Purification and kinetic properties of ox brain histamine *N*-methyltransferase

William L. GITOMER* and Keith F. TIPTON

Department of Biochemistry, Trinity College, Dublin 2, Ireland

Histamine *N*-methyltransferase (EC 2.1.1.8) was purified 1100-fold from ox brain. The native enzyme has an M_r of 34800 ± 2400 as measured by gel filtration on Sephadex G-100. The enzyme is highly specific for histamine. It does not methylate noradrenaline, adrenaline, DL-3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxytyramine or imidazole-4-acetic acid. Unlike the enzyme from rat and mouse brain, ox brain histamine *N*-methyltransferase did not exhibit substrate inhibition by histamine. Initial rate and product inhibition studies were consistent with an ordered steady-state mechanism with *S*-adenosylmethionine being the first substrate to bind to the enzyme and *N*-methylhistamine being the first product to dissociate.

INTRODUCTION

In brain, methylation of histamine by histamine *N*-methyltransferase (EC 2.1.1.8) is the first step in the inactivation of the putative neurotransmitter histamine (Schayer, 1959; Green, 1970). This enzyme is highly specific for histamine and only interacts with other metabolic pathways via the shared methyl-donating substrate *S*-adenosylmethionine and its demethylated product *S*-adenosylhomocysteine (see, e.g., Schayer, 1959). Thus the activity of this enzyme is important in controlling the steady-state concentration of histamine in brain. A number of workers have studied this enzyme (Brown *et al.*, 1959; Gustafsson & Forshell, 1964; Sellinger *et al.*, 1978; Thithapandha & Cohn, 1978; Bowsher *et al.*, 1983; Matuszewska & Borchardt, 1983); however there were several differences in the properties, both physical and kinetic, of the enzyme reported by these different groups.

Conflicting kinetic mechanisms have also been reported to be followed by different preparations of the enzyme. Baudry *et al.* (1973) reported the enzyme from guinea pig brain to obey a rapid-equilibrium random-order mechanism whereas Thithapandha & Cohn (1978), using a more highly purified enzyme preparation from the same source, found a double-displacement mechanism to be followed. In contrast, an ordered-binding steady-state mechanism was reported for the enzymes from rat brain (Orr & Quay, 1978) and human skin (Francis *et al.*, 1980).

This paper reports the preparation of highly purified, although not completely homogeneous, and stable samples of histamine *N*-methyltransferase from ox brain together with studies on its kinetic behaviour.

MATERIALS AND METHODS

Materials

AdoMet hydrochloride, adrenaline bitartrate, specially purified acrylamide and *NNN'*-tetramethylethylenediamine were from BDH; methylimidazole acetic acid,

histamine and 2,5-diphenyloxazole were from Koch-Light Laboratories. 3-Hydroxytyramine hydrochloride, DL-3,4-dihydroxymandelic acid and 3,4-dihydroxyphenylacetic acid were from Calbiochem-Behring; imidazoleacetic acid, (–)-noradrenaline bitartrate, phenylmethane sulphonyl fluoride, bovine serum albumin (crystalline, fraction V) cytochrome *c*, myoglobin, ovalbumin, Coomassie Brilliant Blue R and 2-mercaptoethanol were from Sigma Chemical Co. Trien hydrochloride was from Aldrich; Blue Dextran 2000 and Sephadex G-100 were from Pharmacia and *S*-adenosylhomocysteine was from Boehringer. Chloroplatinic acid was from Johnson Matthey Chemicals. Ultrafiltration membranes were from Amicon and *NN'*-methylenebisacrylamide was from Eastman Kodak. Chymotrypsinogen A was from Seravac Laboratories; carboxymethyl-cellulose (CM-52) and diethylaminoethyl-cellulose (DE-52) were from Whatman Biochemicals. *S*-Adenosyl-L-[methyl- 3 H]methionine was from Amersham International; Polygram cel 300 0.1 mm cellulose thin layer plates were from Camlab. *N'*-Methylhistamine was a kind gift from Dr I. R. Smith, Smith, Kline & French Ltd. All other compounds were analytical grade or better.

Ox brains were obtained from the local abattoir within 2 h of the death of the animal and transported to the laboratory on ice. The rats used were male Wistars.

