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SHORT COMMUNICATIONS

Sidechain-modified histamine analogues as substrates for histamine *N*-methyltransferase*

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A major route of catabolism of histamine is by methylation at the ring N^{τ}-nitrogen (nomenclature of Black and Ganellin [1]). This reaction is catalysed by the enzyme histamine *N*-methyltransferase† (HMT, *S*-adenosyl-methionine: histamine *N*-methyltransferase, EC 2.1.1.8) [2]. The enzyme has been regarded as having high specificity for histamine as substrate, but some further substrates have been identified by Barth *et al.* [3] recently, using the enzyme purified from pig fundic mucosa. Substrates identified by these workers included N^{α}-methylhistamine, N^{α}, N^{α}-dimethylhistamine, spinaceamine and the thio-ether 4-[(2-aminoethylthio)methyl]imidazole. However, none of these compounds is methylated as efficiently as histamine (extracted radioactivity was never greater than 21% of that achieved with optimal concentrations of histamine).

In this communication, we described several sidechain-modified analogues of histamine with substrate activity at the enzyme found in guinea-pig brain high speed supernatant. The dimaprit analogue SK&F 91488 was shown to be a competitive inhibitor of the methylation of each of these compounds.

Materials and methods

Substrates and inhibitors. Histamine dihydrochloride was obtained from Sigma Chemical Co. (London, U.K.). 4-(3-Aminopropyl)imidazole dihydrobromide (homohistamine), (±)-α-methylhistamine dihydrobromide, (±)-β-methylhistamine dihydrochloride, and *S*-[3-(*N*,*N*-dimethylamino)butyl]isothiourea dihydrobromide (SK&F 91488) were synthesized in the Chemistry Department at these Laboratories. *S*-Adenosyl-L-[methyl-^{14}C]methionine (0.5 mCi/mmole) was obtained from the Radiochemical Centre (Amersham, U.K.).

Reagents. All reagents were of analytical grade. TLC plates were 0.25 mm silica gel from Merck (Darmstadt, F.R.G.).

Enzyme preparation. Whole brain from adult male Dunkin–Hartley guinea-pigs was homogenized (Polytron, setting 6, 2 × 10 sec) in 10 vol. ice-cold sodium phosphate buffer, 0.1 M, pH 7.4. The homogenate was spun at 60,000 *g* (av.) for 1.5 hr. The supernatant was flash-frozen and stored at −20°.

Enzyme activity. For investigation of substrate activity and its inhibition by SK&F 91488, a modification of the procedure of Snyder and Axelrod [4] was used. An aliquot (75 μl) of the enzyme preparation was pre-incubated at 37° with histamine or an analogue and the inhibitor as appropriate in sodium phosphate buffer, 0.1 M, pH 7.4. After the 15 min pre-incubation period, the reaction was started by addition of *S*-adenosyl-[methyl-^{14}C]methionine, 0.5 mCi/mmole, 10 μM final concentration. Total volume of the incubation mixture was 2.5 ml. At the end of the 20 min incubation period, aqueous NaOH (3.3 M, 1 ml) was added and the product was extracted with a 1:1 by volume mixture of isoamyl alcohol–toluene (4.25 ml). An aliquot (3.5 ml) of the organic phase was counted for ^{14}C in 10 ml of Pico-fluor 15 (Packard). Incubations were carried out in duplicate; the duplicate dpm values rarely differed by more than 5%. Blank (no substrate) dpm values were *ca.* 50 dpm, and optimal concentrations of histamine gave *ca.* 1000 dpm.

It was shown, using histamine as substrate, that the amount of methylation observed was linear with respect to enzyme concentration and incubation time. Initial rate data were analysed by the method of Wilkinson [5]. The inhibitor constant K_i for SK&F 91488 was calculated from the relationship K_m (apparent) = K_m $(1 + {}^{I}/K_i)$ either by direct substitution or by re-plot of K_m (apparent) vs inhibitor concentration I. In the case of (±)-α- and (±)-β-methylhistamine, it was assumed for the purpose of determination of the K_m value that substrate activity resides in one isomer only of the racemic mixture.

