The presence of cytosolic S-adenosylmethionine-dependent N-methyltransferase(s) activity(ies) capable of converting phosphoethanolamine into phosphocholine has been recently demonstrated in the rat brain. At least two enzymes are involved in the methylation of phosphoethanolamine to phosphocholine and these are separable by ammonium sulphate fractionation. The enzyme catalysing the last step of this methylation process is present in the 50–80% ammonium sulphate fraction. A 220-fold purified enzyme has been obtained with sequentially employed Q-Sepharose fast flow and octyl-Sepharose CL4B column chromatography. The maximum enzyme activity was at pH 9.5. The K_m values for S-adenosylmethionine, the methyl donor, and phosphodimethylethanolamine, the methyl acceptor, were 125 μM and 750 μM respectively. This phosphodimethylethanolamine N-methyltransferase was found to be calcium-dependent, with a 4-fold increase in activity at 0.5 mM-CaCl_2. S-Adenosylhomocysteine at 0.5 mM caused 100% inhibition of the activity. The effects of various structural analogues on the phosphodimethylethanolamine N-methyltransferase activity were also investigated and these results suggest that the enzyme is specific to the substrate. These results provide evidence for the existence of the pathway for the methylation of phosphoethanolamine to phosphocholine in rat brain cytosol.

INTRODUCTION

Choline (Cho) is the polar head group characterizing the Cho-containing phospholipids, which are components of all mammalian membranes. In brain tissue Cho also serves as a precursor for acetylcholine (AcCho) synthesis by the cholinergic neurons. The major source of Cho for the brain is its uptake from the blood circulation [1–5]. However, the brain is also capable of producing free Cho through the catabolism of membrane phosphatidylcholine (PtdCho) [6–8]. PtdCho is synthesized primarily by a pathway involving phosphorylated and cytidine nucleotide intermediates [9], but can also be synthesized by the sequential methylation of phosphatidylethanolamine (PtdEtn) [10–12], catalysed by PtdEtn N-methyltransferase (N-MT; EC 2.1.1.17) [13,14] and by the base-exchange reaction [15–17].

These pathways contribute to the maintenance of the PtdCho content of the brain at a relatively constant level, and it has been shown that a co-ordinate relationship may exist between them. Indeed, it has been reported that the inhibition by 3-deazaribosine (3-DZA) of the methylation of PtdEtn to PtdCho led to an increase in PtdCho biosynthesis by the de novo pathway in hepatocytes [18,19]. A 2-fold increase in the PtdEtn N-MT activity of a rat hepatoma cell line was observed when the cells were grown in the absence of Cho compared with the activity in cells grown in the presence of Cho [20]. This observation would suggest that when the uptake of Cho is impaired, or unavailable, thus restricting PtdCho synthesis by the de novo pathway, cells compensate for this decrease by increasing the PtdEtn N-MT pathway. However, in brain tissue only 5% of total PtdCho synthesis can be provided by the methylation of PtdEtn [21].

Another mechanism of PtdCho production might be through the formation of phosphocholine (PCho) by the sequential methylation of phosphoethanolamine (PEtN). We have previously reported the existence of such a pathway both in primary neuronal cultures and in the intact rat brain [22]. These studies suggested that PEtN was a preferred substrate for methylation(s) compared with either free Etn or PtdEtn. The potential contribution of this methylation pathway in providing Cho and/or Cho-containing compounds may play a part in the maintenance of the Cho steady-state concentration, and therefore in the regulation of PtdCho biosynthesis. Moreover, in investigations utilizing pharmacological agents, it was observed that the methyltransferase(s) responsible for the conversion of PEtN into PCho was (were) different from that (those) for the conversion of PtdEtn to PtdCho [23]. We have demonstrated the existence of a cytosolic enzyme system present in rat brain which converts PEtN into PCho by sequential methylation [24]. These preliminary observations prompted us to further investigate this novel methylation pathway and to attempt to purify the PEtN N-MT. We now describe the partial purification of phosphodimethylethanolamine (PM_e2Etn) N-MT from rat brain cytosol; this enzyme catalyses the final step in the methylation pathway beginning with PEtN and yielding PCho.

