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BRAIN HISTAMINE *N*-METHYLTRANSFERASE PURIFICATION, MECHANISM OF ACTION, AND INHIBITION BY DRUGS*

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Abstract—Histamine *N*-methyltransferase (E.C., 2.1.1.8; HMT) has been purified approximately 200-fold with about 20 per cent yield from guinea pig brain. The purification steps involved centrifugation at 225,000 *g* for 12 hr, calcium phosphate gel adsorption, DEAE-cellulose chromatography and final chromatography on hydroxylapatite. The enzyme obtained is the most highly purified brain HMT so far prepared. It is specific for histamine with a K_m of $(4.30 \pm 0.14) \times 10^{-5}$ M; it fails to methylate norepinephrine, serotonin and betazole, a histamine analogue. The kinetics and mechanism of action of histamine *N*-methyltransferase were investigated. Initial velocity studies at low substrate concentrations suggested that this methyltransferase action proceeded through two half-reactions in a ping pong mechanism with the intermediate formation of a methylated enzyme. This type of mechanism was also supported by results of product inhibition studies, in which methylhistamine was found to inhibit the enzyme competitively with respect to *S*-adenosylmethionine and noncompetitively with respect to histamine.

Several drugs and chemicals were found to inhibit the enzyme; they included mercurial diuretics, antimalarials, antihistaminics, local anesthetics and ethylamine derivatives. The inhibition by each of these compounds, usually at a concentration of 10^{-4} M or lower, was competitive with respect to histamine and appeared to be mixed with respect to *S*-adenosylmethionine. These patterns of inhibition also have a strong support for the ping pong mechanism.

Methylation of histamine by histamine *N*-methyltransferase (HMT; EC 2.1.1.8) is one of the two dominant modes of inactivation of this amine in most animal species [1–3]. The enzyme occurs ubiquitously in animal species but does not appear to be present in invertebrates, plants or microorganisms [4–7]. In mammals, HMT has been found associated with nervous tissues and most organs [4]. Guinea pig brain, which was used by Brown *et al.* [4] as the source for the purification of this enzyme, has been reported to have one of the highest levels of HMT activity [4]. Further, several drugs, including chlorpromazine, antimalarials, 5-hydroxytryptamine, and antihistamines and their analogues, are potent inhibitors of partially purified preparations of histamine *N*-methyltransferase [4, 8, 9].

Brown *et al.* [4], Lindahl [10] and Gustafsson and Forshell [11] determined that K_m values for HMT substrates, histamine and *S*-adenosylmethionine (SAM), pH optimum, and inhibition by several compounds. However, no information was available on the mechanism of action of HMT and the mechanism of its inhibition by drugs. Accordingly, in this paper we have reported on a method for the purification of HMT from guinea pig brain, the mechanism of histamine methylation by this enzyme, and the interactions and inhibition of a variety of drugs and chemicals on our enzyme preparation.

MATERIALS AND METHODS

Guinea pig brains used for the preparation of the enzyme were purchased from Pel-Freeze Biologicals, Inc. The guinea pigs were killed, exsanguinated, and their brains removed and immediately frozen; the brains were then packed and shipped. On receipt they were stored at −30° until used. No loss in enzymatic activity was apparent for at least 6 weeks when the brains were stored at this temperature.

The following chemicals were obtained commercially and used without further purification: histamine dihydrochloride from Mann Research Laboratories; *S*-adenosyl-L-methionine iodide (SAM), 1,4-methylhistamine from Calbiochem; *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (50 mC/m-mole) from Amersham/Searle Corporation; calcium phosphate gel from Sigma Chemical Company; and DEAE-cellulose from Bio-Rad Laboratories. Other drugs and chemicals including antimalarials, antihistaminics, local anesthetics, diurectics were either gifts or purchased from various chemical manufacturers. They were dissolved in distilled water just before use.

The activity of HMT was measured by the method described in detail by Cohn [8]. Each incubation mixture contained 0.09 μmoles histamine, 0.10 μmoles *S*-adenosyl-L-[*methyl*-^{14}C]methionine ([^{14}C]SAM, 0.25 μC/μmole; approximately 4×10^4 cpm), 40 μmoles

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sodium-potassium phosphate buffer, pH 7.4, 0.1 μmoles aminoguanidine sulfate (to inhibit diamine oxidase), 0.1 ml of the enzyme, and sufficient water to give a final volume of 1.01 ml. Unless otherwise indicated, all incubations were carried out at 37° in a Dubnoff-type metabolic incubator with shaking (90 oscillations/min) and under an air atmosphere. The incubation was run in duplicate with either histamine or [^{14}C]SAM as the last addition. Blanks were also incubated containing no enzyme. The reaction was linear for at least 60 min and over a wide range of enzyme protein concentrations. The reaction was terminated after 20 min incubation by the addition of 0.5 ml 5 N NaOH. The enzymatically formed [^{14}C]-methylhistamine was then extracted with 10 ml of toulene-isoamyl alcohol (3:2 by volume) by shaking for 5 min. After centrifugation, a 3 ml aliquot of the organic phase was withdrawn and added to 10 ml of a liquid scintillation "cocktail" containing 4 g Omnifluor per liter of toluene. Radioactivity was determined in a Nuclear Chicago liquid scintillation spectrometer and to approximately the same counting error (<2%). The counting efficiency within each particular set of experiments was relatively constant, and varied only between 70–77% among different sets of experiments.