Extraction efficiency. An extraction efficiency was obtained for each product to allow conversion of recovered dpm into units of pmole min^{-1} μg^{-1} protein. This was done by re-partitioning an aliquot (containing a known number of dpm) of the extract of each of the products between the aqueous and organic phases as before. A similar procedure was carried out for a control incubation. The extraction efficiency for each product was calculated from the recovered dpm, and represents the proportion of product dpm formed which was extracted and counted.

TLC. The R_f value of each of the radio-labelled products was determined as follows. A saturating concentration of each substrate was incubated with the enzyme and *S*-adenosyl-[methyl-^{14}C]methionine and the product extracted as described above. The product was then back-extracted into HCl (0.1 M, 2 × 2 ml) which was freeze-dried. The residue was re-dissolved in water and run on silica gel TLC plates in $CHCl_3/CH_3OH$/aq. NH_3 (60:30:6 by volume). The plate was divided into fractions and the radioactivity counted using the method described previously [6].

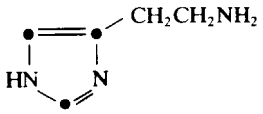
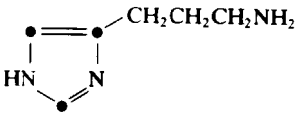
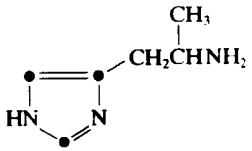
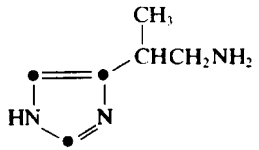
Results and discussion

By the procedure outlined above, several sidechain-modified analogues of histamine were identified as substrates for HMT, viz. 4-(3-aminopropyl) imidazole (homohistamine, the higher homologue of histamine), (±)-α-methylhistamine and (±)-β-methylhistamine (Table 1). In general, substrate activity was comparable in terms of both K_m and V_{max} values with that shown by the natural substrate histamine under identical conditions although a substantially lower K_m was noted for (±)-β-methylhistamine. These observations thus confirm those of Barth *et al.* [3] that HMT does not have an absolute specificity for histamine as substrate. α- and β-Methylhistamine were tested as racemic mixtures, and the K_m and V_{max} values for these compounds have been calculated assuming that the substrate activity resides in one enantiomer only of the mixture. In fact the values will be affected by substrate or inhibitor activity of the other enantiomer. This situation has been analysed by Dixon and Webb [7]. In particular, in the case where the second enantiomer has only inhibitor but no substrate activity, the true K_m and V_{max} values will be

* A report of this study was presented at the European Histamine Research Society Meeting, Hannover, F.R.G., May 1981.

† Abbreviation: HMT, histamine *N*-methyltransferase.

Table 1. Substrates for histamine *N*-methyltransferase identified in this study*

Compound	Structure	K_m (μM)	V_{max} (pmole/min/μg protein)	Extraction efficiency	R_f of product	SK&F 91488 K_i (μM)
Histamine		13.0 ± 0.9	0.34	0.42	0.62	1.23
Homohistamine		9.4 ± 0.7	0.22	0.81	0.69	1.63
(±)-α-Methylhistamine		8.4 ± 0.3	0.40	0.59	0.74	1.54
(±)-β-Methylhistamine		0.94 ± 0.08	0.21	0.69	0.74	1.49

* Experimental details are given in Materials and Methods.