Assay methods

The assay of histamine *N*-methyltransferase was a modification of the method of Taylor & Synder (1972a). The reaction was carried out at 37 °C in plastic Minifuge tubes (1.5 ml capacity), containing a total volume of 100 μ l. Each assay contained 160 mM-sodium phosphate buffer, pH 7.4, 100 μ M-histamine, 100 μ M-[3 H]AdoMet (0.1 μ Ci) and enzyme. After 10 min preincubation, the reaction was started by the addition of 10 μ l of 1.0 mM-AdoMet (0.1 μ Ci) in 10 mM-acetic acid. After incubation for an appropriate time, usually 10 min, the

Abbreviations used: AdoMet, *S*-adenosylmethionine; Trien, triethylene tetramine.

* Present address: Laboratory of Metabolism, NIAAA, 12501 Washington Avenue, Rockville, MD 20852, U.S.A.

reaction was stopped by the addition of 100 μ l of 0.5 M-sodium borate buffer, pH 10.5. Toluene/isoamyl alcohol (1:1, v/v; 1 ml) was then added to each tube; the tubes were vortex-mixed for 10 s and then centrifuged in a Minifuge for 30 s to separate the phases. A 0.9 ml aliquot of the organic phase was then placed in a scintillation vial with 1.0 ml of absolute ethanol and 10 ml of toluene/2,5-diphenyloxazole (0.4%) scintillation fluid and counted in a Packard 3375 or 3385 liquid-scintillation counter. Counting efficiency was 20–30% for ^3H and was corrected for by using the external standard ratio method. Blanks determined in the absence of histamine or enzyme gave the same values.

The reaction was linear as a function of enzyme concentration and as a function of time up to 20 min. Attempts to extend the time-dependent linearity of the assay were made by the addition of adenosine deaminase, purified from *Aspergillus oryzae* by the method of Sharpless & Wolfenden (1967) up to and including the hydroxyapatite column step (about 1400-fold purification). This enzyme converts *S*-adenosylhomocysteine to *S*-inosylhomocysteine (see, e.g., Zappia *et al.*, 1974) and has been shown to prolong the linearity of catechol *O*-methyltransferase assays by relieving product inhibition (Gulliver & Tipton, 1978). The addition of this enzyme to the assays, however, inhibited the observed activity, possibly by the binding of histamine. For the determination of the effects of pH the enzyme was assayed in the presence of the appropriate buffer and the reaction was stopped with 100 μ l of 1 M-NaOH to ensure that the final pH of all assays was the same so that there would be no variation in extraction efficiencies. Stability of the enzyme at these pH values was checked by adjusting a sample to each value and incubating at 37 °C for 20 min before readjusting to pH 7.4 for assay. No attempt was made to maintain a constant ionic strength during these assays, since the enzyme activity was found to be unaffected by ionic strength over the range studied.

Assays to determine substrate specificity were performed as set out above except that the appropriate alternative compound replaced histamine. The assays of noradrenaline, adrenaline and 3-hydroxytyramine were treated identically to those containing histamine. Assays containing DL-3,4-dihydroxymandelic acid and 3,4-dihydroxyphenylacetic acid were stopped with 1.0 M-HCl and then extracted as above. Activity towards imidazoleacetic acid was measured by stopping the reaction with 100 μ l of 0.4 M-HClO₄ containing 1 mg of methylimidazole acetic acid/ml, centrifuging off the protein precipitate and then spotting 10 μ l of the resulting solution on cellulose thin layer plates. The plates were then developed by the method of Aures *et al.* (1968). Pre-poured cellulose thin layer plates, with plastic backing, were used. Prior to use, about 2 mm of cellulose was removed from the edges of the plates, and the plates were pre-run in the solvent system [chloroform/methanol/NH₃ (sp. gr. 0.880) (7:2:1, by vol.)]. The thin layer plates were dried thoroughly and the samples and markers applied (1–10 μ l) and the plates run. Marker compounds were detected with potassium iodoplatinate spray, which was prepared by mixing 5 ml of 5% (w/v) chloroplatinic acid with 45 ml of 10% (w/v) KI and 100 ml of distilled deionized water. Radioactive compounds were detected by cutting the plate into 5 mm slices and placing these slices into scintillation vials with 10 ml of toluene/2,5-diphenyloxazole scintillation fluid. Counting efficiency was 10–

20%. No attempts were made to determine the extraction efficiencies of the products of these reactions. Protein concentration in crude extracts was measured by the microbiuret method (Itzhaki & Gill, 1964). A standard curve was constructed using crystalline bovine serum albumin over the range 0.1–1.0 mg/ml. An A_{330} of 0.67 corresponded to a 1 mg/ml protein concentration. Otherwise, the A_{280} of the sample was measured and protein concentration was calculated assuming $A_{280}^{1\% \cdot 1 \text{ cm}} = 10$.