For kinetic studies, the concentration of histamine was varied between 2.25×10^{-5} M and 1.35×10^{-4} M, while the concentration of SAM was held fixed at 5, 10, 20 or 30 μM. In other experiments, the concentration of SAM was varied between 5×10^{-6} M and 3×10^{-5} M while the concentration of histamine was held fixed at 22.5, 45, 90 or 135 μM. The specific activity of [^{14}C]SAM used in these kinetic experiments was 2.5 μC/μmole. For studies on the substrate specificity of HMT, the incubation mixture remained the same, except that histamine was replaced by 0.09 μmole of norepinephrine, serotonin or betazole. Any radioactive methylated product formed was extracted into 10 ml of water-saturated isoamyl alcohol by shaking for 5 min, according to the method of Morgan and Mandell [12]. This technique has been shown to be able to extract the methyl derivatives of norepinephrine and serotonin into isoamyl alcohol, because when catechol *O*-methyltransferase from rat liver was incubated with norepinephrine or chick brain indole (ethyl) amine *N*-methyltransferase incubated with serotonin, a significant amount of the radioactivity was found in the isoamyl alcohol extract. After centrifugation, a 3 ml aliquot of the organic phase was withdrawn for determining its radioactivity by liquid scintillation spectrometry as described above.

To test the influence of an inhibitor, the inhibitor, in a series of concentrations ranging from 10^{-3} to 10^{-8} M, was preincubated with the enzyme for 10 min before adding histamine and [^{14}C]SAM. It was found that the inhibitory effect was not increased by increasing the preincubation time beyond 10 min. The final incubation volume remained 1.01 ml.

For kinetic analysis, the notation and terminology proposed by Cleland [13] have been used. The initial velocity data were plotted graphically in double reciprocal form to check the linearity of the plots. The lines drawn were calculated by the method of least squares with the use of an Olivetti Underwood Programma 101 Computer. When the plots of initial velocity data were linear, each set was fitted to Equation (I) (see below) to determine the slope and Y-axis intercept. Since the analyses of initial velocity data in these studies were consistent with a ping pong mechanism, the data were fitted to Equation (II). A family of parallel straight lines was obtained when $1/v$ is plotted against $1/A$ or $1/B$ at various constant concentrations of B or A respectively. Secondary plots were then constructed in which the Y-axis intercepts of these lines were plotted against the reciprocals of the fixed substrate concentrations. From these secondary plots one can obtain the best estimates of K_m and V_{max} (13).

$$V = \frac{V_1 A}{K_m + A} \qquad \text{(I)}$$

$$V = \frac{V_1 AB}{K_a B + K_b A + AB} \qquad \text{(II)}$$

As a check for the ping pong mechanism, the rates of reaction were determined while varying both substrate concentrations in a constant ratio. If the concentrations of substrates A and B are varied in a constant ratio, $1/v$ is a linear function of $1/A$ or $1/B$, and the Y-axis intercept of the double reciprocal plot gives $1/V_1$ (the reciprocal of the maximum velocity) [14]. Data which conformed to this linear double reciprocal plot were fitted to Equation (III). Equation (III) is derived from Equation (II) when $A = xB$, where x is any constant.

$$\frac{1}{v} = \left(\frac{K_a + xK_b}{V_1}\right)\frac{1}{A} + \frac{1}{V_1} \qquad \text{(III)}$$

In inhibition experiments, the type of inhibition was first determined from the nature of the double reciprocal plot, e.g., linear, hyperbolic or parabolic. Since all plots in these inhibition studies were linear, data conforming to competitive inhibition were fitted to Equation (IV), and data conforming to noncompetitive inhibition were fitted to Equation (V).

$$v = \frac{V_1 A}{K_m\left(1 + \frac{I}{K_{is}}\right) + A} \qquad \text{(IV)}$$

$$v = \frac{V_1 A}{K_m\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \qquad \text{(V)}$$

To obtain slope and intercept inhibition constants (K_{is} and K_{ii}) secondary plots of slopes and intercepts were plotted as a function of inhibitor concentrations [13]. These inhibition constants are then defined by the points of intersection of the lines with the X-axis [13, 15].

Histamine *N*-methyltransferase was prepared at 0–4° as follow:

Step 1. *Soluble supernatant fraction from brain.* Whole guinea pig brains were thawed, weighed, minced and then homogenized in 4 volumes of ice-cold 0.01 M sodium–potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 37,000 *g* for 1 hr in a Sorvall refrigerated centrifuge. The supernatant was carefully collected and centrifuged at

225,000 *g* in a Spinco refrigerated centrifuge Model L 2-65 for 12 hr.

Step 2. Purification with calcium phosphate gel. The 225,000 *g* supernatant fraction was then mixed with calcium phosphate gel (4 mg of gel per mg of protein). After 15 min of mixing and stirring the suspension was centrifuged at 37,000 *g* for 15–20 min and the precipitate discarded. The supernatant fraction obtained was placed on a DEAE-cellulose column.