MATERIALS AND METHODS

Materials

S-[3H]Adenosylmethionine ([3H]Adomet) was purchased from Amersham Corp. Unlabelled Adomet and EDTA were from Boehringer Mannheim Canada. Dowex 50W × 8 (H+ form), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), Bicine, Heps, phenylmethanesulphonyl fluoride (PMSF), PEtN, PCho, noradrenaline, adrenaline, catechol, 2-hydroxyethyl-hydrazine, β-phenethylamine, nicotinamide, choline, S-adenosylhomocysteine (AdoHcy), L-homocysteine thiolactone, poly-

Abbreviations used: Adomet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; AcCho, acetylcholine; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Cho, choline; 3-DZA, 3-deazaribosine; Etn, ethanolamine; N-MT, N-methyltransferase; PEG, poly(ethylene glycol); PMSF, phenylmethanesulphonyl fluoride; PEtN, phosphoethanolamine; PCho, phosphocholine; PM_e2Etn, phosphodimethylethanolamine; PM_e3Etn, phosphonomonomethylethanolamine; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine.

Vol. 288
(ethylene glycol) (PEG), sucrose and BSA, were obtained from Sigma, St. Louis, MO, U.S.A. Ammonium sulphate was purchased from ICN Biomedicals Inc., Costa Mesa, OH, U.S.A. Q-Sepharose fast flow and octyl-Sepharose gels were supplied by Pharmacia–LKB Biotechnology. Scintiverse II was supplied locally by Fisher Scientific. 2-Aminoethylphosphonic acid, diethylyamine hydrochloride, 2-aminoethanethiol hydrochloride, 2-dimethylaminoethanethiol hydrochloride, ethanolamine, monomethylethanolamine, dimethylethanolamine, N-methylphénylaméthylamine and N,N-dimethylphenethylamine were purchased from Aldrich, Milwaukee, WI, U.S.A. 2-Amino-3-phosphonopropionic acid was purchased from Research Biochemicals. N-Phenylethanolamine was obtained from Fluka, and 3-DZA was from Southern Research Institute, Birmingham, AL, U.S.A. The Coomassie Blue protein assay reagent was purchased from Pierce Chemical Co.

Phosphonomethylethanolamine (PMeEtN) and PMeEtN were chemically synthesized according to the method of Weisburger & Schneider [25] with a slight modification. Briefly, an equimolar mixture of H$_2$PO$_4$ and monomethylethanolamine (or dimethylethanolamine) was heated overnight at 170°C. The sample was allowed to cool, diluted 5-fold with water, the pH adjusted to 6 and the sample loaded on to a Dowex 50W x 8 H$^+$ column. Under these conditions the unreacted free base was retained on the column and the phosphobase was recovered in the water effluent. This effluent was evaporated to dryness and dissolved in water. Samples of the solution containing these products were heated at 110°C in 6 M HCl for 18 h and separate aliquots of the hydrolysate were used to quantify the phosphorus content by the procedure of Bartlett [26] and to quantify the amino alcohol with choline oxidase employing the individual amino alcohol as standard [27,28]. The mol ratios of phosphorus and amino alcohol for both PMeEtN and PMeEtN were approximately 1.0. T.L.c. analysis of the products was performed using butanol/methanol/HCl/water (10:10:1:1, by vol.) as solvent, and the individual spots were located by exposure to iodine vapour. The $R_f$ values were 0.67 for PEtn, 0.57 for PMeEtN, 0.42 for PMeEtN and 0.28 for PMC. The pH of the phosphobase solution to be employed as substrate was adjusted to 9.5 before use. All other materials were of reagent grade.

**Purification of PMeEtN N-MT**

**Preparation of the cytosolic fraction.** Male Sprague–Dawley rats (75–90 g) were killed and their brain tissue was homogenized in 0.32 mM-sucrose containing 10 mM-CAPS and 100 μM-PMFSF at pH 9.5 (10:1, v/wt). The homogenate was centrifuged at 20000 g for 20 min and the resulting supernatant was centrifuged at 105000 g for 60 min. The supernatant (S3) was removed and dialysed overnight against the homogenizing buffer.

**Ammonium sulphate precipitation.** The dialysed S3 fraction was precipitated stepwise with increasing concentrations of ammonium sulphate of 0–30%, 30–40%, 40–50% and 50–80%. The individual precipitates were suspended in 10 mM-CAPS buffer containing 100 μM-PMFSF, pH 9.5, and dialysed overnight against the same buffer.

