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N-METHYLATION OF BIOGENIC AMINES—I

CHARACTERIZATION AND PROPERTIES OF AN *N*-METHYLTRANSFERASE IN RAT BRAIN USING 5-METHYLTETRAHYDROFOLIC ACID AS THE METHYL DONOR

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Abstract—Some properties and kinetics of a recently identified brain *N*-methyltransferase requiring 5-methyltetrahydrofolic acid as the methyl donor are described in this paper. In addition to the assay using dopamine as substrate, the more stable substrate, 3-hydroxy-4-methoxyphenethylamine with a rapid extraction procedure was also used. The pH optimum of the enzyme, about pH 6·4, was found to differ from that previously found with dopamine in the presence of metabisulfite. The reaction products were identified by column and thin layer chromatography and indirectly by testing various *N*-methyl-, dimethyl- and dimethoxy-derivatives of dopamine for their ability to be *N*-methylated. The reaction was a linear function of time and enzyme concentration. The K_m for 5-methyltetrahydrofolic acid was $2{\cdot}5 \times 10^{-5}$ M. The kinetic experiments to determine the K_m for dopamine and 3-hydroxy-4-methoxyphenethylamine showed an anomalous behaviour of the saturation curve. When the results were plotted S/v vs S, a non-linear curve was obtained suggesting that the enzyme was behaving as an allosteric protein. Although further kinetic experiments are needed to confirm these results, a possible regulatory function can be postulated for this enzyme. In addition to dopamine and its derivatives, amines such as tryptamine, serotonin and amphetamine were also *N*-methylated under the same conditions.

S-ADENOSYLMETHIONINE is generally considered the common methyl donor for the *O*- as well as the *N*-methylation of biogenic amines.[1] Consequently the transfer of the methyl group from *S*-adenosylmethionine to amines may occur in a biosynthetic process as well as in a degradative one. In the adrenal medulla, for instance, Kirshner and Goodall[2] have shown that the formation of adrenaline requires the transfer of a methyl group from *S*-adenosylmethionine to the amine nitrogen of noradrenaline. The enzyme responsible for this reaction has been extensively studied by Axelrod who named it phenylethanolamine *N*-methyltransferase (PNMT) because of its specificity toward phenylethanolamine derivatives.[1,3]

However, it has been demonstrated recently that a phenylethylamine (dopamine) can be *N*-methylated *in vitro* by an enzyme from adrenal medulla.[4] This led us to propose a new model for the biosynthesis of catecholamines, in which epinine rather than noradrenaline is the immediate precursor of adrenaline.[4–5]

Abbreviations: 5-methyltetrahydrofolic acid, 5-Me-H_4-folate; 3-hydroxy-4-methoxyphenethylamine, 4-MDA.

Although different types of *N*-methyltransferase requiring *S*-adenosylmethionine as the methyl donor have already been found in the brain,[3,6–10] the *N*-methylation of dopamine in this organ has never been considered. Recent work from this laboratory provided the first evidence that another methyl donor, 5-methyltetrahydrofolic acid (Fig. 1), commonly involved in single-carbon transfer reactions but not with biogenic amines, is able to transfer its methyl group to dopamine by means of an *N*-methylating enzyme from rat brain.[11]

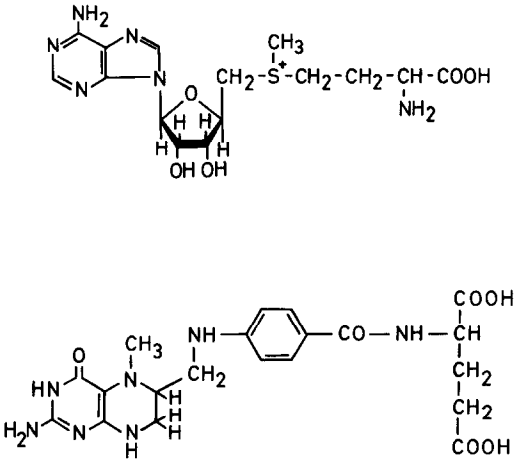

FIG. 1. Chemical structure of *S*-adenosylmethionine (above) and 5-methyltetrahydrofolic acid (below).