Step 3. Chromatography on DEAE-cellulose column. The supernatant fraction obtained from the previous step was placed on this 45 ml column (1.5 × 30 cm) previously equilibrated with 0.01 M sodium–potassium phosphate buffer, pH 7.5. After application of the enzyme solution, containing approximately 50 mg protein in 50 ml, to the column, elution was begun with 100 ml of 0.05 M sodium–potassium buffer, pH 7.5, and followed by 0.15 M sodium acetate buffer, pH 4.6. The column was operated at a flow rate of about 50 ml/hr. Fractions, either 2 or 5 ml, were collected with the aid of an ISCO Model 400 fraction collector. Each fraction was assayed for both protein content and HMT activity. Elution with the pH 7.5 buffer removed approximately 90 per cent of all the protein applied to the column, but none of the HMT. Almost all of the HMT were removed with 10–12 ml of the pH 4.6 buffer.

Step 4. Hydroxylapatite chromatography. A 30 ml syringe was used as a column. Fractions from the DEAE-cellulose column which contained HMT activity were pooled and immediately applied to the hydroxylapatite column (10 ml bed volume) previously equilibrated with 0.01 M sodium–potassium phosphate buffer, pH 7.5. The enzyme was eluted directly with 0.05 M sodium–potassium phosphate buffer, pH 7.5. Collection of 2 ml fractions was made at a flow rate of 18 ml/hr. Fractions 10–14 with the highest specific activity were pooled and used as the enzyme source in all subsequent studies. Prepared in this way, HMT was stable for at least 6 weeks at −30°.

Protein was determined by the method of Miller [16]. For small quantities pf protein the method of Bailey was used [17]. Crystalline bovine serum albumin was used as the protein standard. Polyacrylamide gel electrophoresis of the enzyme preparation was performed by the method of Wright *et al.* [18].

RESULTS

Purification of histamine N-methyltransferase. Histamine *N*-methyltransferase is a soluble enzyme not associated with any subcellular particles [4]. Removal of non-HMT proteins from the whole homogenate of guinea pig brain by centrifugation provided a very convenient method for obtaaning a soluble fraction for further purification. Table 1 presents typical results of the purification procedures. As shown in column 3 of Table 1, enzyme activity was confined essentially to the nonparticulate fraction. Centrifugation at 225,000 *g* for 12 hr increased the specific activity about 11-fold with about 50 per cent recovery of enzyme activity. It was possible to remove additional non-HMT protein in the 225,000 *g* supernatant fraction by negative adsorption on calcium phosphate gel. After this treatment, the specific activity was increased about 15 times that of the original homogenate. The preparation at this stage could be stored at 0–4° for several days without loss of enzymatic activity.

The supernatant fraction obtained after calcium phosphate gel treatment was applied to a DEAE-cellulose column. The elution pattern of HMT from the DEAE-cellulose column is shown in Fig. 1. As can be seen, elution with the pH 7.5 phosphate buffer removed approximately 90 per cent of all the proteins on the column but not HMT. The enzyme was eluted by the pH 4.6 acetate buffer and appeared in fractions 41–43. This step represents a purification of about 160–200 fold increase from the crude homogenate. However, the eluate obtained was unstable. Activity was lost within 24 hr even though the eluate was stored at 0–4°. Attempts to attain stability by the addition of sulfhydryl compounds, bovine serum albumin, substrate histamine or dialysis with change of pH were unsuccessful. Further attempts to purify the enzyme by rechromatography on DEAE-cellulose led to a considerable loss of enzyme activity. However, by passing the DEAE-cellulose eluate through a hydroxylapatite column a stable enzyme preparation was obtained.

The active fractions from the DEAE-cellulose column were pooled and applied directly to a hydroxylapatite column. The enzyme was eluted with 0.05 M sodium–potassium phosphate buffer, pH 7.5, HMT obtained in this way was stable at −30° for at least 6 weeks. This hydroxylapatite eluate was used as the enzyme source in subsequent studies. Attempt to further purify this enzyme by sephadex gel filtration (G-200) and by affinity chromatography was unfruitful.

The enzyme preparation obtained was by no means homogenous. At least three slightly visible and five

Table 1. Purification of histamine *N*-methyltransferase from guinea pig brain

Fraction	Volume ml	Activity DPM/ml/min	Total activity DPM/min	Protein mg/ml	Total protein mg	Sp. Act. DPM/mg/min	Purification	Recovery* %
Whole homogenate	240†	326	78,240	21.3	5112	15	1	100
37,000 *g* Supernatant	180	312	56,000	6.0	1080	52	3.5	72
225,000 *g* Supernatant	160	252	40,320	1.5	240	169	11.2	52
$Ca_3(PO_4)_2$ gel. negative adsorption	150	231	34,650	1.0	150	231	15.4	44
DEAE-cellulose eluate	18	1475	26,550	0.62	11.2	2371	158	34
Hydroxylapatite eluate	24	595	14,300	0.20	4.8	2979	199	18

* Based on total activity recovered.

† Approximately 14 guines pig brains weighing 48 g were homogenized in 192 ml of 0.01 M sodium–potassium phosphate buffer, pH 7.4.

The results in this table are typical of many preparations of the enzyme.