Analysis of kinetic data

Primary data was analysed by the direct linear plot method of Eisenthal & Cornish-Bowden (1974). A 68% confidence limit was obtained for the apparent K_m and V values (Cornish-Bowden & Eisenthal, 1974). These limits were used to calculate weighting factors for the determination of the true values of the kinetic constants as follows. For any set of data each value was divided into the range of its error to give fractional errors for each point. Each fractional error was then divided into the smallest fractional error of the group, and the resulting values were each multiplied by the same suitable factor to give integral values. These integral values were then used as weighting factors.

For the determination of K_m and K_s values from the primary plot, the data was treated as suggested by Eisenthal & Cornish-Bowden (1974), using the appropriate weighting factors. Inhibitor constants were determined by fitting apparent K_m , K_m/V for $1/V$ values to straight line functions of the inhibitor concentration using the appropriate weighting factors. For illustrative purposes, data are displayed as double-reciprocal plots.

Addition of phenylmethanesulphonyl fluoride to buffers

An appropriate volume of 100 mM-phenylmethanesulphonyl fluoride in acetone was added to a small quantity of the boiling buffer (about one-tenth volume). This solution was rapidly mixed with the remainder of the ice-cold buffer and used immediately.

Polyacrylamide gel electrophoresis

Electrophoresis in 7.5% polyacrylamide gels was carried out by the method of Davis (1964). Gels to be stained for protein were first placed in methanol/acetic acid/water (50:7:43, by vol.) (fixer) for 30 min and then stained for protein in the fixer made 2% (w/v) in Coomassie Brilliant Blue R for 30 min. The gels were destained first in the fixer for 30 min, and then with methanol/acetic acid/water (10:7:83, by vol.) that was changed frequently until the protein bands were clearly visible. For the detection of enzyme activity a gel was sliced into 2 mm discs and each disc was assayed for histamine *N*-methyltransferase activity in a final volume of 100 μ l.

Determination of apparent M_r

The apparent M_r of ox brain histamine *N*-methyltransferase was determined by gel-filtration through Sephadex G-100. The column was calibrated by determining the elution volumes of the following proteins: bovine serum albumin (67000), ovalbumin (45000), chymotrypsinogen A (25000), myoglobin (17500) and cytochrome *c* (12400). The relative M_r values shown in parentheses were taken from Andrews (1965).

Preparation of partially purified rat brain histamine *N*-methyltransferase

This was purified by a modification of the method of Taylor & Snyder (1972*b*). Two different preparations were made, one with and one without 0.1 mM-phenylmethanesulphonyl fluoride in the homogenization buffer. Otherwise the procedures were identical. All steps were carried out at 4 °C. One rat brain was homogenized in 4 vol. of 0.25 M-sucrose/4 mM-sodium phosphate, pH 7.4, in a Dounce homogenizer. The homogenate was centrifuged at 48000 *g* for 2 h. (NH₄)₂SO₄ was added to the supernatant to achieve 45% saturation, followed by centrifugation (10000 *g* for 20 min) to remove the resulting precipitate. The supernatant was then brought to 75% saturation with more (NH₄)₂SO₄. The precipitate, collected by centrifugation (10000 *g* for 20 min), was resuspended in a minimum volume of 10 mM-sodium phosphate buffer, pH 7.4, and dialysed overnight against 1 litre of the same buffer with one change. The enzyme prepared in this way was used immediately.

Purification of ox brain histamine *N*-methyltransferase

All steps were carried out at 0–4 °C.

Step 1: homogenization. One ox brain was found to be a convenient amount of starting material to use. The brain was collected from the slaughterhouse within 2 h of death, and transported to the laboratory on ice. Excess blood was removed and it was then homogenized in 4 vol. (4:1, v/w) of 10 mM-sodium citrate buffer, pH 5.0, containing 0.1 mM-phenylmethanesulphonyl fluoride (added just prior to use) and 0.1% 2-mercaptoethanol in a Kenwood Chef blender at maximum speed for 30 s, and half-speed until it was creamy pink.