In this paper, one of a series dealing with the *N*-methylation of biogenic amines in the adrenal medulla and in the brain, we describe some properties and kinetics of the *N*-methyltransferase from rat brain. An unusual methyl donor, 5-methyltetrahydrofolic acid, was used, and the reaction products were identified by means of chromatographic analysis and confirmed by using various *O*- and *N*-methylated biogenic amines as substrates.

MATERIALS AND METHODS

Chemicals. 5-(Methyl-C^{14})-tetrahydrofolic acid barium salt (spec. act. 54 mCi/mmole) was obtained from the Radiochemical Centre Amersham, England. The unlabelled compound was supplied by Sigma Chemical Co., St. Louis, Missouri, U.S.A. *N,N'*-Dimethyldopamine hydrobromide and all the methoxylated derivatives of catecholamines were synthesized for us by H. Verhoeven and *N,N'*-dimethylamphetamine hydrochloride by A. Knaeps (Janssen Pharmaceutica, Beerse). Bufotenine was generously donated by Dr. J. Jacob, Institut Pasteur, Paris. Epinine hydrobromide, dopamine hydrochloride and serotonin creatinine sulphate were obtained from Aldrich Europe, B-2340 Beerse, Belgium. All the other amines used were purchased from Sigma Chemical Co.

Enzyme preparations. Male Wistar rats, 200–250 g body wt, were decapitated, the brains were removed, rinsed in isotonic sucrose and then homogenized with an Ultra-Turrax in 5 vol. distilled water. The homogenate was centrifuged, at low speed. The supernatant was centrifuged 130,000 *g* for 90 min to obtain the final supernatant.

The enzyme was purified by a two-step procedure using ammonium sulphate. The pellet obtained at 30% ammonium sulphate saturation was discarded, whilst the second one (P_2) obtained at a 65 per cent saturation was retained and suspended in 0·02 M phosphate buffer pH 6·8. This P_2 enzyme preparation was dialyzed overnight against the same buffer, centrifuged at 20,000 *g* for 20 min and then stored at −10° until required.

N-*Methyltransferase assay.* The *N*-methyltransferase activity was measured according to the method of Laduron,[11] with the following modifications. The incubation mixture contained in a total vol. 0·5 ml: 0·5 μCi 5-(methyl-C^{14})-tetrahydrofolic acid, 50 μmoles phosphate buffer pH 6·4, 10 μmoles substrate (dopamine or 4-MDA) and enzyme. A blank was prepared using boiled enzyme in the incubation mixture. After incubation at 37° for 2 hr, the reaction products were extracted by two different procedures according to the kind of substrate used.

When dopamine was used as the substrate, the reaction was stopped by adding 3 ml of 10% trichloroacetic acid. After centrifuging, 10 ml of 0·2 M sodium acetate, 1 ml of 10 mM sodium metabisulfite and 1 ml of 0·2 M EDTA were added to the supernatant. This mixture was brought to pH 8·6 with ammonia and loaded onto Al_2O_3 columns (2 × 1 cm). After washing with distilled water, the amines were eluted with 6 ml of 1 N HCl. The radioactivity of a 1 ml aliquot in 10 ml of scintillation fluid (Instagel, Packard), was measured in a liquid scintillation spectrometer.

When 4-MDA was used as the substrate, the reaction was stopped by bringing the incubation mixture to pH 10 with 0·5 ml of 0·5 M borate buffer at 0°. The reaction products were extracted in 10 ml of an isoamyl alcohol–toluene mixture 2:3 from the aqueous phase previously saturated with 1 g NaCl. After shaking and centrifuging, the radioactivity of a 4 ml aliquot was measured in a scintillation spectrometer.

Chromatographic analysis. The reaction products formed in the incubation mixtures were identified using, column and paper chromatography. Amines were separated on Dowex 50W × 5 (Na^+ form) columns (3 × 1 cm) as described previously.[4] After washing with 20 ml of 0·2% EDTA pH 6·0 and distilled water, the columns were eluted with 0·7 N HCl and 1·5 ml fractions were collected. The optical density of each fraction was measured at 280 nm and the radioactivity of an 0·5 ml aliquot was determined. The radioactive fractions were evaporated *in vacuo* and suspended in 100 μl of 0·01 N HCl. Twenty-five microlitres were spotted on thin layer plates (cellulose, F Merck) and developed using either phenol–water–HCl (80:20:1) or butanol–water–acetic acid (40:50:10).