**Q-Sepharose fast flow chromatography.** The 50–80% ammonium sulphate precipitate containing the bulk of the PMeEtN N-MT activity was applied to a Q-Sepharose fast flow anion-exchange column (10 ml bed volume) previously equilibrated with 10 mM-CAPS containing 100 μM-PMFSF, pH 9.5. The column was first washed with 50 ml of the equilibrating buffer and then eluted with 200 ml of a linear gradient of NaCl from 0 to 0.6 M at a flow rate of 20 ml/h and individual 2 ml fractions were collected. The fractions that possessed the PMeEtN N-MT activity were pooled, dialysed against 10 mM-CAPS containing 100 μM-PMFSF, pH 9.5, and concentrated using PEG.

**Octyl-Sepharose CL4B chromatography.** The pooled sample from the Q-Sepharose column was loaded on to an octyl-Sepharose CL4B hydrophobic column of 2 ml bed volume previously equilibrated with 10 mM-CAPS containing 100 μM-PMFSF and 0.6 M NaCl, pH 9.5. The column was washed with 10 ml of the starting buffer and eluted with 40 ml of a linear reverse gradient of NaCl from 0.6 to 0 M, followed by 50% ethylene glycol in the buffer at a flow rate of 20 ml/h; 1 ml fractions were collected. The fractions containing the PMeEtN N-MT activity were pooled, dialysed against 10 mM-CAPS containing 100 μM-PMFSF, pH 9.5, and concentrated with PEG.

**Determination of PMeEtN N-MT activity**

To 24 μl of an incubation solution containing 1 mM-PMeEtN, 0.5 mM-CaCl$_2$, 66.67 mM-CAPS buffer, pH 9.5, and 250 μM-[3H]Adomet (specific radioactivity 12500 d.p.m./nmol) was added 36 μl of the enzyme preparation. Controls were prepared with heat-inactivated enzyme samples. After 60 min of incubation at 37°C, the reaction was stopped by the addition of 20 μl of cold 1 M HCl. The samples remained on ice for 10 min and were then centrifuged at 5000 rev./min for 10 min. The resulting supernatant was added 20 μl of 1.2 M cold KOH, and after 10 min on ice the sample was centrifuged again at 5000 rev./min for 10 min. An aliquot of the supernatant was diluted 5-fold with water and applied to a Dowex 50W x 8 H$^+$ form with a 0.5 ml bed volume previously washed with 1 M HCl and then washed with water until neutral. After the application of the sample, the column was washed with 1 bed vol. of water and with another 10 ml of water. An aliquot of the water effluent was added to 10 ml of Scintiverse II and the radioactivity was determined.

The incubation conditions and the assays for PEtn N-MT and PMeEtN N-MT were similar to that described for PMeEt N-MT.

**Other methods**

The protein concentrations were determined by the method of Lowry et al. [29], except for the column fractions where the Coomassie Blue protein assay procedure was used [30]. The $K_m$ and $V_{max}$ values were determined according to the Line-Weaver–Burk procedure. The noradrenaline N-MT assay was performed according to a previously described method [31].

**RESULTS**

**Ammonium sulphate precipitation**

As shown in Table 1, there are at least two enzymes involved in the methylation of PEtn to PCho, and these are separable by ammonium sulphate fractionation. The methyltransferase(s) responsible for the conversion of PEtn to PMeEtN, and of PMeEtN to PMeEtN, is (are) recovered in the 0–40% ammonium sulphate precipitate. The enzyme responsible for converting PMeEtN to PCho is recovered principally in the 50–80% ammonium sulphate fractions.

**Q-Sepharose fast flow column**

The protein elution profile and the enzyme activities of the Q-Sepharose column are shown in Fig. 1. The peak of PMeEtN N-MT activity appears at approx. 0.3 M NaCl. The contents of tubes 50–65 containing the activity were pooled, dialysed overnight, concentrated and found to have a specific activity of about 0.25 nmol/min per mg of protein.

**Octyl-Sepharose CL4B column**

The pooled sample from the Q-Sepharose column was loaded
The cytosol prepared from rat brain homogenate was subjected to ammonium sulphate fractionation and assayed with PEtn, PMeEtn or Pme2EtN as substrate as described in the Materials and methods section. Values are the average of four separate experiments. Each assay was performed in duplicate. WH, whole homogenate; S3, cytosol.

