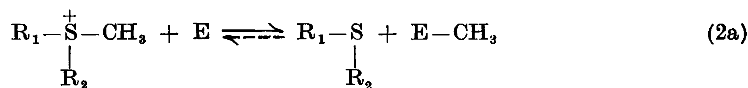
$$R_1-\overset{+}{S}(R_2)-CH_3 + Hi \longrightarrow R_1-S(R_2) + Hi-CH_3 + H^+ \quad (1)$$

Table 3. Histamine analogues as inhibitors and as substrates for histamine-N-methyltransferase.

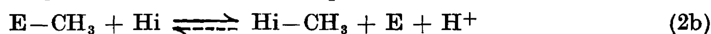
No.	Compound	% inhibition at an inhibitor conc. of			% methylation as substrate
		10^{-5} M	10^{-4} M	10^{-3} M	
1	4-Hydroxymethyl-imidazole	0	0	52	9
2	4-Methylaminomethyl-imidazole	37	43	67	0
3	4-Dimethylaminomethyl-imidazole	29	29	49	0
4	4-(1-Piperidylmethyl)-imidazole	0	0	44	0
5	4-[β(4-Morpholinyl)ethyl]-imidazole	16	10	44	0
6	4-Dimethylaminoethyl-imidazole	8	10	39	31
7	4-Ethylaminoethyl-imidazole	4	11	24	6
8	4-Piperidinoethyl-imidazole	8	8	40	3
9	Histidine	2	0	0	1
10	α-Methylhistidine	4	0	0	4
11	1-Methyl-4(β-aminoethyl)-imidazole (Reaction product)	8	38	74	0
12	3-β-Aminoethyl-pyrazole	13	20	55	9
13	3-β-Aminoethyl-1,2,4-triazole	20	17	55	2
14	4-(β-Aminoethyl)-thiazole	3	29	30	9
15	2-Methyl-imidazole	3	14	38	0
16	2-(β-Aminoethyl)-pyrazine	8	16	56	3
17	3-(β-Aminoethyl)-pyridazine	17	28	50	—
18	2-(β-Aminoethyl)-pyridine	5	7	23	2
19	2-(β-Aminoethyl)-quinoline	10	13	35	0
20	2-(β-Aminoethyl)-quinoxaline	17	28	61	—

where R_1 = the adenosine moiety of AMe, R_2 = $-CH_2-CH_2-CH(NH_2)-COOH$, Hi = histamine and $Hi-CH_3$ = methylhistamine.

Experiments have been performed to test the possibility that the reaction proceeds stepwise as indicated in eqns. (2a) and (2b).



$$R_1-\overset{+}{S}(R_2)-CH_3 + E \rightleftharpoons R_1-S(R_2) + E-CH_3 \quad (2a)$$



$$E-CH_3 + Hi \rightleftharpoons Hi-CH_3 + E + H^+ \quad (2b)$$

where E = histamine-N-methyltransferase.

The sum of eqns. (2a) and (2b) is identical with eqn. (1). It has been shown that the overall reaction is essentially irreversible, but this does not exclude the possibility that either of the reactions in eqns. (2a) or (2b) is reversible.

To test for the reversibility of reaction (2a) the following experiment was undertaken. A reaction mixture containing 2.3 μmoles S-adenosyl-^{3}H-homocysteine, 2.5 μmoles AMe, 1350 μl of 0.5 M Tris buffer, pH 8.1, and 50 μl enzyme solution (1 mg protein/ml) in a total volume of 3000 μl was incubated at 37°C. 500 μl of the reaction mixture were withdrawn after 0, 5, 30, and 60 min incubation. The reaction was terminated by adding 200 μl 25 % trichloroacetic acid, and after centrifugation the protein-free supernatant was decanted. The pH of the supernatant was adjusted to 7.0 by adding N sodiumhydroxide and 0.01 M phosphate buffer, pH 7.0. The mixture was then applied to an Amberlite-IRC 50 (XE—64) column, previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, as described by de la Haba *et al.*[9] At this pH S-adenosylhomocysteine can be washed out from the column by the same buffer while AMe remains adsorbed. In order to remove the last traces of S-adenosyl-^{3}H-homocysteine the column was washed with 20 ml of 0.25 N acetic acid. AMe was then eluted with 4 N acetic acid, and this eluate as well as the S-adenosylhomocysteine eluate were lyophilized. The solid residue was dissolved in distilled water and 200 μl of this solution were transferred to vials containing 2 ml of ethanol and 5 ml of phosphor solution. The radioactivity was measured in a liquid scintillation counter. As can be seen in Table 4 incorporation of S-adenosyl-^{3}H-homocysteine into AMe was insignificant, which indicates that reaction (2a) is essentially irreversible. A reaction mixture identical with the one described with a further addition of 0.16 μmole histamine-^{14}C was incubated for 60 min as a control of enzyme activity under the conditions used. 0.04 μmole methylhistamine was formed indicating that no inactivation of the enzyme had occurred.

In order to test the reversibility of reaction (2b) the following incubation mixture was used: 0.0164 μmole histamine-^{14}C, 0.0134 μmole methylhistamine, 100 μl enzyme solution and 100 μl 0.5 M Tris buffer, pH 8.1, in a total volume of 220 μl. Incubation times 30, 60, and 120 min. The results of these experiments (Table 5) do not agree with the reaction mechanism proposed in eqn. (2b) and consequently methyl group transfer from AMe to histamine seems to occur directly on to the substrate without forming a methylated enzyme as an intermediate.

Table 4. Experiments on the possible incorporation of S-adenosyl-^{3}H-homocysteine into AMe. For conditions used, see text.