Step 2: pH treatment. The homogenate was adjusted to pH 4.0 with 2 M-citric acid and left stirring on ice for 15 min. The pH of the homogenate was then adjusted to 4.8 with 1 M-NaOH and it was immediately centrifuged at 23000 *g* for 1 h.

Step 3: (NH₄)₂SO₄ fractionation. The resulting supernatant was adjusted to 40% saturation with the slow addition of finely ground (NH₄)₂SO₄. During the addition no attempt was made to maintain the pH. The suspension was left stirring for 30 min and then centrifuged at 16000 *g* for 20 min. The pellet was discarded and the supernatant was brought to 75% saturation by the slow addition of finely ground (NH₄)₂SO₄. During the addition of (NH₄)₂SO₄, no attempt was made to maintain the pH of the suspension. The suspension was left stirring for 30 min and then centrifuged at 16000 *g* for 20 min. The supernatant was discarded and the pellet was resuspended in a minimal volume of the homogenization buffer to which phenylmethanesulphonyl fluoride was added just prior to use. The resuspended pellet was then dialysed overnight against 2 litres of 10 mM-sodium citrate buffer, pH 5.0, containing 0.1% (v/v) 2-mercaptoethanol (buffer A) with three changes. Gel filtration on Sephadex G-25 to change buffers was not possible due to marked precipitation of proteins at this stage.

Step 4: CM-cellulose chromatography. The dialysed enzyme solution was centrifuged at 16000 *g* for 20 min to remove precipitated protein. The ionic strength of the en-

zyme solution was adjusted with cold distilled-deionized water to be less than or equal to that of buffer A, and it was then applied to a column of CM-cellulose (Whatman CM-52) (8 cm × 4.2 cm diam.) equilibrated in buffer A. The column was then washed with buffer A until the *A*₂₈₀ of the eluent was less than 0.1 (measured in a 1 cm path-length cuvette). The enzyme was eluted from the column with a linear gradient of 0–200 mM-NaCl in buffer A (total volume 4 litres). The flow rate was about 8 ml/min and fractions of about 24 ml were collected. The enzyme was eluted as a single peak and all fractions containing greater than half the activity of the peak fraction were pooled and concentrated to about 40 ml in an Amicon ultrafiltration apparatus fitted with a XM 50 membrane. The enzyme could not be concentrated by precipitation with (NH₄)₂SO₄ at this stage, as this resulted in a considerable loss of enzyme activity.

Step 5: DEAE-cellulose chromatography. The concentrated enzyme solution was dialysed overnight against 2 litres of 10 mM-triethanolamine/HCl/2 mM-Trien, pH 7.6, containing 0.1% 2-mercaptoethanol (buffer B) with two changes. The conductivity of the dialysed enzyme solution was checked and adjusted, if necessary, with cold distilled deionized water to that of buffer B. The enzyme solution was then applied to a column of DEAE-cellulose (Whatman DE-52) (11 cm × 3.2 cm diam.) equilibrated in buffer B and the column was washed with buffer B until the *A*₂₈₀ of the eluate was less than 0.1 (measured in a 1 cm pathlength cuvette). The enzyme was then eluted from the column with a linear gradient, total volume 2 litres, of 0–100 mM-NaCl in buffer B. Fractions (approx. 23 ml) were collected at a flow rate of about 3.3 ml/min. All fractions containing at least 25% of the activity of the peak fractions were pooled and concentrated as before to a final volume of about 20 ml.

Step 6: Sephadex G-100 chromatography. Prior to application to the Sephadex G-100 column, the enzyme solution was further concentrated by placing the enzyme solution in dialysis tubing and then surrounding the tubing with cold granular sucrose. The sucrose was replaced as it became damp. After the volume of the enzyme solution had been reduced to about 7 ml, it was applied to a column of Sephadex G-100 (84 cm × 3.3 cm diam.) equilibrated in 50 mM-sodium phosphate/1 mM-EDTA, pH 7.5, containing 0.1% 2-mercaptoethanol. The column was eluted with the same buffer and 10 ml fractions were collected in plastic tubes. This is essential, since enzyme activity was rapidly lost if the fractions were collected in glass tubes. After passage through the G-100 column only plastic containers were used to hold the enzyme solution. The fractions of highest specific activity were pooled and concentrated in an Amicon ultrafiltration apparatus fitted with a UM 10 membrane. The concentrated enzyme solution was then made 10% (v/v) in glycerol. The enzyme prepared in this way was stable for about 3 weeks at 4 °C, and for at least 4 months at –20 °C with no apparent loss of activity. The results of a typical purification are given in Table 1.