### Table 1. Distribution of phosphobase N-MT activities in various ammonium sulphate fractionations

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity (pmol/mg per min)</th>
<th>Total activity (pmol/min)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEtn</td>
<td>PMeEtn</td>
<td>Pme2EtN</td>
</tr>
<tr>
<td>WH</td>
<td>1527</td>
<td>5.0</td>
<td>4.8</td>
<td>4.7</td>
</tr>
<tr>
<td>S3</td>
<td>560</td>
<td>11.5</td>
<td>12.7</td>
<td>12.9</td>
</tr>
<tr>
<td>0-30%-(NH4)2SO4</td>
<td>124</td>
<td>14.2</td>
<td>38.4</td>
<td>4.0</td>
</tr>
<tr>
<td>30-40%-(NH4)2SO4</td>
<td>96</td>
<td>42.9</td>
<td>22.9</td>
<td>5.2</td>
</tr>
<tr>
<td>40-50%-(NH4)2SO4</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50-80%-(NH4)2SO4</td>
<td>125</td>
<td>0</td>
<td>37.8</td>
<td>37.8</td>
</tr>
</tbody>
</table>

Fig. 1. Q-Sepharose fast flow column chromatography of Pme2EtN N-MT activity

The 50-80% ammonium sulphate fraction was applied to a Q-Sepharose fast flow column as described in the Materials and methods section. ○, Protein concentration was determined by the Coomassie Blue method; ●, enzyme activity was measured in the presence of 1 mM-Pme2EtN; ---, NaCl gradient.

Purification of Pme2EtN N-MT

Purification of the Pme2EtN N-MT from rat brain homogenate is summarized in Table 2. A 220-fold purified enzyme with a specific activity of 1 nmol/min per mg of protein was obtained from the octyl-Sepharose column.

General properties

pH curve. Pme2EtN N-MT activity was measured as a function of pH and it was found that the optimum was at pH 9.5. At neutral pH the activity was almost undetectable, and it was decreased by 80% at pH 11 (results not shown).

Effect of bivalent cations. For these studies the enzyme sample had been dialysed against a solution containing 10 mM-CAPS, 5 mM-EDTA and 100 μM-PMSF, pH 9.5, overnight, and then dialysed against the same solution without EDTA overnight prior to investigating the effect of cations. The Pme2EtN N-MT activity was increased 4-fold by the presence of 0.5 mM-CaCl2 in the incubation mixture, compared with the control incubated in the absence of calcium. In the presence of 0.5 mM-MgCl2 there

was a 1.5-fold stimulation of activity. In contrast, ZnCl2 at the same concentration caused a slight inhibition and MnCl2 dramatically decreased the activity by 90% (results not shown).

Affinity for Adomet and Pme2EtN. When the concentration of Pme2EtN was held constant and the concentration of Adomet was varied, the Kₘ for Adomet, the methyl donor, was found to be 125 μM and the Vₘₚₙ was 1.33 nmol/min per mg of protein (Fig. 3). In a similar manner, the Kₘ for Pme2EtN, the methyl

Vol. 288
The results showed that the phosphobases PteN, PMeEtN and PCho, the free bases Etn, MeEtN, MeEtN and Cho, the phospholipids PtdEtn, PtdMeEtN and PtdMeEtN, and the analogues 2-amino-3-phosphopropionic acid, 2-aminoethylphosphonic acid, 2-aminoethanethiol, 2-dimethylaminomethanol, diethylamine and 2-hydroxyethylhydrazine did not affect the PMeEtN N-MT activity. The other compounds examined are either the substrates for or the products of other previously described mammalian cytosolic methyltransferases, and included noradrenaline, adrenaline, phenylethanolamine, phenylethyamine, N-methylphenethylamine, N,N-dimethylphenethylamine, catechol, nicotine and histamine. These compounds had no effect on PMeEtN N-MT activity when present in the incubation medium at 4 mM, except for noradrenaline and adrenaline which caused a slight decrease in activity (results not shown). However, this inhibitory effect was not dose-dependent and, moreover, the purified PMeEtN N-MT preparation failed to catalyse the methylation of noradrenaline to adrenaline (results not shown).

**Effects of AdoHcy and 3-DZA.** The inhibitory effect of 3-DZA on PtdEtn N-MT activity is due to its conversion into a deaza-analogue of AdoHcy which becomes a strong inhibitor of AdoHcy hydrolase. 3-DZA had only a slight effect on the PMeEtN N-MT activity that did not appear to be concentration-dependent. However, when 50 μM-AdoHcy was added to the incubation, a 75% decrease in the PMeEtN N-MT activity was observed, and a concentration of 0.5 μM caused complete inhibition (Table 3).