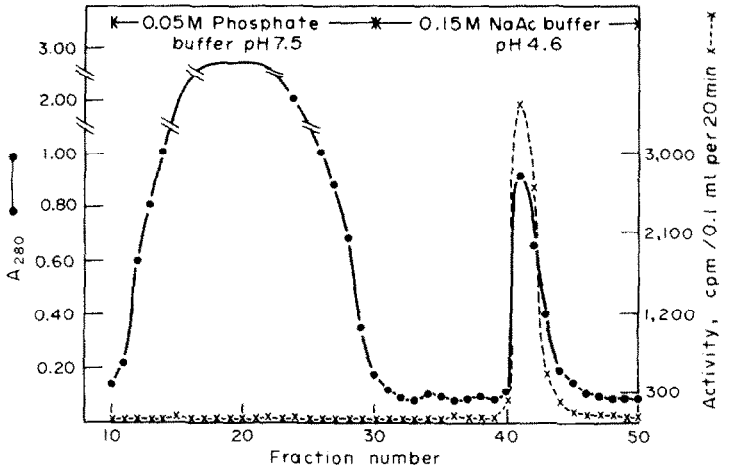

Fig. 1. Elution pattern of histamine *N*-methyltransferase from DEAE-cellulose. The column dimensions were 1.5 × 30 cm. Fifty ml of calcium phosphate gel supernatant containing 50 mg of protein was applied to the column. Elution was carried out with two buffers as indicated. Five ml fractions were collected for fractions 1–38, and two ml fractions for the remaining. Fractions 41–43 containing almost all of the HMT activity were pooled and applied directly onto a hydroxylapatite column.

clearly visible bands were discerned when the hydroxylapatite eluate was subject to polyacrylamide gel electrophoresis. Nevertheless, this is the most highly purified brain enzyme so far obtained; the yield was fairly good (20 per cent recovery). Although data are not presented, our partially purified HMT exhibited a high degree of substrate specificity; it catalyzed only the *N*-methylation of histamine but not of its closely related analogue, betazole. It failed to either *O*-methylate norepinephrine or to *N*-methylate serotonin.

Mechanism of histamine methylation. Histamine *N*-methyltransferase requires two substrates, histamine and *S*-adenosylmethionine. The mechanism of enzyme-catalyzed reaction involving two or more substrates falls essentially into two general types, and the steady state kinetics of the two types has been developed and reviewed by Alberty [15], Dalziel [19] and Cleland [13]. In the first type, called "sequential," all substrates must combine with the enzyme before any product is released. In the second type, called "ping pong," one or more products are released before all substrates have been added to the enzyme.

Performing initial velocity experiments where one substrate is varied at several fixed concentrations of the other, one can differentiate between sequential and ping pong mechanisms [14]. When results of such experiments are shown as double reciprocal plots, a sequential mechanism yields a family of straight lines which intersects to the left of the *Y*-axis, whereas a ping pong mechanism yields a family of parallel straight lines.

Such initial velocity studies for partially HMT are shown in Figs 2 and 3. In each case, a family of parallel straight lines was observed, thus suggesting a ping pong mechanism. This same pattern was also observed when the enzyme reaction was carried out at any pH between 6.0 and 9.0. The apparent Michaelis constants for each substrate can be calculated from these data by constructiong secondary plots of the *Y*-intercepts as a function of the reciprocal of the fixed substrate concentrations [13]. Examples of these secondary plots are shown in Figs 4 and 5, from which K_m for histamine from 3 sets of experiments was found to be $(4.30 \pm 0.14) \times 10^{-5}$ M; the K_m for SAM was $(0.97 \pm 0.10) \times 10^{-5}$ M and the V_{max} was (65.6 ± 2.3) nmoles of methylhistamine formed per mg protein per min.

A further check on this type of mechanism can be made by studying the initial velocity of the reaction as a function of the concentration of one substrate while maintaining constant the ratio of the two substrates. Such a plot was obtained when histamine and *S*-adenosylmethionine were varied in a constant ratio of 4.5:1 as shown in Fig. 6. As predicted for the ping pong mechanism [14, 20], this gives a straight line cutting the ordinate at $1/V_1$. The maximum velocity determined by this technique was found to be

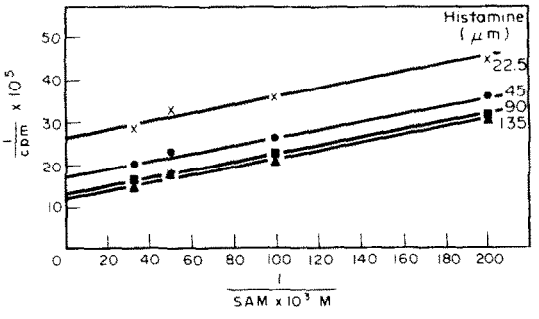

Fig. 2. Double reciprocal plot of initial velocity versus *S*-adenosylmethionine concentration. The concentration of SAM (2.5 μC/μmole) was varied between 5×10^{-6} M and 3×10^{-5} M while the concentration of histamine was held fixed at 22.5, 45, 90 or 135 μM as indicated. In addition to SAM and histamine, each incubation contained 20 μg protein (active hydroxylapatite eluate), 40 μmoles sodium potassium phosphate buffer, pH 7.4, and 0.1 μmoles aminoguanidine in a final volume of 1.01 ml. Incubation was carried out for 20 min at 37° in an air atmosphere. Initial velocities are based on the cpm of [^{14}C]methylhistamine extracted at the end of the incubation, as described in Methods. All lines in this and subsequent figures were fitted to the data by the method of least squares.