RESULTS

The purity of the enzyme preparation was assessed electrophoretically on 7.5% polyacrylamide gels. Staining

Table 1. Summary of a typical purification of ox brain histamine *N*-methyltransferase

Protein concentration was determined spectrophotometrically assuming A_{280} 1%, 1 cm = 10 except where noted (*), when it was determined by biuret assay

Purification step	Volume (ml)	Protein (mg/ml)	Enzyme activity (nmol/min per ml)	Enzyme specific activity (nmol/min per mg)	Purification (-fold)	Recovery (%)
1. Homogenate	2000	26*	1.5	0.058	—	100
2. Low pH supernatant	1520	4.4	0.79	0.18	3	40
3. 40–75% (NH ₄) ₂ SO ₄ fraction	440	3.0	2.3	0.77	13	33
4. Concentrated CM-cellulose eluate	57	2.1	23	11	190	43
5. DEAE-cellulose eluate	300	0.21	2.8	13	220	28
6. Concentrated Sephadex G-100 eluate	23	0.17	11	65	1100	8

with Coomassie Brilliant Blue R revealed two major bands, and one or two minor bands, with the more intense major band corresponding to histamine *N*-methyltransferase activity.

Properties of ox brain histamine *N*-methyltransferase

The enzyme had an M_r of 34800 ± 2400 as measured by gel filtration on Sephadex G-100. No activity could be detected when histamine was replaced with noradrenaline, adrenaline, 3-hydroxytyramine, DL-3,4-dihydroxy mandelic acid, 3,4-dihydroxyphenylacetic acid or imidazoleacetic acid.

Unlike the enzyme from rat, guinea pig and mouse brain (Taylor & Snyder, 1972*b*; Sellinger, *et al.*, 1978) that from ox brain did not exhibit significant high substrate inhibition at histamine concentrations of up to about $10 \times K_m$ (Fig. 1). It was thought possible that the reported inhibition of the rat and mouse enzyme might be due to some artifact in either the preparation (possible proteolysis) or assay (inhibitory impurities in substrate) of the enzyme. However, rat brain histamine *N*-methyltransferase purified in a similar manner to that of Taylor & Snyder (1972*b*) with and without phenylmethanesulphonyl fluoride present in the homogenization buffer, did exhibit high substrate inhibition (Fig. 1) when assayed under conditions identical to those used for the ox brain enzyme.

The effects of pH on the activity of ox brain histamine *N*-methyltransferase are shown in Fig. 2. Determinations were not made above pH 9.0 owing to instability of the enzyme. The lack of any clear optimum in this range contrasts with that of the enzyme from guinea pig brain where an optimum at pH 7.2–7.4 (Brown *et al.*, 1959) and optima at pH 7.4 and pH 9.0 (Matuszewska & Borchardt, 1983) have been reported. A narrow pH profile with an optimum in the region of pH 8.0–8.25 has been reported for the enzyme from rat kidney (Bowsher *et al.*, 1983).

Effects of substrate concentration

When the concentration of histamine or AdoMet was varied at a fixed concentration of the other substrate, an intersecting pattern of lines was observed for the double

Fig. 1. Dependence of the initial velocity of the reaction catalysed by ox brain (●) and rat brain (○) histamine *N*-methyltransferase on the concentration of histamine

The enzyme samples were prepared and assayed as described in the text. Initial velocity values have been scaled to allow the activities of the two preparations to be represented on the same graph.

reciprocal plots, as shown in Fig. 3. These plots were linear over a range of 5–100 μM for both substrates and the slope and intercept replots (not shown) were also linear in all cases. These plots rule out double-displacement mechanisms and also a compulsory-order mechanism in which the first substrate binds under conditions of thermodynamic equilibrium.

Product inhibition studies

The inhibition due to *S*-adenosylhomocysteine with respect to AdoMet at 10 μM -histamine was found to be

Fig. 2. Effects of pH on the activity of ox brain histamine *N*-methyltransferase

The enzyme activity was assayed, as described in the text, in either 50 mM-sodium phosphate buffer (●) or 50 mM-Tris/HCl buffer (○).

competitive (Fig. 4a) and a secondary plot of the slopes of the lines against the inhibitor concentration (not shown) was linear.