**Distribution of PMeEtN N-MT activity in various rat tissues.** PMeEtN N-MT activity in the cytosolic portions of the brain, liver, lung, spleen, kidney, heart, muscle and adrenals was determined (Table 4). The lowest activity was found in lung tissue and the highest in brain and adrenals. An investigation of the cytosolic PMeEtN N-MT activity in various regions of the rat brain revealed that the activity in the hippocampus was 2.5-fold and that of the hypothalamus was 1.5-fold greater than in the whole rat brain. The frontal cortex, the caudate putamen, the cerebellum and the medial septum had the same range of activity as in the cytosol from the whole brain (results not shown).
DISCUSSION

The pathways have been described for PtdCho synthesis in the rat brain which are also present in other tissues. The main metabolic route in mammalian tissues is the de novo pathway [9]. The two other minor pathways are the methylation of PtdEtn [13,14] and the base-exchange reactions [15–17]. However, studies demonstrated that in neuronal cells, as well as in rat brain in vivo, PEtN could be converted into PCho by a stepwise methylation process [22] which is catalysed by a cytosolic N-MT [24].

In the present report we describe the partial purification and properties of a PME2Etn N-MT from rat brain cytosol which catalyses the final step in the progressive PEn methylation pathway leading to the synthesis of PCho. This is the first experimental evidence documenting the existence of such a conversion in mammalian tissue. Although its existence had been suggested [32], the pathway had only been described to be present in plants. It was shown that the Adomet-dependent N-methylation of PEn is the sole pathway for PtdCho synthesis in Lemna (duckweed), but in the carrot both phosphobase and phospholipid methylations occurred [33].

We investigated the distribution of PME2Etn N-MT in different rat tissues (Table 4). In the liver, which is the tissue containing the greatest activity of PtdEtn N-MT, accounting for up to 20 % of total hepatic PtdCho synthesis, the activity of the cytosolic PME2Etn N-MT is only 20 % of that present in the brain cytosol. There seems to be a regional variation of this PEn N-MT activity in brain tissue, with enrichments in the hippocampus and the hypothalamus. A major concern was whether or not the PME2Etn N-MT was similar to any previously described cytosolic methyltransferase activity. A variety of potential substrates for these previously described methyltransferases were examined and these did not affect, nor were they substrates for, the PME2Etn N-MT activity. These observations suggest that this enzyme is specific for PME2Etn as the methyl acceptor. Another unusual property of PME2Etn N-MT is its calcium-dependency. The cytosolic methyltransferases that have been described were not found to be metal-dependent with the exception of the catechol O-methyltransferase, which is inhibited by calcium [34]. The alkaline pH optimum of 9.5 for the PME2Etn N-MT is similar to that of the PtdEtn N-MT but different from those of other cytosolic methyltransferases.

These studies demonstrate the existence of cytosolic enzymes capable of carrying out the progressive methylation of PEn to PCho in the rat brain. They also provide direct evidence that the methylation of Etn-containing compounds into Cho-containing compounds is not restricted to the phospholipid level. However, the biological significance of such a pathway is unexplored.

Numerous studies focused at the phospholipid level reported the effects of hormones on PtdEtn N-MT activity. Conflicting results were obtained and, in general, no correlation was shown between effects of hormones and PtdEtn N-MT activity when studied in vitro, or in intact cells [35–38]. Several explanations were offered for these differences, and one of the contributing factors was the controversy about the nature of the PtdEtn N-MT [39,40]. Pajares and co-workers claimed that the methylation of PtdEtn into PtdCho was catalysed by a methyltransferase with a molecular mass of 50 kDa, and several in vitro studies have been carried out using this enzyme preparation [41–43]. However, Ridgway & Vance have provided evidence that the 50 kDa protein is not PtdEtn N-MT, since they successfully purified the enzyme to apparent homogeneity [44]. Therefore most of the hormonal studies on PtdEtn N-MT should be re-evaluated.

Based on the fact that the levels of PEn N-MT and PtdEtn N-MT appear to have reciprocal relationships, it will be interesting to investigate the possible involvement of P Etn N-MT in these hormonal regulation and signal transduction phenomena. The role of this enzyme in the regulation of AcCho and PtdCho syntheses must also be considered.

This work was supported by grants from the Medical Research Council of Canada.

REFERENCES


Received 10 April 1992/27 May 1992; accepted 9 June 1992