After development, radioactivity was measured with a Berthold Scanner and the unlabelled amines (standard) were detected by spraying the plates with 2% potassium iodate in 0·2 M phosphate buffer pH 6.

Protein determination. Protein was estimated by the method of Lowry *et al.*[12] using bovine serum albumin as standard.

RESULTS

Influence of metabisulfite on pH optimum. In preliminary work[11] the maximum activity of *N*-methyltransferase in the rat brain was found to occur between pH 8·2 and 8·4 with dopamine as the substrate and 5-Me-H_4-folate as the methyl donor. However, at that time, the enzyme activity was measured in the presence of 0·5 μmoles sodium metabisulfite and 2·5 μmoles EDTA in the incubation mixture.

Recently more extensive experiments have shown that EDTA and metabisulfite are not needed to prevent the oxidation of dopamine during the incubation period, as had previously been suggested for the adrenal medulla.[4] Figure 2 illustrates the effect of metabisulfite on the pH curves of rat brain *N*-methyltransferase by using three different substrates. For dopamine and noradrenaline, a considerable shift of pH values from about 8 to 6·4 together with a much higher enzyme activity was

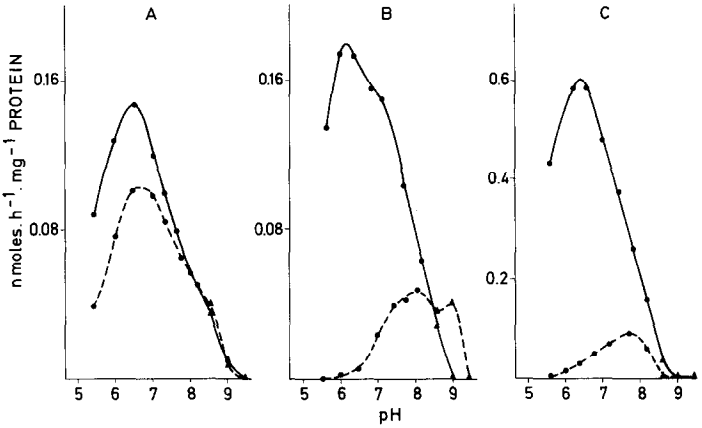

FIG. 2. Influence of pH on the *N*-methylation of 4-MDA (A), dopamine (B) and noradrenaline (C) by means of a P_2 fraction of rat brain in the presence (– – –) and absence (——) of metabisulfite. Incubations were carried out under standard conditions in 0·1 M sodium phosphate (●) and 0·1 M glycine-NaOH buffer (▲). The *N*-methylated compounds were extracted from samples incubated with 4-MDA using toluene-isoamyl alcohol and from those incubated with dopamine and noradrenaline on Al_2O_3 columns.

observed when metabisulfite was omitted from the incubation mixture. In contrast, only a small difference in activity without any change of pH optimum was detected when 4-MDA was used as the substrate. The maximum activity of the enzyme at about pH 6·4 was common for these three substrates in the absence of metabisulfite.

The same pH optimum was also observed for other substrates such as amphetamine, serotonin and tryptamine (unpublished results).

Identification of reaction products. Although some preliminary attempts to identify the reaction products have been described, the introduction of improved experimental conditions (pH 6·4 and no metabisulfite) have necessitated a reinvestigation of this problem.

The column chromatographic analysis of the *in vitro* *N*-methylation of dopamine is shown in Fig. 3. A very high peak of radioactivity, overlapping that of unlabelled dopamine was eluted from the Dowex column for the assay while no significant radioactivity was detected for the blank (boiled enzyme) sample.

According to previous data[4,11] the elution pattern of the radioactivity corresponded to that of epinine. In order to confirm this, chromatographic analyses were carried out on thin layer plates using two different solvents. As illustrated in Fig. 4 the pooled amines, dopamine and epinine, eluted previously from the column, were completely separated on cellulose plates using phenol as a solvent.