At non-saturating concentrations of AdoMet (10 μ M), inhibition by *S*-adenosylhomocysteine was found to be mixed with respect to histamine (Fig. 4b) with the slope and intercept replots of these data being linear; however, at saturating concentrations of AdoMet (100 μ M) no inhibition was observed. This finding is not consistent with a random mechanism in which this product could bind only to the free enzyme, since such a mechanism would give competitive inhibition at non-saturating concentrations of AdoMet. A mechanism of this type, in which the binding of histamine could lead to the formation of an abortive ternary complex with *S*-adenosylhomocysteine would, however, lead to the observed inhibition pattern as would an ordered mechanism in which AdoMet was the first substrate to bind.

Mixed inhibition of histamine *N*-methyltransferase by methylhistamine was observed when histamine was the variable substrate and AdoMet was held constant at a non-saturating concentration (10 μ M) (Fig. 5a). This is consistent with an ordered-binding steady-state mechanism, while one would expect competitive inhibition with a Theorell-Chance mechanism and with a rapid-equilibrium random mechanism. At non-saturating concentrations of histamine (10 μ M) inhibition by methylhistamine was found to be mixed with respect to AdoMet (Fig. 5b). Inhibition by this product was still mixed when the histamine concentration was 100 μ M, but the apparent K_i values determined from slope and intercept replots were both increased by approx. 2-fold.

Fig. 3. Double-reciprocal plots of initial velocity versus substrate concentration for the reaction catalysed by ox brain histamine *N*-methyltransferase

Experimental details are given in the text. Initial velocities are expressed in arbitrary units. (a) Determinations with varying concentrations of AdoMet at different histamine concentrations: 5 μ M (■), 10 μ M (▲), 20 μ M (△), 30 μ M (●) and 100 μ M (○). (b) Determinations with varying concentrations of histamine and different AdoMet concentrations: 5 μ M (■), 15 μ M (▲), 25 μ M (△), 40 μ M (●) and 100 μ M (○).

DISCUSSION

The procedure for the purification of ox brain histamine *N*-methyltransferase described in this paper gives a highly purified and stable preparation of the enzyme. Its apparent lack of inhibition by high ($\sim 10 K_m$) concentrations of histamine contrasted with the behaviour reported for this enzyme from some other sources. The M_r of the enzyme was similar to the value of 33 400 reported for the enzyme purified from rat kidney (Bowsher *et al.*, 1983) but somewhat higher than those of 25 000 reported for the enzymes from mouse and rat brain

Fig. 4. Inhibition of ox brain histamine *N*-methyltransferase by *S*-adenosylhomocysteine

Experimental details are described in the text. Initial velocities are expressed in arbitrary units. (a) Inhibition with respect to AdoMet at a fixed histamine concentration of 10 μM and the following inhibitor concentrations: 0 (▲), 2 μM (△), 5 μM (●) and 15 μM (○). (b) Inhibition with respect to histamine at a fixed AdoMet concentration of 10 μM and the following inhibitor concentrations: 0 (▲), 2 μM (△), 5 μM (●) and 15 μM (○).

(Sellinger *et al.*, 1978) and from guinea pig brain (Matuszewska & Borchardt, 1983).

Prior to passage through Sephadex G-100, histamine *N*-methyltransferase activity did not permeate a XM 50 ultrafiltration membrane, which excludes globular proteins of M_r 50000 or greater. Such behaviour of impure preparations was also observed by Sellinger *et al.* (1978) in their studies of the enzyme from rat and mouse brain,

Fig. 5. Inhibition of ox brain histamine *N*-methyltransferase by *N*^ε-methylhistamine

Experimental details are described in the text. Initial velocities are expressed in arbitrary units. (a) Inhibition with respect to histamine at a fixed AdoMet concentration of 10 μM and the following inhibitor concentrations: 0 (■), 20 μM (▲), 50 μM (△), 80 μM (●) and 150 μM (○). (b) Inhibition with respect to AdoMet at a fixed histamine concentration of 10 μM and the following inhibitor concentrations: 0 (■), 20 μM (△), 50 μM (●) and 150 μM (○).

although they did not comment upon it, and by Bowsher *et al.* (1983) for the enzyme from rat kidney. After passage of the ox brain preparation through Sephadex G-100 it no longer exhibited this anomalous behaviour. This change in the apparent M_r with purification may explain the high value reported of 100000 for the less highly purified enzyme preparation from guinea pig brain (Thithapandha & Cohn, 1978). Glycoproteins have been reported to behave anomalously on filtration systems, but attempts to detect carbohydrate moieties associated with the purified enzyme by staining, after polyacrylamide-gel electrophoresis, by the method of Fairbanks *et al.* (1971) did not yield a positive result.