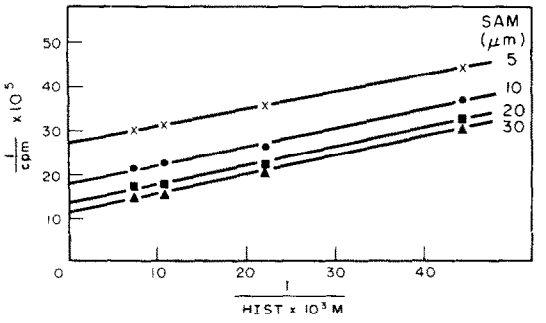

Fig. 3. Double reciprocal plot of initial velocity versus histamine concentration. The concentration of histamine was varied between 2.25×10^{-5} M and 1.35×10^{-4} M, while the concentration of SAM (2.5 μC/μmole) was held fixed at 5, 10, 20 or 30 μM as indicated. Other details are identical to those in Fig. 2.

65.6 nmoles of methylhistamine formed per mg protein per min, in exact agreement with that previously determined (i.e. from Figs 4 and 5).

Product inhibition studies. The evidence discussed above is consistent with a ping pong mechanism:

$$E + S_1 \rightleftharpoons [E\text{---}S_1] \rightleftharpoons E' + P_1$$
$$E' + S_2 \rightleftharpoons [E'\text{---}S_2] \rightleftharpoons E + P_2$$

This mechanism implies that one substrate (S_1) reacts with the enzyme (E) forming an altered enzyme (E') and product (P_1) which is released. The second substrate (S_2) then reacts with the altered enzyme (E') releasing a second product (P_2) and the regenerated eznyme (E). For HMT, S_1 and S_2 are *S*-adenosylmethionine and histamine, respectively; P_1 and P_2 are *S*-adenosylhomocysteine and methylhistamine, respectively; and E' represents a methylated enzyme. This scheme predicts that methylhistamine (P_2) should inhibit the enzyme competitively with respect to SAM (S_1) and non-competitively with respect to histamine (S_2) [13]. Figs 7 and 8 show that these predictions were, in fact, borne out experimentally, and thus gave further support to the suggested mechanism.

Inhibition of HMT by drugs. The partially purified brain HMT was found to be quite sensitive to inhibition by several drugs including *p*-chloromercuribenzoate, *N*-ethylmaleimide, and chlorpromazine (Table 2). However, among the four classes of drugs tested (Table 3), the antimalarials were found to be the most effective inhibitors of the HMT, in agreement with

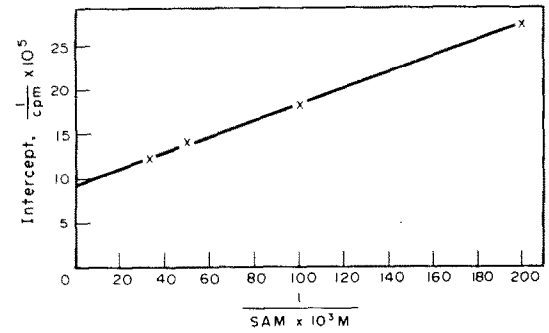

Fig. 4. Secondary plot of *Y*-axis intercepts from Fig. 3 versus reciprocal concentration of *S*-adenosylmethionine.

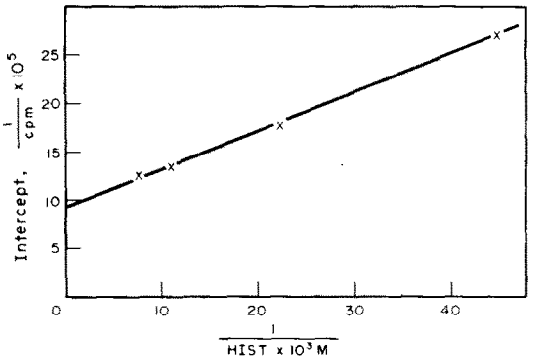

Fig. 5. Secondary plot of *Y*-axis intercepts from Fig. 2 versus reciprocal concentration of histamine.

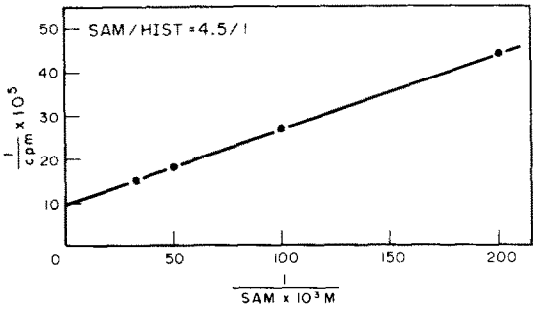

Fig. 6. Double reciprocal plot of initial velocity versus *S*-adenosylmethionine concentration while maintaining the SAM/HIST ratio constant at 4.5/1. In addition to SAM (2.5 μC/μmole) and histamine, each incubation contained 20 μg protein (active hydroxylapatite eluate), 40 μmoles sodium–potassium phosphate buffer, pH 7.4, 0.1 μmole aminoguanidine and water in a final volume of 1.01 ml. Incubation was carried out for 20 min at 37° in an air atmosphere. Initial velocities are based on the cpm of [^{14}C]methylhistamine extracted at the end of the incubation, as described in Methods.