Epinine was identified as a reaction product also by mass-fragmentography (unpublished results).

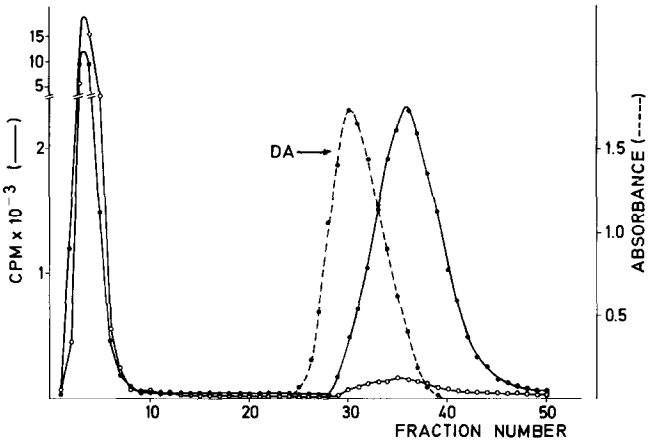

FIG. 3. Elution pattern of radioactivity from Dowex 50 W × 5 columns loaded with reaction mixtures incubated with 5-Me^{C14}-H_4-folate and dopamine as substrate. The P_2 fraction of rat brain was used in the assay sample (●) while boiled enzyme was substituted for active enzyme in the blank (○).

Kinetic experiments. In order to assess the enzymatic formation of *N*-methylated amines various enzyme kinetics were investigated. As shown in Fig. 5 the rate of dopamine conversion to epinine was linear up to 120 min. Similarly, the reaction was also linear as a function of enzyme concentration (Fig. 6). The K_m value for 5-Me-H_4-folate with 4-MDA as the substrate was $2{\cdot}5 \times 10^{-5}$ M (Fig. 7) which is similar to that previously found with dopamine as a substrate although under different experimental conditions.[11]

On increasing the 5-Me-H_4-folate concentration, the enzymatic reaction exhibited a typical hyperbolic curve of saturation, but the attempts to determine the K_m values for dopamine and 4-MDA showed an anomalous type of kinetic behaviour (Figs. 8 and 9). A concave downward curve instead of a straight line was obtained with dopamine was well as with 4-MDA when the velocity was plotted as S/v vs S. Similarly,

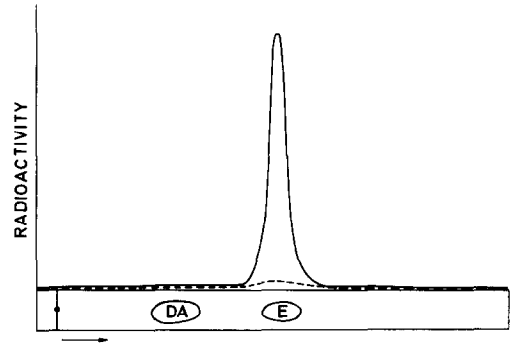

FIG. 4. Radiochromatogram of the main peak isolated by column chromatography in Fig. 3. Eluate fractions 30 to 45 were pooled and reduced to a small volume, assay (——) and blank (– – – –). An aliquot of each was spotted on thin layer (cf. Methods). Radioactivity was measured and the position of the compounds was compared with unlabelled dopamine (DA) and epinine (E) used as standards. Solvent: phenol–water–HCl (80:20:1).

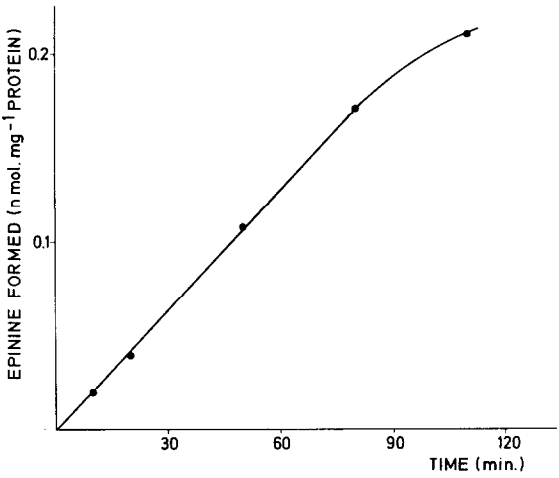

FIG. 5. *N*-Methylation rate of dopamine as a function of time.

plots of velocity versus substrate concentration were hyperbolic at low substrate concentration and sigmoidal at high substrate concentration. The significance of these apparently anomalous kinetics will be discussed later.