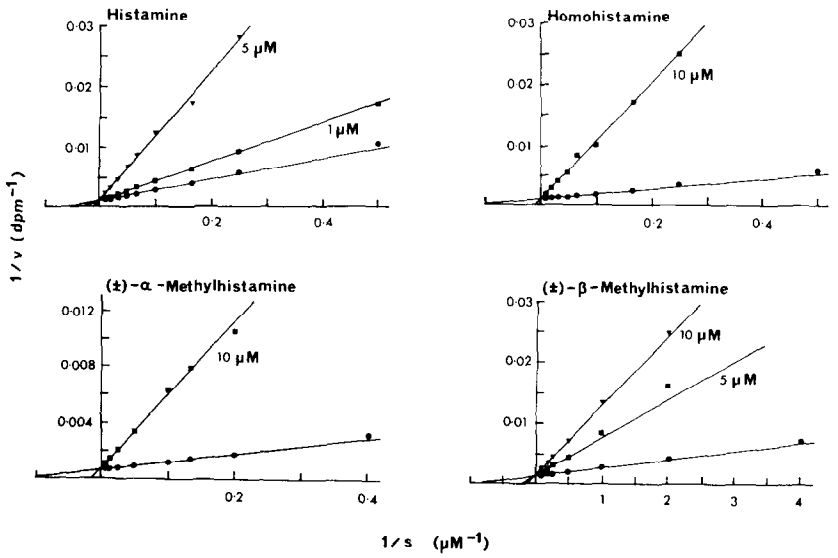


Fig. 1. Double reciprocal plots indicating competitive inhibition by SK&F 91488 of the methylation of histamine, homohistamine, (±)-α-methylhistamine and (±)-β-methylhistamine. Substrate concentration (s) in units of μM. Velocity (v) in units of dpm product formed during incubation. Inhibitor concentration for each line is shown.

reduced by the factor $(1+K_m/K_i)$, thus producing an apparantly lower K_m and V_{max}. Therefore, no firm conclusion can be drawn as to whether the low K_m value for (±)-β-methylhistamine represents either higher affinity than histamine at the active site or inhibition by the second isomer. This situation can be resolved only by testing each isomer separately.

For each substrate, the product ran as a single spot on the TLC system. See Table 1. The R_f (0.62) of the product from histamine was identical to that of authentic N^τ-methylhistamine under the same conditions, but in other cases the authentic N^τ-methyl derivatives were not available for comparison. In theory, the observed methylation of these further substrates might be due to other brain methyltransferases such as catechol O-methyltransferase or phenylethanolamine O-methyltransferase present in the enzyme preparation. However, confirmation of the nature of the enzyme catalysing the methylation has been obtained by use of the inhibitor SK&F 91488. This compound has been shown by Beaven *et al.* [8, 9] to be a potent inhibitor of rat kidney HMT, apparently in a non-competitive manner when tested against low concentrations of substrate histamine. However, we have consistently found SK&F 91488 to be a competitive inhibitor against histamine at this enzyme when tested using a range of substrate concentrations around the K_m (see Fig. 1). SK&F 91488 was shown also to be a competitive inhibitor against the synthetic substrates identified in this study, and K_i values were similar to that obtained against histamine (see Fig. 1 and Table 1). This suggests that the enzyme catalysing the methylation is indeed HMT in each case. The use of a purified enzyme will clarify this situation further.

Since completion of this work, Hough *et al.* [10] have described substrate activity of α-methylhistamine (presumably racemic) at the enzyme prepared from rat kidney. The mass spectrum of the derivatized product was consistent with the predicted fragmentation pattern of the bis-heptafluorobutyryl derivative of α,N^τ-dimethylhistamine. These workers found that α-methylhistamine and histamine have similar V_{max} values at this enzyme, but the apparent K_m for α-methylhistamine (30.35 ± 5.45 μM) was some ten-fold higher than the K_m for histamine (2.88 ± 0.62 μM). This difference in K_m values is in contrast with the similarity of K_m values for the two compounds in the present study. This may be due to the use of different types of enzyme preparation (dialysed 45–70% ammonium sulphate fraction from high-speed supernatant of rat kidney homogenate or, in the present study, high speed supernatant from guinea-pig brain homogenate). Alternatively the difference may be due to different tissue sources. In this respect it is interesting that Watanabe [11] has recently described an apparent difference between the enzymes prepared from rat kidney and guinea-pig brain based on the inhibitory potency of quinacrine, which was some 54-fold less potent against the rat kidney enzyme compared with the enzyme from other tissue sources, including guinea pig brain.

One aspect of substrate non-specificity of HMT is that such substrates could interfere with the histamine radioenzymatic assay, in which histamine to be assayed is converted by HMT into N^τ-methylhistamine using methyl-labelled *S*-adenosylmethionine as methyl group donor. This problem has been discussed by Barth *et al.* [3] who pointed out that potentially naturally occurring substrates such as N^α-methylhistamine and N^α,N^α-dimethylhistamine could reduce specificity. However, none of the substrates identified in this study is known or likely to be an endogenous substance and should therefore not reduce the specificity of the assay. In any case, the specificity problem can be overcome by incorporating a separation procedure for the labelled N^τ-methylhistamine product of the assay. This may be accomplished by TLC as demonstrated by Dent *et al.* [12] and Brown *et al.* [13]. Such a procedure serves to increase markedly the sensitivity of the assay also. For example, Brown *et al.* [13] have obtained a sensitivity of *ca.*50 pg/ml by this procedure. These workers used N^α-methylhistamine as internal standard in their radio-chromatographic assay. We are currently investigating the usefulness of the new substrates identified in this study for this purpose.