It is possible that in the impure state the enzyme is associated with other macromolecules and that dissociation occurs on purification. If such dissociation occurs, however, it does not appear to be affected by high ionic strength or a decrease in metal ion concentration, since the enzyme eluted from CM-cellulose did not pass

through the XM 50 membrane in the presence of about 0.10 M-NaCl (the NaCl concentration of the pooled CM-cellulose fractions), or in the presence of either of the chelating agents citrate (10 mM) or Trien (2 mM). Protein-concentration-dependent dissociation might be a possible explanation, since the enzyme was eluted from Sephadex G-100 with a protein concentration that was at least five times more dilute than at any other stage in the purification of the enzyme. Such an equilibrium would, however, have to have been very far to the side of the associated state in the post DEAE-cellulose preparation, since no detectable enzyme activity was found to pass through the XM 50 membrane at this stage of the purification procedure.

The simplest mechanism that would be consistent with the initial-rate and product-inhibition data would be an ordered steady-state mechanism involving the formation of ternary complexes in which AdoMet was the first substrate to bind to the enzyme and *N*⁷-methylhistamine was the first product to dissociate. Such a mechanism would predict inhibition by *N*⁷-methylhistamine to be mixed towards both substrates according to the steady-state equation:

$$v = \frac{V}{1 + \frac{[\text{MH}]}{K_{\text{iu}}^{\text{MH}}} + \frac{K_{\text{m}}^{\text{A}}}{[\text{A}]} + \left(\frac{K_{\text{m}}^{\text{H}}}{[\text{H}]} + \frac{K_{\text{s}}^{\text{A}} K_{\text{m}}^{\text{H}}}{[\text{A}][\text{H}]} \right) \left(1 + \frac{[\text{MH}]}{K_{\text{ic}}^{\text{MH}}} \right)} \quad (1)$$

where [A], [H] and [MH] represent the concentrations of AdoMet, histamine and *N*⁷-methylhistamine, K_{m}^{A} and K_{m}^{H} are the true Michaelis constants for the substrates, K_{s}^{A} is the apparent dissociation constant for the interaction of AdoMet with the enzyme in the absence of histamine and $K_{\text{iu}}^{\text{MH}}$ and $K_{\text{ic}}^{\text{MH}}$ are the inhibitor constants for the uncompetitive and competitive components of inhibition by *N*⁷-methylhistamine.

This equation predicts that increasing the concentrations of histamine would change the apparent K_{i} values obtained from slope and intercept replots of data for inhibition by this product, plotted initially in double-reciprocal form, with the former increasing towards infinity, as the inhibition becomes uncompetitive and the latter decreasing towards the value of $K_{\text{iu}}^{\text{MH}}$. From the above equation and the data in Table 2 it can be calculated that a histamine concentration over 10-fold greater than that used here would be necessary to render the competitive element of the inhibition negligible.

However, the effects on the apparent K_{i} values observed when the histamine concentration was raised from 10 μM to 100 μM would not be in accord with this mechanism in its simplest form. It is possible that the formation of an additional abortive complex with this product might account for this behaviour, but further work would be necessary to account for this anomaly.

The corresponding equation for inhibition by *S*-adenosylhomocysteine (S) is

$$v = \frac{V}{1 + \frac{K_{\text{m}}^{\text{A}}}{[\text{A}]} \left(1 + \frac{[\text{S}]}{K_{\text{i}}^{\text{S}}} \right) + \frac{K_{\text{m}}^{\text{H}}}{[\text{H}]} + \frac{K_{\text{s}}^{\text{A}} K_{\text{m}}^{\text{H}}}{[\text{A}][\text{H}]} \left(1 + \frac{[\text{S}]}{K_{\text{i}}^{\text{S}}} \right)} \quad (2)$$

The values of the kinetic constants determined from the initial rate data in the presence and absence of products are shown in Table 2. In common with several other methyltransferases (see Saavedra, 1977), histamine

Table 2. Kinetic constants for the reaction catalysed by ox brain histamine *N*-methyltransferase

The enzyme was assayed and the constants defined by eqns. (1) and (2) were determined as described in the text. Values in parentheses represent the 68% confidence intervals. The precision of the K_{i} values is given as one standard deviation.