Rat brain *N*-methyltransferase (P_2 fraction) was preincubated at 45° in 0·01 M phosphate buffer (pH 6·8) before measuring the activity using 4-MDA as substrate under normal incubation conditions. Figure 10 shows a typical inactivation curve as a function of time, indicating that the enzyme is thermolabile.

Specificity of the substrate. Several substrates were tested for their ability to be *N*-methylated by rat brain *N*-methyltransferase. The enzyme activity was measured by isolating the reaction products according to two different methods. For catecholamines (dopamine, epinine, dimethyldopamine, noradrenaline, and adrenaline) the labelled *N*-methylated amines were absorbed on Al_2O_3 columns. Other amines were

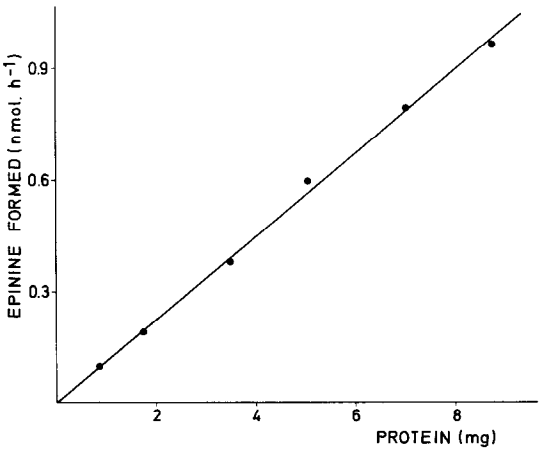

FIG. 6. Activity of *N*-methyltransferase from rat brain as a function of enzyme concentration.

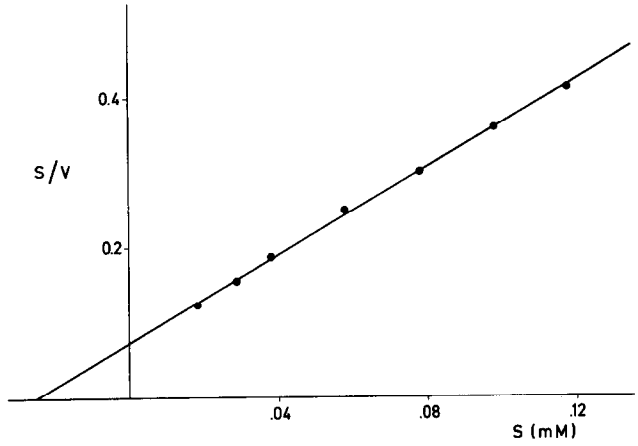

FIG. 7. K_m determination for the methyl donor 5-Me-H_4-folate using 4-MDA as a substrate. The ratio of the 5-Me-H_4-folate (S) concentrations to the velocity (v) is plotted against the 5-Me-H_4-folate concentration (S).

extracted with isoamyl alcohol–toluene. Although the reaction products were not identified except epinine it seems that all the primary and secondary amines can be *N*-methylated (Table 1). Noradrenaline and 5-hydroxytryptamine were the substrates most easily converted into *N*-methylated compounds. This point needs further confirmation.

The data presented in Table 1 rule out the occurrence of an *O*-methylation process and confirm that the methylating enzyme cannot act other than by transferring the methyl group from 5-Me-H_4-folate to the nitrogen of amines.

All the methoxy-derivatives and even the dimethoxy-derivatives could be used as substrates. The tertiary amines (dimethyldopamine, dimethylamphetamine) were not converted but low activity was detected with bufotenine which suggests a possible binding of methyl C^{14} to another position (for instance the nitrogen of the indole nucleus).