In summary, the work described in this study demonstrates that several sidechain-modified analogues of histamine can be methylated by an enzyme identified as histamine *N*-methyltransferase by the use of the competitive inhibitor SK&F 91488.

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Effects of pH on 5-methyltetrahydrofolic acid transport in human erythrocytes

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The effect of pH on membrane transport of folate compounds has been studied, but the results have been conflicting. For example, the optimal pH for uptake of pteroylglutamic acid by rat jejuneum was found to be 6.0 [1] to 6.3 [2], and incorporation of pteroylglutamic acid and methotrexate by brush border membrane vesicles from rat intestine was maximum at around 5.0 [3]. In contrast, the optimal pH for uptake of pteroylglutamic acid by human bone marrow was 7.4 [4], while a broad pH optimum between 4.5 and 7.5 was observed for *Lactobacillus casei* [5]. In human leukocytes, the transport of amethropterin was found to be directly proportional to pH [6], but in Sarcoma-180 cells amethopterin uptake was inversely proportional to the pH of the medium [7]. These diverse results undoubtedly reflect, at least in part, the use of different experimental conditions. In addition, it has been appreciated only recently that most anionic buffers themselves influence the transport of folate compounds, thereby necessitating the use of zwitterionic buffers for studies of pH effect [8].

The relationship between pH and folate compound permeation is of interest because it may provide insights into the mechanism of transport. Studies in a variety of experimental systems, including the human erythrocyte, have established that folate compounds are transferred across the plasma membrane by a saturable, temperature-dependent, concentrative, substrate-specific process which is influenced by heteroexchange [9]. Since folate compounds have pK values of less than 5 (the pK values of the carboxyl groups in *p*-aminobenzoylglutamic acid are 3.76 and 4.83), these compounds should be almost completely dissociated at physiologic pH [7]. Consequently, it seems possible that the folate ion, like other organic anions, may undergo co-transport with H^+ or exchange with OH^- across the membrane. If this is the case, permeation of folate compounds could be expected to be strongly pH dependent. To test this possibility, influx, efflux and steady-state levels of 5-methyltetrahydrofolic acid ($^{14}CH_3H_4PteGlu_1$) were measured in human erythrocytes suspended in media of different pH values.

Human peripheral blood was obtained from normal volunteers and depleted of white cells and platelets by dextran sedimentation as previously described [9]. The erythrocytes were washed three times with the appropriate buffer and resuspended to a hematocrit of approximately 20. The erythrocyte suspension was divided into 1 ml aliquots, and isotope was added in 0.1 ml volumes containing 20 mM ascorbate. All determinations were done in triplicate. Red cell counts were performed with a Coulter z.

Initial rates of uptake were measured by incubating red cells with the appropriate concentration of radioisotope for 45 min at 37°. In previous studies we found that uptake was linear during this period for the entire range of concentrations [9]. Steady-state levels were determined after a 4- to 6-hr incubation at 37° with 30 nM $^{14}CH_3H_4PteGlu_1$. Extracellular volume, as measured by [^{3}H]inulin concentration, does not change during this incubation period [10]. Efflux was measured by incubating washed erythrocytes in phosphate-buffered saline, pH 7.2, containing 5 mM phosphate, with 2 μM $^{14}CH_3H_4PteGlu_1$ for 90 min at 37°. After washing, the red cells were resuspended to hematocrit 20 in the appropriate buffers and divided into 1 ml aliquots. Levels of incorporated radioactivity were determined as previously described, except that protein was precipitated by the addition of 0.1 ml of 100% trichloroacetic acid to the hemolysate rather than by autoclaving [9, 11].

The pH of the erythrocyte suspension was measured before and after the incubation period and never varied by more than 0.1 unit from the indicated pH.

$^{14}CH_3H_4PteGlu_1$ (sp. act. 79–91 μCi/mg) was obtained from the Amersham/Searle Corp. (Arlington Heights, IL). Radiochemical purity was assayed by column chromatography as previously described [9].

The relationship between extracellular pH and initial