Constant	Value (μM)
K_{m}^{A}	6.8 (4.2, 8.1)
K_{m}^{H}	13 (9.6, 16)
K_{s}^{A}	21 (17, 23)
K_{i}^{S}	2.7 ± 0.7
$K_{\text{ic}}^{\text{MH}}$	150 ± 25
$K_{\text{iu}}^{\text{MH}}$	94 ± 12
V	65 (59, 73) nmol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹ of protein

N-methyltransferase is highly sensitive to inhibition by *S*-adenosylhomocysteine. Mudd & Poole (1975) have suggested that alterations in the ratio of the concentration of this product to that of AdoMet resulting from competition amongst methylating enzymes may be an important factor in regulating their activities. The enzyme is much less powerfully inhibited by *N*⁷-methylhistamine but further knowledge of the behaviour of the systems that remove the products of the reaction will be necessary before the significance *in vivo* of such sensitivity can be assessed.

We are grateful to the Medical Research Council of Ireland and to the Trinity College Development Fund for grants towards this work and to Dr I. R. Smith for his kind gift of a sample of *N*⁷-methylhistamine. W. L. G. is also grateful to Corpus Christi College, Cambridge, for the support of a studentship during the early stages of this work.

REFERENCES

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Aures, D., Fleming, R. & Hakanson, R. (1968) *J. Chromatogr.* **33**, 480–493
- Baudry, M., Chast, F. & Schwartz, J. C. (1973) *J. Neurochem.* **20**, 13–21
- Bowsher, K. R., Verburg, K. M. & Henry, D. P. (1983) *J. Biol. Chem.* **258**, 12215–12220
- Brown, D. D., Tomchick, R. & Axelrod, J. (1959) *J. Biol. Chem.* **234**, 2948–2950
- Cornish-Bowden, A. & Eisenthal, R. (1974) *Biochem. J.* **139**, 721–730
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Francis, D. M., Thompson, M. F. & Greaves, M. W. (1980) *Biochem. J.* **187**, 819–828
- Green, J. P. (1970) in *Handbook of Neurochemistry* (Lajtha, A., ed.), pp. 221–250, Plenum Press, New York
- Gulliver, P. A. & Tipton, K. F. (1978) *Biochem. Pharmacol.* **27**, 773–775
- Gustafsson, A. & Forshell, G. P. (1964) *Acta Chem. Scand.* **18**, 2069–2076
- Itzhaki, R. F. & Gill, D. M. (1964) *Anal. Biochem.* **9**, 401–410
- Matuszewska, B. & Borchardt, R. T. (1983) *J. Neurochem.* **41**, 113–118
- Mudd, S. H. & Poole, J. R. (1975) *Metabolism* **24**, 721–735

- Orr, E. L. & Quay, W. B. (1978) *J. Neurochem.* **30**, 1539–1542
- Saavedra, J. M. (1977) *Essays Neurochem. Neuropharmacol.* **1**, 1–41
- Schayer, R. W. (1959) *Physiol. Rev.* **39**, 116–126
- Sellinger, O. N., Schatz, R. A. & Ohlsson, W. G. (1978) *J. Neurochem.* **30**, 437–445
- Sharpless, T. K. & Wolfenden, R. (1967) *Methods Enzymol.* **12**, 126–131
- Taylor, K. M. & Snyder, S. H. (1972*a*) *J. Neurochem.* **19**, 1343–1358
- Taylor, K. M. & Snyder, S. H. (1972*b*) *Mol. Pharmacol.* **8**, 300–310
- Thithapandha, A. & Cohn, V. H. (1978) *Biochem. Pharmacol.* **27**, 263–271
- Zappia, V., Galletti, P., Carteni-Farina, M. & Sevillo, L. (1974) *Anal. Biochem.* **58**, 130–138

Received 15 July 1985/17 September 1985; accepted 1 October 1985