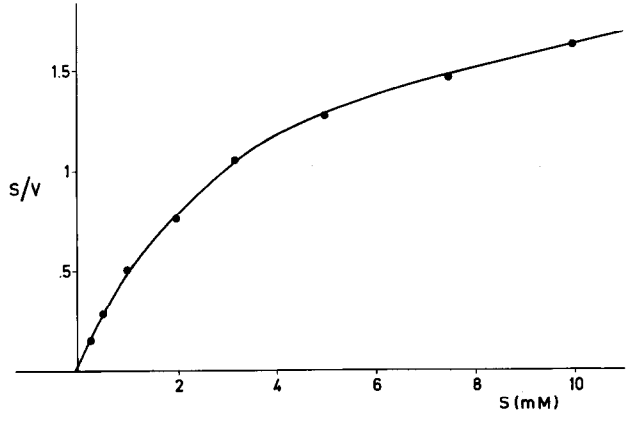

FIG. 8. S/v plots for rat brain *N*-methyltransferase as a function of increasing concentrations of dopamine (S).

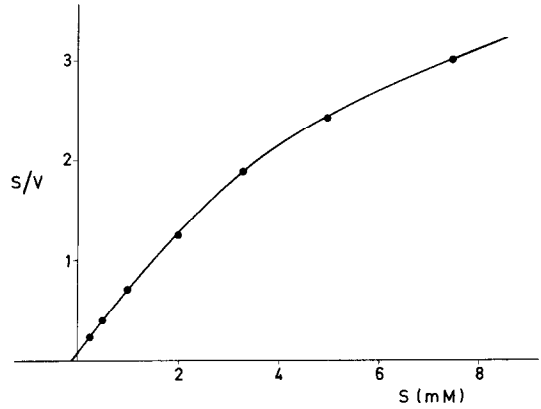

FIG. 9. S/v plots for rat brain N-methyltransferase as a function of increasing concentration of 4-MDA (S).

DISCUSSION

The results presented here, confirm and extend a preliminary report[11] on the occurrence of an N-methyltransferase for biogenic amines in rat brain. This enzyme is unusual since it requires 5-methyltetrahydrofolic acid as the methyl donor.

The use of 4-MDA, a more stable substrate than dopamine, and the omission of metabisulfite and EDTA from the incubation mixture, has improved the enzyme assay.

Under these conditions the pH optimum of this N-methyltransferase is about pH 6·4 rather than pH 8·2–8·4, which had been described previously[11] and again found here when dopamine was incubated *in vitro* in the presence of metabisulfite. Although not observed with 4-MDA, this shift of the pH optimum in the presence or absence of metabisulfite could be due either to a modification of the substrate-enzyme binding or to a conformational modification of the enzyme. As no shift was

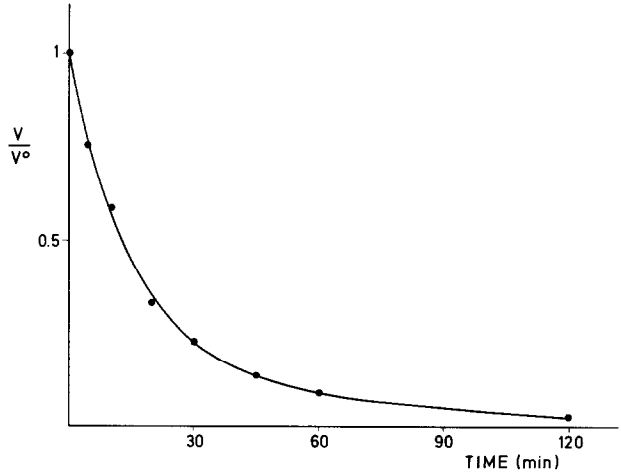

FIG. 10. Thermal inactivation of rat brain N-methyltransferase preincubated at 45° as a function of time. V_o: enzyme activity without preincubation.