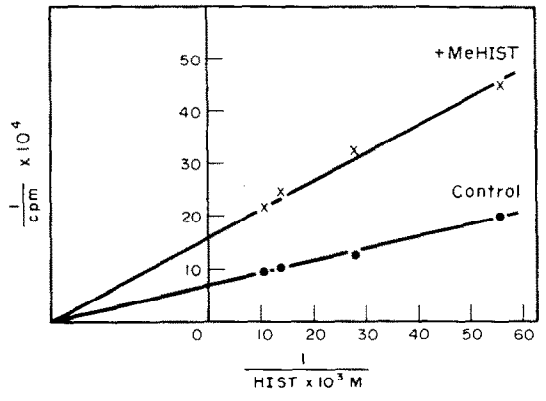

Fig. 7. Inhibition of histamine *N*-methyltransferase by methylhistamine with histamine as the variable substrate. In addition to histamine and methylhistamine, each incubation contained 30 μg protein (hydroxylapatite eluate), 40 μmoles sodium–potassium phosphate buffer, pH 7.4, 0.1 μmole aminoguanidine, 0.1 μmole [^{14}C]SAM (0.25 μC/μmole) and water in a final volume of 1.01. The concentration of methylhistamine was 1×10^{-4} M. Other experimental conditions are described in Methods.

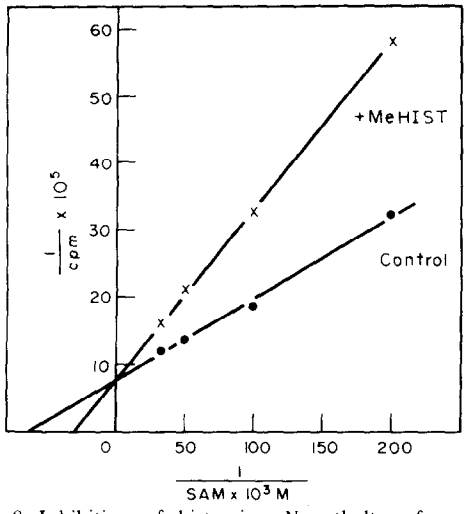

Fig. 8. Inhibition of histamine *N*-methyltransferase by methylhistamine with *S*-adenosylmethionine as the variable substrate. In addition to *S*-adenosylmethionine (2.5 μC/μmole) and methylhistamine, each incubation contained 30 μg protein (hydroxylapatite eluate), 40 μmoles sodium–potassium phosphate buffer, pH 7.4, 0.1 μmole aminoguanidine, 0.09 μmole histamine and water in a final volume of 1.01 ml. The concentration of methylhistamine was 1×10^{-4} M. Other experimental conditions are as described in Methods.

results previously reported with crude enzyme preparation [4, 8, 9].

The mechanism of inhibition produced by a representative member of each class of the drugs was studied. The three classes of drugs were represented by amodiaquine, pheniramine and procaine. All three compounds inhibited the enzyme competitively with respective to histamine, the pattern of inhibition being typified by results shown in Fig. 9. The apparent inhibitory constant (K_i) for each of the inhibitor is tabulated in Table 4. However, the inhibition produced

Table 2. Inhibition of histamine *N*-methyltransferase by chemicals

Compound	Molar inhibitor concentration	% Inhibition*
p-Chloromercuribenzoate	1×10^{-6}	100
	2×10^{-7}	25
N-Ethylmaleimide	1×10^{-4}	100
	1×10^{-5}	67
Phenethylamine	1×10^{-4}	84
Chlorpromazine	1×10^{-4}	92

HMT (50 μg protein) was preincubated with each chemical at the concentrations indicated for 10 min at 37° in 40 μmoles sodium–potassium phosphate buffer, pH 7.4, 0.1 μmoles aminoguanidine and water in a total volume of 0.81 ml. The enzymatic activity was then measured by the addition of 0.1 μmoles [^{14}C]SAM (0.25 μC/μmole) and 0.09 μmole histamine, the final incubation volume being 1.01 ml. The reaction was carried out for another 20 min, at the end of which the [^{14}C]methylhistamine formed was extracted and its radioactivity counted as described in Methods.

* The following compounds, at 10^{-4} M and below, produced no inhibition of the enzyme: 2-phenylethylhydrazine, α-amino-acetophenone, betaxole, histidine, imidazole acetic acid, morphine, strychnine, βphenylacetamide, acetazolamide, chlorothiazide, and hydrochlorothiazide.

Table 3. Inhibition of histamine *N*-methyltransferase by antimalarials, antihistaminics, local anesthetics and diuretics

Compound	% Inhibition Molar inhibitor concentration 1×10^{-7}	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}
Antimalarials					
Amodiaquine	75	93	100		
Chloroquine	19	60	92	100	
Chlorguanide			28	74	90
Cycloguanil			72	96	100
Primaquine	0	0	0	10	71
Pyrimethamine			42	70	95
Quinacrine	20	70	93	100	100
Quinacrine nitrogen mustard	86	100	100		
Antihistaminics					
Antazoline			23	46	97
Cyclizine			5	20	73
Chlorcyclizine			13	41	84
Clemizole			17	72	81
Cyproheptadine			21	56	85
Diphenylpyraline			0	35	92
Doxylamine			4	41	89
Dimethindene			50	90	100
Methapyrilene			17	67	98
Pheniramine			0	37	88
Pyrathiazine			60	90	100
Pyrilamine			30	76	96
Pyrrobutamine			51	90	100
Triprolidine			2	17	86
Local Anesthetics					
Dibucaine			57	87	94
Lidocaine			0	0	10
Procaine			15	48	88
Diuretics					
Mercaptomerin			20	31	
Meralluride			14	100	
Triamterene			5	100	
Ethacrynic acid				44	93

All experimental details were identical to those described in Table 2, except that different drugs were used.