Incubation time min	Radioactivity of AMe, cpm	Percentage of total ^{3}H*
0.5	150	0.33
30.0	83	0.12
60.0	10	0.02

* Each sample contained 0.42 μmole of S-adenosyl-^{3}H-homocysteine corresponding to 45 000 cpm.

Table 5. The non-incorporation of metylhistamine into histamine-^{14}C. For conditions see text.

Incubation time min	Radioactivity of methylhistamine, cpm	Percentage of total ^{14}C*
30	51	0.24
60	53	0.25
120	67	0.32

* Each sample contained 0.064 μmole histamine-^{14}C corresponding to 21 000 cpm.

DISCUSSION

Ring N-methylation is one of the principal pathways of histamine inactivation in many mammals.[10] The product formed, 1-methyl-4-(β-aminoethyl)-imidazole, as well as histamine itself is known to serve as substrate for histaminase, which deaminates the side chain oxidatively.[11]

Histamine-N-methyltransferase shows no requirement for metal ions and differs in that respect from catechol-O-methyltransferase.[12] Copper ions as well as ions of nickel, cobalt, and zinc inhibit the enzyme strongly. White has shown that copper ions inhibit the methylation also *in vivo*, especially in the brain.[13]

There is little doubt that the methyl transferase has SH-groups essential for its enzymic activity. The inhibition caused by SH-inhibitors can in most cases be counteracted by adding reduced glutathione to the reaction mixture. Cystein activates the enzyme but thioglycollate has no effect. Histaminase, on the other hand, is insensitive towards SH-inhibitors but is inhibited by carbonyl reagents.[11]

The inhibition caused by chlorpromazine is partly reversed by reduced glutathione and resembles in that case the effect of common SH-inhibitors. Burton and Salvador [14] have examined the influence of chlorpromazine on the methylation of nicotinamide and found that the drug inhibits the reaction at relatively high concentrations (10^{-2} M) but increases the reaction velocity at a concentration of 10^{-3} M. Lower concentrations have no effect. Khouw *et al.*[15] have examined the relationship between chlorpromazine and alcohol dehydrodehydrogenase. The inhibition caused by the drug could not be counteracted even if a large excess of reduced glutathione was added to the reaction mixture. In both cases the inhibition seems to be of a very complex nature, and it is difficult to compare the influence of chlorpromazine on these enzyme systems with the effect of the same drug on histamine methylation.

Of the histamine analogues tested, the derivatives of aminomethyl-imidazole caused the greatest inhibition of the methylation reaction. These compounds cannot serve as substrates for the enzyme and do not compete with histamine for the AMe added. This, however, may be the case when adding for instance dimethylaminoethyl-imidazole to the reaction mixture. In a

recent publication Lindahl-Kiessling [16] describes inhibition studies with a few histamine analogues. The results are in close agreement with those obtained in our laboratory.

Histamine-N-methyltransferase seems to be very specific for the histamine structure and only minor modifications in the side chain of histamine can be undertaken without loosing the property of accepting methyl groups from AMe. The investigation of the substrate specificity is by no means complete and for a correct interpretation of the results obtained the studies must be extended over a greater number of compounds. It is also necessary to perform the test under a variety of conditions in order to make kinetic evaluation of the data.

The methyl group transfer from AMe to histamine seems to occur directly without any methylated enzyme as an intermediate. A similar mechanism for the methylation of homocysteine (betaine is the methyl donor) has been proposed by Durell and Cantoni.[17] Very unstable intermediates cannot, however, be excluded and more experiments must be done to establish the proper reaction mechanism.

The authors are greatly indebted to Drs. T. White, J. A. Duerre, C. F. Huebner and R. G. Jones as well as Houdry Proc. and Chem. Comp. for gifts of histamine analogues. We also wish to thank Drs. S. Gatenbeck and J. Liljekvist for their interest and helpful suggestions during the course of this work.

REFERENCES

1. Gustafsson, A. and Plym Forshell, G. *Acta Chem. Scand.* **18** (1964) 2098.
2. Brown, D. D., Tomchick, R. and Axelrod, J. *J. Biol. Chem.* **234** (1959) 2948.
3. Lindahl, K. M. *Acta Physiol. Scand.* **49** (1960) 114.
4. Gustafsson, A. and Plym Forshell, G. *Acta Chem. Scand.* **17** (1963) 541.
5. Schlenk, F., Dainko, J. L. and Stanford, S. M. *Arch. Biochem. Biophys.* **83** (1959) 28.
6. Parks, L. W. and Schlenk, F. *J. Biol. Chem.* **230** (1958) 295.
7. Killander, A. *Acta Soc. Med. Uppsalien.* **62** (1957) 39.
8. Lineweaver, H. and Burck, D. *J. Am. Chem. Soc.* **56** (1934) 658.
9. de la Haba, G., Jamieson, G. A., Mudd, S. H. and Richards, H. H. *J. Am. Chem. Soc.* **81** (1959) 3975.
10. Shayer, R. W. *Physiol. Rev.* **39** (1959) 116.
11. Kapeller-Adler, R. and Macfarlane, H. *Biochim. Biophys. Acta* **67** (1963) 542.
12. Axelrod, J. and Tomchick, R. *J. Biol. Chem.* **233** (1958) 702.
13. White, T. *J. Physiol. (London)* **149** (1959) 34.
14. Burton, R. M. and Salvador, R. *Ann. N. Y. Acad. Sci.* **96** (1962) 353.
15. Khouw, L. B., Burbridge, T. N. and Sutherland, V. C. *Biochim. Biophys. Acta* **73** (1963) 173.
16. Lindahl-Kiessling, K. M. *Acta Chem. Scand.* **16** (1962) 2293.
17. Durell, J. and Cantoni, G. L. *Biochim. Biophys. Acta* **35** (1959) 515.

Received June 26, 1964.