TABLE 1. *N*-METHYLTRANSFERASE ACTIVITY IN RAT BRAIN WITH VARIOUS SUBSTRATES

Substrate	Enzyme activity (nmoles hr^{-1} mg^{-1} protein)
Dopamine	0·172
N-Methyldopamine (epinine)	0·119
N,N'-Dimethyldopamine	0
3-Hydroxy-4-methoxyphenethylamine	0·149
3,4,5-Trimethoxyphenethylamine (mescaline)	0·119
3,4-Dimethoxy-*N*-methylphenethylamine	0·099
3-Hydroxy-4-methoxy-*N,N'*-dimethylphenethylamine	0
Amphetamine	0·098
N-Methylamphetamine	0·054
N,N'-Dimethylamphetamine	0·006
Tryptamine	0·094
N-Methyltryptamine	0·052
5-Hydroxytryptamine	0·365
N,N'-Dimethyl-5-hydroxytryptamine (bufotenine)	0·071
Noradrenaline	0·642
Adrenaline	0·145
Normetanephrine	0·059

observed with 4-MDA, this latter possibility seemed rather unlikely, but could not be completely excluded as the anomalous enzyme kinetics have to be considered.

The identification of the reaction products formed under the improved experimental conditions, i.e. pH 6·4 and no metabisulfite, has shown that dopamine is enzymatically converted to epinine. Moreover, a comparison of the enzyme activity with other substrates confirmed the results obtained by chromatographic analysis. No enzyme activity could be detected with *N*-dimethyl derivatives as substrates, whereas *O*-dimethoxy-derivatives did yield enzyme activity.

Therefore the enzymatic reaction involves a transfer of a methyl group from 5-Me-H_4-folate to the nitrogen of the side chain to form *N*-methylated or *N*-dimethylated compounds. Consequently this enzymatic system is able to synthesize *in vitro* hallucinogenic compounds (*N,N'*-dimethyltryptamine and bufotenine) which were found to play a prominent role in schizophrenic disorders.[13–15]

Another striking property of this *N*-methyltransferase was revealed by the anomalous enzyme kinetics as a function of substrate concentration. It seems reasonable to think that this enzyme behaves as an allosteric protein.

The results obtained with two different substrates, described here, and more recent and detailed experiments (work in preparation) clearly show the appearance of an intermediary plateau in the kinetic saturation curve, followed by a sigmoidal section. S/v vs S plots like $1/v$ vs $1/S$ plots, were concave downward where some pseudolinear sections could be discerned (Figs. 8 and 9).

According to Teipel and Koshland[16] such a kinetic behaviour, apparently anomalous, indicates that the enzyme possesses more than two substrate binding-sites. These authors have also postulated that the occurrence of an intermediary plateau in the kinetic saturation curve cannot be explained only by a negative co-operativity but that this transition must also be followed by a positive co-operativity corresponding to the sigmoidal part of the curve.[16,17] Other possible explanations which allow these complex kinetics to be elucidated, cannot be completely excluded for this rat brain *N*-methyltransferase, until the required observations have been completed

with a purified enzyme or the presence of isoenzymes has been entirely excluded. More extensive kinetic studies together with further elucidation of the structural features of this protein are needed to assess its regulatory nature. The regulatory function of the brain *N*-methyltransferase should be of prime importance in schizophrenic disorders by controlling the occurrence of hallucinogenic *N*-methylated compounds.

It is not easy to conciliate our present findings with earlier studies[3,6–10] dealing with the brain *N*-methyltransferase for which *S*-adenosylmethionine was used as the methyl donor since detailed kinetic experiments have never been presented and procedures for measuring the enzyme activity were sophisticated.[7,10]

It is very unlikely that the same enzyme can use two different kinds of methyl donors in the brain. Indeed, this would not agree with other results,[18] since under our experimental conditions almost no enzyme activity was detectable using *S*-adenosylmethionine. Therefore, 5-Me-H_4-folate can be considered as a much more specific methyl donor for *N*-methylation in the brain. A specific methyl donor is required for both the *N*- and *O*-methylations in the brain (unpublished results). Furthermore, because they are endowed with properties which are fundamentally different, the *N*-methyltransferases in the adrenal medulla and the brain constitute two separate enzymatic entities.

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Note added in proof—After this manuscript was submitted for publication, two recent papers [L. L. HSU and A. J. MANDELL, *Life Sci.* **13,** 847 (1973); S. P. BARNAJEE and S. H. SNYDER, *Science N.Y.* **182,** 74 (1973)] have confirmed the existence of an enzyme requiring 5-methyltetrahydrofolic acid as the methyl donor. However, in contrast to their results, we were unable to isolate *N*-methyltryptamine as the reaction product when tryptamine was used as substrate.

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