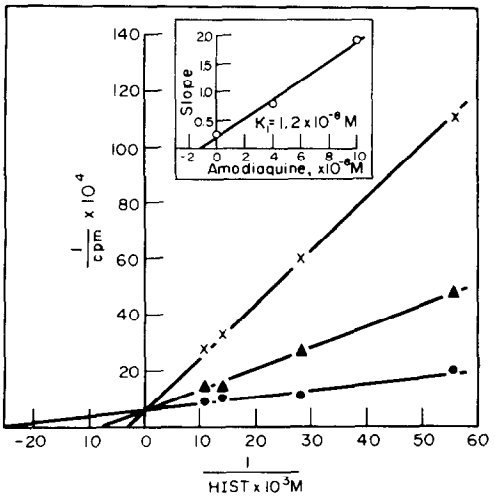

Fig. 9. Double reciprocal plot of initial velocity of histamine N-methyltransferase both in the absence and in the presence of amodiaquine versus histamine concentration. HMT (50 μg protein) was preincubated for 10 min at 37° with amodiaquine at the concentrations indicated in 40 μmoles sodium-phosphate buffer, pH 7.4, 0.1 μmole aminoguanidine and water in a total volume of 0.81 ml. The enzymatic activity was then measured by the addition of 0.1 μmole [^{14}C]SAM (0.25 μC/μmole) and varying concentration of histamine, the final incubation volume being 1.01 ml. The incubation was carried out for another 20 min, at the end of which the [^{14}C]methylhistamine was extracted and its radioactivity counted as described in Methods. The inset plots the slope of these lines against the concentrations of amodiaquine. The intercept on the Y-axis gives an estimate of K_i. ●, Control; ▲, Plus 4×10^{-8} M amodiaquine; ×, Plus 1×10^{-7} M amodiaquine.

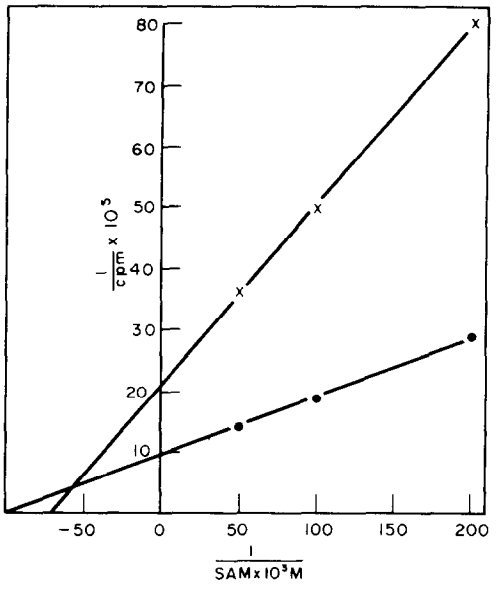

Fig. 10. Double reciprocal plot of initial velocity of histamine N-methyltransferase both in the absence and in the presence of amodiaquine versus S-adenosylmethionine concentration. HMT (50 μg protein) was preincubated for 10 min at 37° with amodiaquine at the concentration indicated in 40 μmoles sodium-potassium phosphate buffer, pH 7.4, 0.1 μmole aminoguanidine and water in a total volume of 0.81 ml. The enzymatic activity was then measured by the addition of 0.09 μmol histamine and varying concentrations of [^{14}C]SAM (2.5 μC/μmole), the final incubation volume being 1.01 ml. The incubation was carried out for another 20 min, at the end of which the [^{14}C]methylhistamine formed was extracted and its radioactivity counted as described in Methods. ●, Control; ×, Plus 10^{-7} M amodiaquine.

by amodiaquine, pheniramine and procaine appeared to be mixed with respect to S-adenosylmethionine (Fig. 10).

DISCUSSION

The method described herein for the preparation of histamine N-methyltransferase from guinea pig brain is relatively simple and has proved to be quite reproducible. The overall purification produces an enzyme approximately 200 times more active than the source material with approximately 20 per cent yield. The enzyme obtained is the most highly purified brain HMT so far prepared. It is stable for at least six weeks when stored at −30°. Based on its behavior on Sephadex G-100 (from which it was excluded) and electrophoretic mobility, the molecular weight of the enzyme is probably greater than 100,000. The only other extensive purification of HMT was accomplished by Gustafsson and Forshell [21] using pig liver as the enzyme source. They were able to effect about a 300-fold increase in specific activity but with very low yield.

Table 4. Apparent inhibition constant (K_i) for histamine N-Methyltransferase

Inhibitor	K_i(M)
Amodiaquine	1.2×10^{-8}
Pheniramine	3.2×10^{-5}
Procaine	3.8×10^{-5}

Each K_i value was obtained from plots as shown in Fig. 9.

The partially purified HMT is completely devoid of activity to methylate either norepinephrine or serotonin. In contrast, Gustafsson and Forshell [5] found that their crude preparation of HMT from rat liver showed ability to methylate tryptamine and serotonin as well as histamine. The discrepancy between their findings and ours reported here may be attributed to their crude preparation being contaminated with indole (ethyl)amine N-methyltransferase or to a different form of HMT. The existence of HMT isozymes is well known [22, 23].

The initial velocity patterns described in the present studies show that guinea pig brain HMT obeys the initial velocity equation described for a ping pong mechanism [13]:

$$\frac{1}{v} = \frac{1}{V_1}\left(\frac{K_a}{A} + \frac{K_b}{B} + 1\right). \quad (1)$$

This mechanism is revealed by the parallel lines obtained when $1/v$ is plotted against $1/A$ or $1/B$ at various constant concentrations of B or A, respectively (Figs 2 and 3). This mechanism means that one or more products are released before all substrates have been added to the enzyme. In other words, no

ternary complex is formed. For HMT, this implies that the enzyme first interacts with *S*-adenosylmethionine to yield a methylated enzyme and the first released product, *S*-adenosylhomocysteine. The methylated enzyme then donates its methyl group to the second substrate, histamine, to give methylhistamine and the free enzyme. If it were essential for both substrates to be bound simultaneously to the enzyme forming a ternary complex, Equation 1 would have to be modified to include an "interaction term," $K_{ia}K_b/AB$ [13]:

$$\frac{1}{v} = \frac{1}{V_1}\left(\frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1\right). \qquad (2)$$

The initial velocity pattern would then be a family of straight lines intersecting to the left of the *Y*-axis [13]. Moreover, the presence of such an interaction term would be apparent as a curvilinear double reciprocal plot when both substrates are varied in a constant ratio [14]. This, however, was not observed (Fig. 6), thus supporting the absence of the interaction term. Alternatively, the formation of a ternary complex which is not required but produces a significant effect on the kinetic behavior of the reaction would yield a rate equation containing exponential functions of both substrate concentrations. The presence of such exponential functions would be reflected as non-linear velocity plots. None of the data observed with our partially purified HMT show any evidence of non-linearity. All of the kinetic data obtained in our studies are consistent with the ping pong mechanism.

The ping pong mechanism is also supported by the product inhibition studies. This mechanism assumes that *S*-adenosylmethionine and methylhistamine react at a common site on one form of the enzyme (E) and that histamine and *S*-adenosylhomocysteine also react at this same site but with the methylated enzyme (E-CH_3):

$$E + \text{SAM} \rightleftharpoons [E\text{---}SAM] \rightleftharpoons E - CH_3 + \text{SAH} \quad (3)$$

$$E - \text{CH}_3 + \text{HIST} \rightleftharpoons [E - \text{CH}_3\text{---HIST}] \rightleftharpoons \text{Me-HIST} + E \quad (4)$$

Consequently, reactants which combine with the same form of the enzyme will be competitive, and conversely, reactants which combine with different forms of the enzyme will be noncompetitive. The results obtained showed that methylhistamine inhibited the enzyme competitively with respect to *S*-adenosylmethionine (Fig. 8) and noncompetitively with respect to histamine (Fig. 7) and thus are consistent with the proposed mechanism. Analogously, *S*-adenosylhomocysteine (SAH) would be expected to inhibit the reaction competively with respect to histamine and noncompetitively with respect to *S*-adenosylmethionine. Zappia *et al.* (24) have shown that *S*-adenysylhomocysteine does inhibit the enzyme, but they did not study the mechanism of inhibition. Recently, Baudry *et al.* [25] found that SAH inhibited guinea pig brain HMT competitively with respect to SAM and noncompetitively with respect to histamine. The discrepancy between their findings and our prediction remains unknown. We do not have SAH in our hands to verify our prediction. However, such different results on product inhibition studies are possible in view of the fact that crude enzyme preparation (only 6 times greater than that of the original 10,000 g supernatant fraction of brain homogenate) was used in their experiments.

Further studies with other inhibitors also support the ping pong mechanism which predicts that an inhibitor combining with only one enzyme form will yield a competitive inhibition pattern with respect to one substrate and a noncompetitive inhibition pattern with respect to the other substrate. The data obtained from inhibition studies with amodiaquine, quinacrine, pheniramine, and procaine indicated that these four drugs react with the methylated enzyme. They inhibited the enzyme competitively with respect to histamine but the inhibition was mixed (one form of noncompetitive inhibition) with respect to *S*-adenosylmethionine (Figs 9 and 10).

The above data are all consistent with and thus provide strong kinetic support for the ping pong mechanism. Attempts to isolate the methylated enzyme by Sephadex G-50 filtration or on a Millipore membrane filter after TCA precipitation were unsuccessful.

Histamine *N*-methyltransferase has been shown to be inhibited *in vitro* by imidazole analogues [11], antimalarials [8], antihistaminics [9, 26], and several other drugs such as chlorpromazine and lysergic acid diethylamide [4]. As an extension of the findings of these investigators [4, 8, 9, 11, 26], we also found that any derivative of ethylamine was a potential inhibitor of the enzyme; histamine analogues, antimalarials, antihistaminics and local anesthetics are just some of the many examples of such derivatives (Tables 2 and 3).

It must be emphasized here that while derivatives of ethylamine may inhibit the enzyme it does not mean that chemicals devoid of this structure will not. Compounds such as amodiaquine, chlorguanide and cycloguanil are not ethylamine derivatives but are all potent inhibitors of HMT. Even vitamins can inhibit the enzyme [27]. This gives emphasis to the fact that our knowledge is still imperfect regarding the requirements for a compound to be an inhibitor of HMT, and this is a subject for future investigation.

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